

THE BIG FISH BANG

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Proceedings of the 26th Annual Larval Fish Conference

Hosted by the Norwegian Institute of Marine Research
and the University of Bergen

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Edited by Howard I. Browman and Anne Berit Skiftesvik

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Complete details of the conference,
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www.fishlarvae.com/lfc

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Foreword

This book contains a selection of the papers that were presented at the 26th Annual Larval Fish Conference (LFC2002). Manuscripts were screened for scientific content by journal-style peer review, but only lightly edited for format, writing style and other technical aspects of presentation. Thus, any errors that remain are the responsibility of the authors.

Technical note about colour plates and micrographs. Some of the figures associated with the articles printed here are colour plates and/or micrographs. The cost of printing these – one page at a time – in the body of each paper was prohibitive. As a compromise, colour plates and micrographs were converted to black and white illustrations and appear as such in the body of the articles. However, all of them are reprinted – in the colours and qualities provided by the authors – in an Appendix at the end of the book. This is noted in the Legends of the relevant Figures.

About the American Fisheries Society's Early Life History Section and the Larval Fish Conferences. The Early Life History Section (ELHS) is an interest-discipline subunit of the American Fisheries Society (AFS – www.fisheries.org) with an international membership of over 350 scientists. It is the only organisation of this kind devoted to interests in the early life history of freshwater, estuarine, and marine fishes, and related matters. Through its newsletter, *Stages*, and now its web page (www2.ncsu.edu/elhs) the AFS's ELHS encourages and facilitates exchange of knowledge and ideas, updates members on current research, publications, meetings and other events, provides feature articles and reviews and communicates Section and pertinent AFS business and concerns.

The annual Larval Fish Conferences (LFC) that serve as the focal point of ELHS activities evolved from a series of informal, freshwater-oriented, symposia that began in 1977. The current LFCs, which are hosted and sponsored by various organisations throughout the world, cover the complete spectrum of research (from all habitats and geographic locations) related to fish early life history. A list of all the LFCs can be found on the ELHS web page (www2.ncsu.edu/elhs/elhspubs.html). The dates, locations, host institutions and publications resulting from these meetings are all available. All LFCs are run under the auspices of the AFS's ELHS.

About LFC2002. LFC2002 marked the first time that the LFC was convened in Europe. The conference was hosted by the Norwegian Institute of Marine Research and the University of Bergen. The organizing committee consisted of Howard I. Browman and Anne Berit Skiftesvik. The meeting, which attracted 215 delegates from 27 countries, was convened at the Solstrand Fjord Hotel, Os, Norway, 22-26 July 2002. There were 120 oral and 53 poster presentations.

The objectives of the 26th Annual Larval Fish Conference were to bring together the research community actively working on, and/or interested in, all aspects of the early life history of fishes under intimate and highly interactive conditions and in pleasant surroundings. Anyone interested in the subject matter was free to attend the conference, and we made a particular effort to ensure and facilitate the participation of students.

The conference was organized around four special theme sessions and several contributed paper sessions. The theme sessions were: “Morphological development and physiological function in fishes: studies in larval fish adaptation”, organized by John Jeffrey Govoni; “Larval growth and survival in varying environments: implications for stock conservation and aquaculture”, organized by C. Tara Marshall; “Essential fatty acids and fish development”, organized by Reiji Masuda; “Developmental neurobiology and sensory biology of fishes”, organized by Howard I. Browman and Thomas Becker.

LFC2002 web site – www.fishlarvae.com/lfc. Organizing and managing a conference such as this one is an enormous task, particularly for amateur organizers with a full slate of other responsibilities. The job would have been impossible without the professionalism and dedication of Ken Elman, proprietor of SimboliQ Technologies Inc. (www.simboliq.com), and web site designer extraordinaire. Ken contributed significantly of his company’s time, with far less than the industry-standard level of compensation (he heard, repeatedly, the standard refrain, “we are poor scientists”...). In addition to the clean and ergonomic public face of the web page, Ken provided us with a full slate of utilities so that the site’s content and databases could be managed easily, and from anywhere. The LFC2002 web site, which will remain online, contains complete details of the conference, including the titles and abstracts of the oral and poster presentations and the complete content of this book. All of this content is freely downloadable.

Other books issuing from LFC2002. In addition to the conference proceedings volume, several of the papers presented at the theme session “Development of Form and Function in Fishes, and the Question of Larval Adaptation” will be published as review-style chapters in a book edited by John Jeffrey Govoni. That book will be published by the American Fisheries Society, and should appear in print sometime in 2004.

Acknowledgements. For their assistance with the conference program, we thank the theme session organizers – Thomas Becker, John Jeffrey Govoni, C. Tara Marshall and Reiji Masuda. For their help as session moderators, thanks go to John Jeffrey Govoni, C. Tara Marshall, Don Hoss, Catriona Clemmesen, Reiji Masuda, Douglas Tocher, Thomas Becker, Jeff Isely, Perce Powles, Arild Folkvord, Art Kendall, Richard Cloutier, and Robert Batty. The following friends and colleagues helped with various aspects of organizing, preparing for, and running the conference: Helene Pedersen, Kirsten Aas, Emmanuelle Babatunde, Syed Yahiya Yacoob, Kirsten Poling, Dennis Higgs, all of the LFC2002 student delegates who operated the multimedia equipment, the staff of Austevoll Aquaculture Research Station (and especially Karin Boxaspen for her help in coordinating and implementing the delegates’ visit to the station), the staff of the Solstrand Fjord Hotel (but particularly the hotel’s owner, Børrea Schau-Larsen, and her daughter). All LFC2002 delegates recognized the contributions and assistance of our son, Petter Skiftesvik, with all aspects of setting up and implementing the computer-based multimedia presentations. Our daughter, Line Skiftesvik, assisted with various organizational tasks. In addition to acknowledging their help, we must also thank our children for their patient acceptance of the unusual time commitment that we were required to make in preparing for the conference. A very special “tusen takk” to Reidun Bjelland, who was

always ready and willing to help, *with anything and everything*. We could not have made it without you Reidun! Penny Kuhn's patient, conscientious and professional assistance with preparing the conference program book and schedule, and with the technical editing of the manuscripts that appear here, is gratefully acknowledged. Special recognition is due John Jeffrey Govoni for his unfailing support and experience-laden advice, and for his long-term devotion and service to the AFS-ELHS.

The numerous financial sponsors of the conference, and of this book, are listed on the pages that follow.

Finally, we wish to thank the 215 LFC2002 delegates, whose participation in the conference was the main reason for its success.

Howard I. Browman and Anne Berit Skiftesvik
Storebø, Norway, 1 September 2003

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Acknowledging the sponsors

The financial support listed below was used to (i) plan and organize the meeting (including development of the conference web page), (ii) ensure the participation of keynote speakers, (iii) facilitate student participation (by reducing their registration fees, accommodation costs and, for some, travel costs), (iv) and publish this proceedings book, which is being distributed free of charge to all delegates and other interested parties.

Funding for the conference was provided by the Norwegian Institute of Marine Research, the Research Council of Norway, the Norwegian Agency for Development Cooperation (NORAD), the Norwegian Ministry of Foreign Affairs, and the European Commission's Fifth Framework Program for Community Research (under the Quality of Life and Management of Living Resources Program). The social events at the conference hotel, and at Austevoll Aquaculture Research Station, were generously supported by The University of Bergen, Sjøtroll, Skretting et Nutreco-selskap, Intervet Norbio, EWOS, Bergen Aqua AS, ARE, Kvernsmolt, Kobbbevik og Furuholmen Oppdrett AS, Strandvik Plast AS, and Reed Mariculture Inc.

The development of the conference web page was only partially supported by extramural funding. Ken Elman, proprietor of SimboliQ Technologies Inc. (www.simboliq.com), contributed significantly of his company's (and his own) time. It would have been impossible to organize this conference without that support.

The theme session on «Morphological development and physiological function in fishes: studies in larval fish adaptation», organized by John Jeffrey Govoni, received funding (to offset the travel costs of invited speakers) from: the U. S. National Oceanic and Atmospheric Administration (Ocean and Atmospheric Research (National Sea Grant Program, New Jersey Sea Grant Program, and Delaware Sea Grant Program), the National Marine Fisheries Service (Southeast Fisheries Science Center), the National Ocean Service (National Centers for Coastal Ocean Science Program Office and the Coastal Ocean Program); the New Jersey Marine Sciences Consortium; and the European Commission's Fifth Framework Program for Community Research (Quality of Life and Management of Living Resources Program). Dr. Michael P. Weinstein coordinated the support from Sea Grant. Administrative support for the preparation of this Book was provided by NOAA's National Ocean Service, Center for Coastal Fisheries and Habitat Research, Dr. Donald E. Hoss, Director.

We are very grateful for all of this support!

About the conference's theme image: "The Big Fish Bang"

Howard I. Browman

The striking image of fish eggs exploding outwards from the centre of the universe that graces the cover of this book was created by the very talented Harald Kryvi, Professor of Zoology at the University of Bergen, Norway. "The Big Fish-Bang", an image commissioned specifically for the 26th Annual Larval Fish Conference, is a print made from a copper etching.

Professor Kryvi took up copper etching as a hobby, although he studied with a graphic artist for a short time in order to acquire the technique. Etching in copper serves as an artistic expression of Harald's interest in zoology and functional morphology. Much of Professor Kryvi's art is inspired by a combination of morphology and his delightfully playful "Larsonesque" sense of humour.

The creative process for this image began with a detailed morphological study of fish embryology, and a thorough knowledge of the conference's theme. This is typical of the approach taken for all of Professor Kryvi's work – he first studies the anatomy and then waits until an image that pleases him appears in his mind. At this point, one or more (very rough) preliminary sketches are produced, from which are decided his approach (size, orientation, extent of etching for each line) to the etching. Once the etching process begins, there is no turning back – etched lines in the copper cannot be corrected, only incorporated into the image creation process.

Etchings are made in 1 mm thick non-elastic copper using an Ash England # 9 Lustra tool. A line drawing of the entire image is completed on the first pass. The copper plate is then sprayed with an under-coating and ceratin areas are then painted with a lacquer coating. The plate is then immersed in nitric acid for 20 – 40 minutes – as a result of this, areas of the surface not painted with lacquer will take on a rougher texture and appearance (and this, therefore, is part of the artist's original vision for the printed image). After acid exposure the plate is rinsed in water and washed in white spirit. The plate then undergoes a second round of etching – the deeper the etched line, the more pronounced the colour in the printed image.

Once the etching is complete, it must be coloured for printing. Professor Kryvi's creative process is unusual in that the images come to his mind without colour. It is only at the end of the etching process that he begins to contemplate colours for the image. The colours chosen are always complementary so that the transitions blend smoothly. Also unusual is Professor Kryvi's application of all colours to the plate simultaneously – in printing from copper etchings it is more common to apply colours one-at-a-time and to make a print to the paper for each colour.

Colours – usually a mix of 1-4 etching inks -- are applied to the plate by dabbing. It is the pressure of dabbing that eventually wears down the etching and limits the number of prints that can be produced from any one plate. Excess ink (approximately 90% of the amount applied) is removed by repeatedly placing (and removing) porous wrapping paper onto the plate surface. Only one

print is made from each application of ink – thus, no two prints are the same. Finally, a cotton swab is used to remove all of the ink from certain areas (in the case of "The Big Fish-Bang, the clear areas in some of the eggs).

Water-saturated etching paper ("Vangerov") – dried to a very precise level of humidity (the ink will not be satisfactorily transferred if the paper is too wet or too dry) – is used for printing. Prints are made using a small press in the basement studio of Professor Kryvi's residence. To prevent shrinking, the fresh print is pinned to a cork board until dry. It is then ready for delivery and framing.

The amount of time Professor Kryvi uses to create an image is highly variable, but depends upon the level of detail. Clearly, the creation of such images represents many hours of work: the printing process alone takes more than one hour.

To date, Professor Kryvi has produced approximately 200 images. Many of these hang in the corridors outside his office at the University of Bergen and we strongly encourage visitors to stop by and enjoy viewing his work. Signed and numbered artist's prints of these works can be obtained by contacting Professor Harald Kryvi at the University of Bergen, Institute of Zoology, Allégaten 41, N-5007 Bergen, Norway, Email: harald.kryvi@zoo.uib.no. Other works of art by Professor Kryvi can be viewed at our online gallery: www.fishlarvae.com/photos.asp?GID=29&AID=6

Additive budgeting of metabolic costs in larval coregonids

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Key words: *energy allocation, larval whitefish, additive budgeting, costs of digestion, costs of activity*

Abstract

Commonly used bioenergetics models of fish assume that the energy budget is additive, that is, each component of the budget has its own allocation and at least the basal level of this specific capacity cannot be used for other purposes. However, it has been proposed that rapidly growing fish larvae with a narrow metabolic scope may have to reduce the energy allocated to activity or maintenance in order to maintain high growth rates. This has been called previously compensatory budgeting. In the present study, we addressed this important issue by conducting swimming respirometer experiments and reanalysing previous feeding respirometer data using larval whitefish. In the swimming respirometer, the total metabolic rate of unfed larvae was lower than the total rate of fed larvae, in which the total rate increased with increasing ration. The swimming respirometer experiments suggested that the costs of digestion and activity have their own basal requirements and are at least to some extent independent. The reanalysis of the feeding respirometer data showed that up to a specific growth rate of about 16% day⁻¹ no suppression of maintenance functions is needed to achieve high growth rates. These results together suggest that the energy budget of larval whitefish is indeed additive, and that this assumption is therefore justified as a theoretical base in modelling larval coregonid energetics.

Introduction

In fish energetics, interaction between activity and digestion is important because both components influence greatly the growth rate and survival of fish (Blaikie and Kerr 1996). Traditionally, the energy budgeting of fish is assumed to be additive (Winberg 1956) and this assumption underpins commonly applied bioenergetics models (e.g. Kitchell et al. 1977; Karjalainen et al. 1997).

Additive budgeting assumes that each component has its own scope and that at least the basal level of this specific capacity cannot be used for other purposes. This means also that to some extent costs of digestion and activity must be independent. Blaikie and Kerr (1996) observed that different organ systems (muscle vs. gastrointestinal) of adult cod (*Gadus morhua*) may have independent maximum metabolic capacities which can operate without interference. They argued that the digestive system has its own blood circulation and capacity to transport gases, and that muscles for swimming are a similarly independent system, and that the respiratory system can deliver enough gas even when both systems work at their highest rate. Although this may not be true in juvenile and adult fish of many species under variable feeding conditions (Farrell et al. 2001; Hunt von Herbing and White 2002), the oxygen supply to tissues of larval fish is suggested to be limited by the mitochondrial capacity of each tissue rather than by the capacity of the respiratory system (Wieser 1995).

An alternative allocation model, the compensatory (Rombough 1994) budgeting, assumes that the total aerobic capacity is divided between different functions by priority rules and that maintenance is usually found to have a higher priority than growth and reproduction (Wieser 1994). Priede (1985) proposed that the costs of digestion and swimming activity are mutually exclusive; so that if a fish is swimming at its maximum speed, it cannot digest food simultaneously. This may be especially inevitable under unfavourable conditions where the metabolic scope is not sufficient to cover all metabolic activities running simultaneously. Thus, the metabolic budgeting may be additive under favourable conditions, but compensatory under adverse conditions. Another implication of compensatory budgeting could be in the energy allocation of larval fish (Wieser and Medgyesy 1990a, b; Rombough 1994; Wieser 1994, 1995): if fish larvae consume food and grow at a very high rate (as they can) they must reduce the energy allocated to activity or maintenance since growth has the highest priority. Positively phototactic larvae of many fish species, however, perform continuous swimming behaviour and the priority of activity must also be high.

This critical allocation issue was examined in this study by carrying out a series of swimming respirometer experiments and reanalysing the feeding respirometry data of Wieser and Medgyesy (1990b) and Huuskonen et al. (1998). In the swimming experiments, the effect of meal size on energy allocation of larval whitefish (*Coregonus lavaretus* L.) at near-maximum expenditures was studied. The feeding respirometry experiments allowed us to estimate the magnitude of digestion costs in whitefish larvae in relation to their growth rate.

Materials and Methods

Swimming respirometry. The eggs of whitefish were obtained from the hatchery of the Finnish Game and Fisheries Institute in Laukaa where they were incubated over winter in glass jars. Three weeks before hatching the eggs were transported to the hatchery of University of Jyväskylä and water temperature was slowly increased from 2°C to 6–8°C which induced the start of the hatching. Newly-hatched whitefish larvae were reared at 12°C in 45 dm³ glass aquaria and fed *Artemia* nauplii. The larvae were given nauplii by an automatic feeding system once every hour during the light period (from 0730 to 2330). The flow-through aquaria were cleaned twice a day by siphoning faeces and food particles from the bottom.

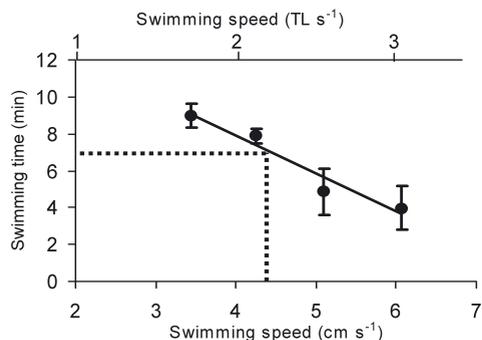


Figure 1. The maximum sustained swimming time (mean \pm se) of unfed larval whitefish (total length 19-20 mm) swimming against a current at variable speed (cm s⁻¹ and body length s⁻¹) in the swimming respirometer at 12°C. The dotted line indicates the swimming speed used in the 7 min swimming respirometer experiments.

graphical oxygen sensors (POS, YSI 5750). Each respirometer system included three parallel acrylic swimming chambers (length 20 cm, diameter 1 cm). The oxygen consumption of each chamber was recorded for 7 minutes and the average rate during this period extrapolated to an hourly value. The oxygen electrode chamber and the fish chambers were flushed after each measurement with fully aerated water. Bacterial oxygen consumption in the respirometers was measured at the beginning and at the end of each experiment and was subtracted from the total decline of oxygen. Water temperature and changes in air pressure during the measuring period were also taken into account for the calculations (according to Forstner and Gnaiger 1983).

The allocation of energy costs of active larval whitefish was examined in three separate swimming respirometer experiments. In the swimming respirometer, fish were forced to swim against a current at 12 or 14°C for 7 minutes in order to reach their maximum aerobic performance and, thus, metabolic rates. The duration of the exercise and the water current were adjusted according to preliminary swimming tests with unfed larvae (Fig. 1).

In all experiments, half of the fish were fed for 2-3 hours on *Artemia* nauplii before the respirometry while the rest of the larvae remained without food for 18-20 hours before the oxygen consumption measurements. During the feeding period, both treatments were replicated (four aquaria per treatment) and the oxygen consumption of 6-24 schools (5 larvae per school) randomly sampled from the aquaria was measured in the respirometer. In experiments 1 and 2, fish were fed in excess and in experiment 3, high (100% of fresh mass of fish) and low \pm 10% of FM) rations were offered. High ration corresponded to the ad libitum feeding in experiments 1 and 2. The fresh mass and total length of the larvae varied from 25-55 mg and 19-25 mm, respectively.

During the measurement period, each school of 5 fish swam against a current (4-6 cm s⁻¹) and the peak rates (total metabolic rate, R_T) of the fed and unfed fish were recorded. In experiments 1 and 2, the same school of fish was placed into the chamber and measured three times, but because there was no difference between three sequential measurements, in experiment 3 only one measurement was carried out with each school. Experiment 1 was carried out in 2001 and experiment 2 in 2002. The costs of digestion (R_{SDA} = the post-prandial rise in the metabolic rate, SDA as in Jobling 1983) was calculated by subtracting the mean R_T of unfed fish from the mean R_T of fed fish. In the swimming experiments, the standard metabolic rates (R_M) of whitefish larvae were calculated according to the mass- and temperature-dependent oxygen consumption functions of Karjalainen et al. (1995). R_M was measured from unfed fish and thus was lower than the R_M of fed fish.

Oxygen consumption was measured in two intermittent-flow respirometers (Forstner 1983; Forstner et al. 1983) equipped with polarographic oxygen sensors (POS, YSI 5750).

Feeding respirometry. The feeding respirometry data of Wieser and Medgyesy (1990b) and of Huuskonen et al. (1998) were reanalysed to try to identify which cost budgeting model is appropriate for larval coregonids. Detailed descriptions of the feeding respirometer (Wieser and Medgyesy 1990a) and experimental protocols (Huuskonen et al. 1998) are given elsewhere. The experiments at 10°C (n=9) and 15°C (n=13) were carried out in Innsbruck in 1991, and those at 12°C (n=2) in Joensuu in 1996 (Huuskonen 1997; Huuskonen et al. 1998).

In the feeding respirometer, three components of the energy budget could be determined directly: food consumption (C), total metabolism (R_T) and growth (P_G). Further, it was possible to separate total metabolic rate (R_T) into standard metabolic rate (R_M) and feeding-induced thermogenesis (R_F); i.e. the energy used for maintenance was distinguished from costs related to activity and digestion, absorption and processing of food (Wieser and Medgyesy 1990b). R_F and R_{SDA} have thus slightly different meanings: R_{SDA} includes no feeding costs, and R_F is practically synonymous with the apparent heat increment (AHI, Beamish and Trippel 1990). Metabolic rates were calculated as in Wieser and Medgyesy (1990b): 1) R_T = sum of all oxygen consumption measurements recorded hourly; 2) R_M = the average of the three lowest values during the dark period multiplied by the number of hours during an experiment; and 3) $R_F = R_T - R_M$. The R_F could be further divided into R_{SDA} and R_{ACT} (the costs of activity). The following calculations were used to convert oxygen consumption and somatic growth into energy units: 1 $\mu\text{mol O}_2 = 0.45 \text{ J}$ and 1 mg DM = 22.7 J (Wieser and Medgyesy 1990b).

R_T , R_M , R_F and P_G were inversely correlated with fish body mass (Wieser and Medgyesy 1990b) and, due to the high size differences (fresh body mass 12.5–210 mg) of the fish in the separate experiments, R_T , R_M , R_F and P_G were mass-corrected. The mass-dependent power functions used for the conversion were calculated according to the original feeding respirometry data: $R_T = 34.7 \text{ FM}^{-0.132}$, $R_M = 57.09 \text{ FM}^{-0.160}$ and $P_G = 62.2 \text{ FM}^{-0.576}$. Furthermore, the true R_F was calculated in order to correct the observed overestimation of the R_M in the feeding respirometer experiments. The overestimation of R_M was feeding induced and this part of the costs was added to R_F in order to obtain true R_F . These calculations are explained in detail in the next section.

Results

Like many other positively phototactic fish larvae, after hatching, whitefish larvae have very vigorous swimming behaviour, high metabolic costs and extremely narrow scope for the physiological performance. In our four feeding respirometry experiments, the costs of the maintenance (R_M), digestion (R_{SDA}) and activity (R_{ACT}) were on average 68%, 22% and 10% of the total metabolic costs, respectively. Note, however, that R_M here evidently included some level of activity due to the continuous swimming of larval fish.

In the swimming respirometer experiments, the total metabolic rate (R_T) of unfed larvae was lower than the total rate of fed larvae, and both total costs and digestion costs (R_{SDA}) of the larvae fed on a high ration were higher than the rates of larvae fed on a low ration. Food ration significantly affected the total costs and digestion costs (Table 1, Fig. 2). The constant swimming speed in the swimming chambers kept the activity and maintenance costs at a constant level.

Table 1. Fresh mass (mg, mean \pm standard deviation) and oxygen consumption rates (mean \pm standard deviation, n = number of cases in parentheses) of the fed (low or high ration) and unfed (no food) larval whitefish in the swimming respirometer experiments at 12 and 14°C. Asterisks and dashes indicate the significant (*, $p < 0.05$) and insignificant (-, $p > 0.05$) difference between the food rations, respectively.

| Experiment | Fresh mass mg \pm SD | Temperature °C | Total metabolic rate R_T $\mu\text{mol g}^{-1} \text{h}^{-1} \pm$ SD (n) | | |
|------------|---------------------------|-------------------|---|------------------------|----------------------|
| | | | No food | Low Ration | High Ration |
| 1 | 33.7 \pm 4.1 | 14 | 21.0 \pm 3.0 (18) | * no data | 26.3 \pm 5.6 (18) |
| 2 | 57.6 \pm 4.0 | 14 | 19.4 \pm 2.0 (6) | * no data | 26.5 \pm 2.3 (6) |
| 3 | 25.6 \pm 4.8 | 12 | 17.5 \pm 4.1 (8) | ns 20.9 \pm 4.9 (11) | * 25.0 \pm 1.6 (4) |

Wieser and Medgyesy (1990b) observed that when whitefish larvae grew at a very high rate the costs of feeding and digestion (R_F) remained nearly constant or were independent of the growth rate (P_G) (Fig. 3A, dotted regression lines were originally based on data at 15°C). In the original form of the data, R_F indeed increased more slowly among the fast-growing larvae. However, because the costs of maintenance (mass-corrected R_M in Fig. 3B) correlated positively with P_G at 15°C, the R_M was obviously overestimated and R_F underestimated in the high ration experiments. Thus, the “true” R_F was calculated as follows: firstly, the intercept constant (17.22 and 14.20 $\mu\text{mol g}^{-1} \text{h}^{-1}$ at 15°C and at 10–12°C, respectively) was subtracted from the each R_M and then, the difference was added to the R_F . The maximum difference at 15°C was 9.4 $\mu\text{mol g}^{-1} \text{h}^{-1}$. The relationships between the mass-corrected “true” R_F and P_G were linear and significant at all temperatures (Fig. 3C). The R_F correction was also made for low temperature experiments, although the regression between R_M and P_G was not significant at 10 and 12°C. After the correction, the R_F differed between the temperatures, which was not observed in the original data.

R_F and food consumption (C) also had a linear relationship: higher C produced higher R_F . (Fig. 3D). The high variation in this relationship was mainly due to inaccurate estimation of the number of *Artemia* during each feeding period.

The summary of the results from all experiments are repre-

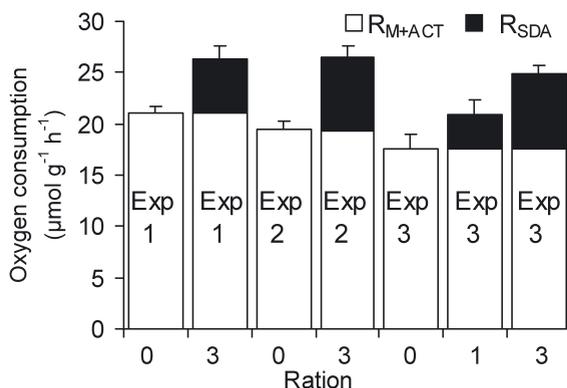


Figure 2. The oxygen consumption ($\mu\text{mol g}^{-1} \text{h}^{-1}$, based on fresh body mass) of fed (ration 1 = low and ration 3 = ad libitum) and unfed (ration=0) larval whitefish in the swimming respirometer experiments 1 and 2 at 14°C and experiment 3 at 12°C. The costs of digestion (R_{SDA}), activity and maintenance (R_{M+ACT}) are given separately and the vertical lines represent the standard errors of the mean of total oxygen consumption rates (R_T).

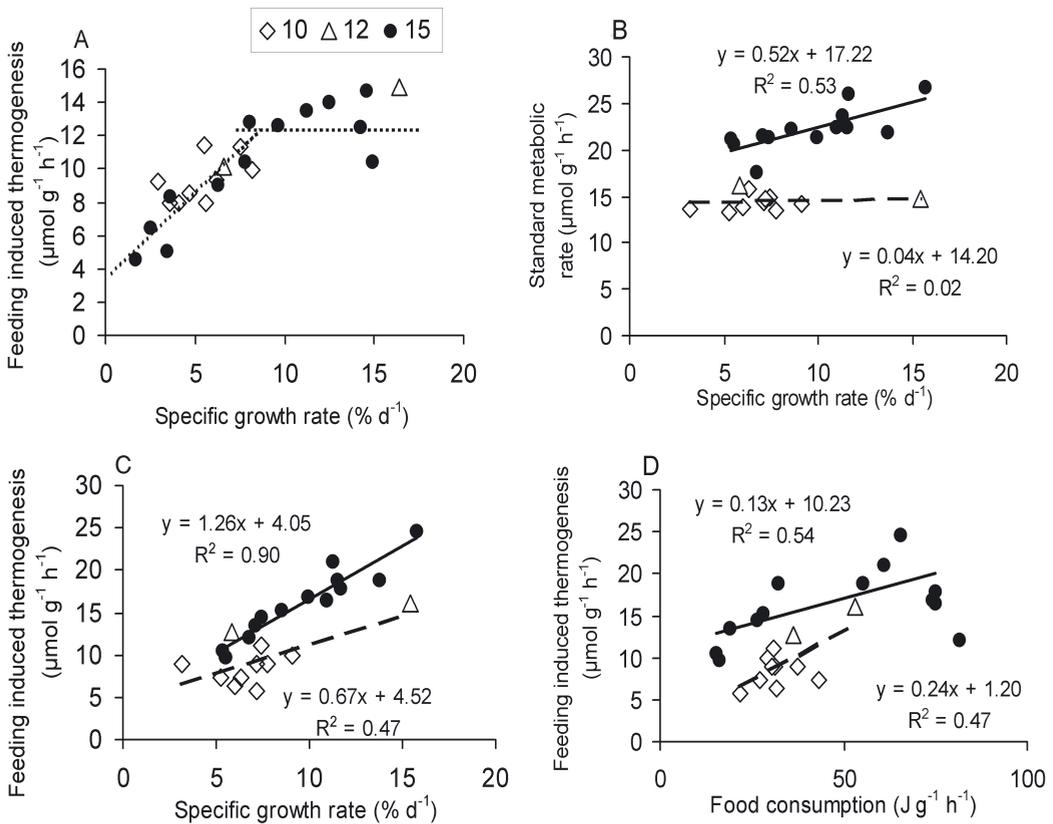


Figure 3. The oxygen consumption ($\mu\text{mol g}^{-1}\text{h}^{-1}$, based on fresh body mass) of larval whitefish in the feeding respirometer experiments. The relationship A. between the costs of digestion (feeding induced thermogenesis R_F) and the specific growth rate (P_G , $\% \text{d}^{-1}$), B. between the mass-corrected maintenance costs (standard metabolic rate R_M) and P_G , C. between the true R_F and P_G and D. between the true R_F and food consumption rate (C , $\text{J g}^{-1} \text{h}^{-1}$) at 15°C (black circle, solid line) and at 10 and 12°C (blank symbol, dashed line). The dotted lines (linear regressions) in section A were fitted according to Wieser and Medgyesy (1990b, see text). All mass-corrected rates correspond the rates of a larva at the size of 30 mg fresh body mass.

sented in Fig. 4. In the swimming experiments, although the fish swam at high speed, it was the higher ration that produced the higher total costs. The predicted maintenance costs were fairly constant, as was the proportion of energy allocated to the forced activity. The R_M (on average $17.6 \mu\text{mol g}^{-1} \text{h}^{-1}$ at 14°C) calculated for the swimming respirometer experiments corresponded well to the “true” R_M (17.22 at 15°C) extrapolated from the measurements of the feeding experiments. The proportion of the activity costs was smaller than in the feeding respirometer experiments which may be due to the oxygen debt after swimming.

In the feeding experiments, the activity costs (R_{ACT}) increased in relation to the ration (more food, more time and energy in feeding) and likewise the R_{SDA} and the total costs were higher in the

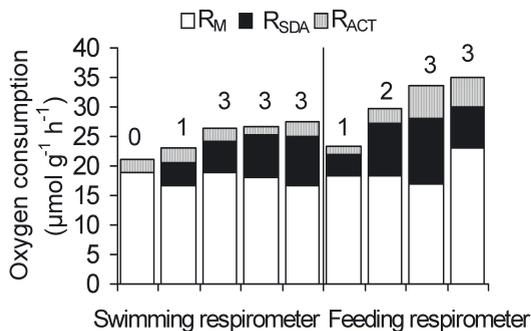


Figure 4. The oxygen consumption ($\mu\text{mol g}^{-1}\text{h}^{-1}$, based on fresh body mass) of fed and unfed larval whitefish in the swimming and feeding respirometers. The costs of digestion (R_{SDA}), activity (R_{ACT}) and maintenance (R_M) are given separately. The rates were converted to correspond with the rates at 14°C . The temperature conversions (12°C to 14°C and 15°C to 14°C) were made according to temperature-dependent function in Karjalainen et al. (1995). The numbers above bars indicate ration levels (0=unfed, 1=low ration, 2= medium ration, 3= fed ad libitum).

high ration experiments. Unfortunately, in only four of the 20 feeding experiments were R_{ACT} data available for this analysis. The R_M was again fairly constant except in the last experiment, when it seemed too high and probably included either digestion or activity costs. In the high ration experiments, the specific growth rates were from 14 to 16%.

Discussion

Our reanalysis of the feeding respirometer data of larval whitefish suggested that up to a growth rate of $16\% \text{d}^{-1}$ there was a positive linear correlation between the costs of growth and the growth rate. Hence, the aerobic capacity available for digestion is sufficient and there is no need for compensatory budgeting. The higher ration produced higher costs of feeding and digestion as well as higher growth rate, and thus, the energy budget of the larval whitefish seems to be additive. In his review, Wieser (1994) concluded that, “the non-linear relationship between cost and rate of growth may be predominantly due to the changes in protein turnover”. However, he still argued the possibility of the compensatory budgeting and represented the alternative model of Pirt (1982) which assumes that “maintenance metabolism is not only dependent on experimental conditions but also on the rate of growth itself”. This maintenance model has two components: a constant component and a component decreasing proportionally with growth rate (Wieser 1994). We suggest that, in the case of larval whitefish, this is mainly a methodological problem. If the cost components could be measured precisely (i.e. the maintenance component includes only maintenance costs) then at least in long-term budgets (Lucas 1996) the simple additive energy budget model proposed by Winberg (1956) is suitable also for larval coregonids. The duration of the apparent heat increment after feeding is several hours to several days depending on fish size, temperature, ration etc. (Jobling 1994; Blaikie and Kerr 1996), and the increased maintenance costs in the feeding respirometer experiments were likely due to the longer duration of the apparent heat increment when whitefish larvae were fed on higher number of *Artemia* nauplii. In other words, the heat increment of feeding was not finished before the next feeding period and some part of the R_F was measured in R_M , thus overestimating its proportion especially in the high ration experiments.

The slopes of the regression between R_F and P_G expressed the costs of growth for larval coregonids (R_F/P_G , Wieser 1994) and at 15°C the slope represented $17.3 \mu\text{molO}_2 \text{mg}^{-1}$ dry body mass

(using the dry body mass : fresh body mass ratio of 0.173 from Wieser and Medgyesy (1990b) and 30 mg fresh body mass in the beginning of the growth period). Our R_F/P_G is, therefore, only slightly higher than the “consensus value” ($16 \mu\text{molO}_2 \text{ mg}^{-1}$ dry body mass) of Wieser (1994) for the average efficiency of the transformation of metabolizable energy into production in a wide range of organisms. If the feeding activity (on average 31% of total R_F , $n=4$) is subtracted from the R_F the cost of growth equals $12.3 \mu\text{mol O}_2 \text{ mg}^{-1}$ dry body mass. At lower temperature the R_F/P_G appears to be lower, but due to higher variation in the data the exact values were not calculated.

The additive energy budget model assumes the independence of the different components. The swimming respirometer experiments were designed to test this assumption and they confirmed that the digestion and swimming costs were independent. In spite of the high swimming speed, the costs of digestion increased in relation to the food ration. Active metabolic rates remained fairly low in our swimming experiments and were slightly higher in the feeding respirometer experiments. However, the swimming tests showed that whitefish larvae managed to maintain their position in the current for no longer than 6-7 minutes, and thus they had to perform at their maximum capacity. The oxygen debt (the oxygen consumption was recorded only for 7 minutes) may partly explain the relatively low activity costs observed and total metabolic costs were possibly slightly underestimated.

The duration of the swimming experiments was short and the effects of activity on the growth rates and growth efficiencies could not be estimated. Simultaneous long-term swimming and feeding experiments would be needed to clarify the long-term relation between activity and digestion costs. The effect of the unfavourable feeding and growing conditions as well as individual growth strategies and growth efficiencies of larval fish also need further research. Conover and Schultz (1997) proposed a possible trade-off between predator avoidance probability and growth investment; then a larva with a more conservative energetic investment in growth would have a higher capacity to allocate energy to predator evasion.

Several bioenergetics models constructed for juvenile and larval fish (e.g. Dabrowski et al. 1989; Post 1990; Karjalainen et al. 1997; Huuskonen et al. 1998) are based on the additive model originally developed for adult fish. Our results suggest that the theoretical base of modelling larval coregonid fish energetics under favourable feeding conditions is firm. Huuskonen et al. (1998) evaluated the validity of a bioenergetics model constructed for whitefish using the same feeding respirometer data as in this study. They found that the model was able to predict the food consumption of whitefish reasonably well under experimental conditions. The consumption rates of fast-growing larvae were also estimated satisfactorily. Furthermore, our results show that the scope for activity of larval whitefish is tight and the effects of changes in this component (e.g. due to predator avoidance or wind-induced currents) on the food consumption estimates seem to be negligible. In general, Kooijman (2001) argued that “costs of feeding and movements that are part of the routine repertoire are usually insignificant with respect to the total energy budget”. On the contrary, the unknown activity costs are considered to be one of the weakest points in the current bioenergetics models of adult fish (Ney 1993).

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Temperature-induced changes in viscosity and its effects on swimming speed in larval haddock

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Key words: *temperature, viscosity, larval fish, swimming*

Abstract

The effects of temperature-induced changes in the kinematic viscosity of water on the swimming speed of Atlantic haddock (*Melanogrammus aeglefinus*) larvae was examined at four different ages and sizes, from 5 to 25 days post hatch (dph) (5 mm to 8 mm total length). In order to separately observe the effects of temperature on the physiology of fish larvae from its effects on the physics of water flow, the viscosity of the water was artificially increased by adding methyl cellulose to the seawater. Experiments were conducted once a week as larvae grew, in temperature controlled conditions and swimming speed was determined using a high-speed digital video camera mounted on a dissecting microscope. For small larvae, <6 mm in length, swimming in the natural viscosity range of the ocean ($\leq 1.82 \times 10^{-6} \text{ m}^2\text{s}^{-1}$), both temperature and viscosity explained 40% of the variance in speed, but physical effects, relative to physiological effects decreased with increasing fish size. In larger larvae, >7 mm in length, both viscosity and temperature explained 30% of the variation in swimming speed, but, viscosity was not a significant factor and physiological effects were much greater than physical effects on swimming speed. Thus, for the smallest haddock larvae, changes in kinematic viscosity had the greatest influence on swimming speed. For larger larvae, both physiological and physical factors were important and together accounted for 41% of the variation in swimming speed, suggesting that physical factors become less important as fish larvae grow and swim faster.

Introduction

Temperature has a profound effect on physiology through its effects on enzyme reaction rates (Hochachka and Somero 2001). This is particularly true because most fish are ectotherms and depend on the environment for temperature regulation. For fish larvae, which are very small in size, temperature can also influence the physics of the water in which they swim. This is because fish larvae inhabit very different hydrodynamic environments compared to their juvenile and adult counterparts. The larval fish environment is often characterized as a transitional or intermediate Reynolds number regime ($(Re_L; 10 < Re_L < 1000)$), which is affected by both viscous and inertial forces and where Re_L is the characteristic length or total length of the fish (Webb and Weihs 1996). In this intermediate hydrodynamic regime temperature-induced changes in the physics of water flow have a large influence on the swimming performances of microscopic organisms (Webb and Wiehs 1986; Podolsky and Emler 1993). Larger fish larvae, juveniles and adults, inhabit higher Re_L regimes ($1000 < Re_L < 10,000$) which are thought to be entirely dominated by inertial forces and where changes in the viscosity of water have little effect on swimming performances. Thus, as larvae grow larger and swim faster, temperature-induced changes in viscosity become less and physiological effects become more important (Fuiman and Batty 1997).

A change in temperature has a higher impact on the kinematic viscosity of water than on its density or dynamic viscosity. The kinematic viscosity can double over a change of 30°C , which is approximately the temperature difference between the poles and tropical oceans (Dorsey 1968).

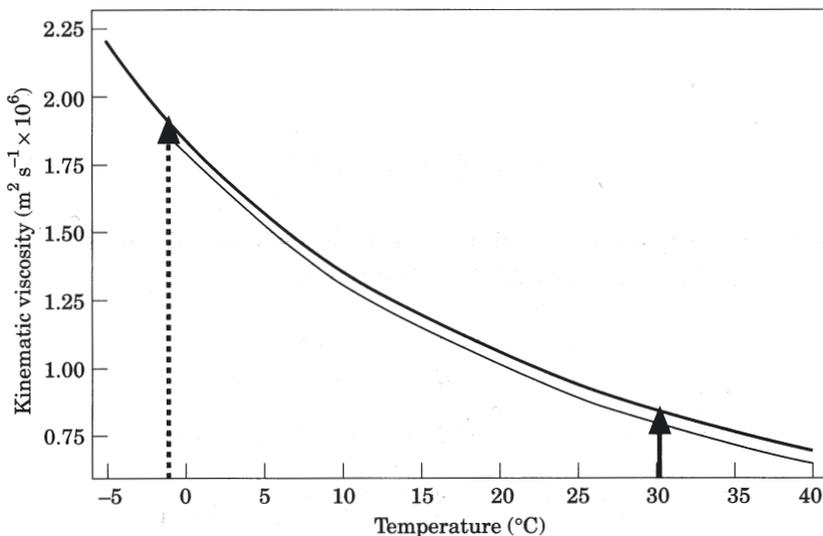


Figure 1. Relationship between kinematic viscosity (m^2s^{-1}) and temperature ($^\circ\text{C}$) as calculated from equations in Sengers and Watson (1986). The upper curve represents changes in kinematic viscosity with temperature in seawater, while the lower curve represents changes in freshwater (after Jumars et al. 1993). Arrow (dashed) indicates the viscosity at -2.0°C and arrow (solid) indicates the viscosity at 30°C .

For larvae, the size of the physical effects of density and viscosity depends on the natural range of temperatures that larvae may experience in the ocean. For the cold-water fish species, Atlantic haddock (*Melanogrammus aeglefinus*), spawning occurs in the boreal winter (February and March) and larvae develop in water that can be as low at 0°C in mid-winter and rise to 10°C in spring (Scott and Scott 1988). At these cold temperatures (0°-10°C), changes in water viscosity are greater per degree temperature change than at higher temperatures (after Jumars et al. 1993; Fig.1). Consequently, for larval haddock, temperature-induced changes in both the physiology and physics are likely to play a large part in influencing its swimming performance.

Two decades of research on the effects of temperature on small aquatic invertebrate larvae have shown that swimming performance are significantly affected by small-scale changes of viscosity in the water column (La Barbera 1984; Strickler 1984; Emler and Strathman 1985; Power 1989; Denny 1990; Podolsky and Emler 1993). Within the last decade a few studies have examined the effect of temperature on the physiology of fish larvae and on the flow conditions in which they swim (Kauffman and Wieser 1992; Yin et al. 1995; Fuiman and Batty 1997; Wieser and Kauffman 1998, Batty and Fuiman 2003). Two of these studies separated the effects of temperature on the physiology from its effects on the physics of water flow by artificially increasing water viscosity through the addition of methyl cellulose, an osmotically inert substance, to sea water (Yin et al. 1995; Fuiman and Batty 1997). These studies showed that when viscosity was increased independently of temperature, 40% of the variation in voluntary swimming performance in small larval herring (*Clupea harengus*) (9.6 mm TL) was explained by changes in viscosity while temperature did not have a significant effect (Fuiman and Batty 1997). In contrast, in larger larvae (18.2 mm), both physiological and physical effects were important and together could account for 48 % of the variation in swimming speed, suggesting that physical factors became less important as larvae grew and swam faster.

In the present study, the effects of temperatures on physical and physiological factors were separated by observing voluntary swimming in larval haddock in sea water solutions containing methyl cellulose. In this study swimming speed was measured in larvae at four different ages and sizes and at colder temperatures than previously used. As a result, this study was able to determine how all three factors – larval size and temperature-induced changes in physical and physiological factors – affect the swimming performances in a larval cold-water fish species. In addition, data from this study supports previous work that emphasizes the importance of considering the effects of temperature on physical as well as physiological factors when studying swimming in small micro- and macroscopic animals.

Methods and Materials

Fish. Fertilized haddock eggs were obtained from St. Andrews Biological Station, St. Andrews, New Brunswick, Canada. Eggs were incubated, hatched and raised through the larval stages at 8°C at the University of Maine's Aquaculture Research Center at Orono, Maine. All eggs and larvae were cultured in artificial sea water (Forty Fathoms Crystal Sea ()) in flow-through 20 l plastic tanks until they reached sizes of 15-20 mm (60-70 days post-hatching (dph)), when they were moved to larger

40 l tanks. Larvae were fed rotifers (*Branchionus* sp.) and *Artemia* sp. ad libitum and larger larvae (>15 mm) and juveniles were fed to satiation on Biokyowa –700 and –1000 pellet food 3-4 times daily.

Experiments were performed on four age/size groups which were approximately 1 week apart in age; 1) 5 dph, 2) 12 dph, 3) 19 dph and 4) 25 dph. At 5 dph, the haddock larva reached the end of its yolk-sac stage and for the next 20 days developed from a simple larval body shape with a continuous median finfold to a late larval stage which had begun to show differentiation of median fins.

Experimental protocol. Routine (voluntary, spontaneous) swimming behavior was observed in seawater at 3 temperatures (0°, 5°, 10°C) that spanned the natural temperature range of haddock larvae in the ocean. The kinematic viscosity was adjusted by dissolving methyl cellulose (Sigma Chemical Company, M0262, mol. Wt. 41,000) in seawater. A stock solution (1.4 gl⁻¹ methyl cellulose in seawater) was then diluted to give a series of different viscosities at each of the 3 test temperatures (see Table 1). The kinematic viscosity (ν) (dynamic viscosity/density) at the test temperatures were measured using a glass capillary viscometer (Cole-Palmer A-98934).

The methyl cellulose solutions used in all the experiments were all less than 0.014 mM and had a minimum density of 0.02 % less than sea water. As a result there was likely to have been little increased osmoregulatory stress on the fish larvae.

All experiments were performed in a small petri dish (diameter = 4 cm, Volume=30 ml) placed in a temperature-controlled slide \pm 0.1°C, on an Olympus dissecting microscope (SZH10) stage. On the morning of each experiment, 200 ml of each test solution was prepared and brought to experimental temperature. Ten larvae were removed from their rearing tanks and individually pipetted into the test solution for 3 hours to recover from handling and to acclimate to the change in temperature and viscosity. Temperature was monitored continuously using a digital thermometer and did not vary more than \pm 0.1°C. For each temperature-viscosity treatment routine swimming was recorded for 5 larvae individually (that is five bouts of routine swimming were recorded for each temperature-viscosity treatment). Although every attempt was made to select different larvae, there was no way to differentiate among larvae and pseudoreplication was a possibility.

Filming and Analysis. Routine swimming of haddock larvae was recorded at 250 frames s⁻¹ using a digital high speed video recording system (Motionscope 1000 S) which fed directly into a linked personal computer equipped with software to analyze the kinematic variables. Larvae in the dish

Table 1. Experimental conditions of temperature (°C) and kinematic viscosity (ν) ($\times 10^6$ m²s⁻¹) for larval haddock swimming experiments. Tabulated values are calculated kinematic viscosities for each solution (after Sengers and Watson 1986; Jumars et al. 1993).

| Methyl Cellulose Concentration (gl ⁻¹) | Temperature (°C) | | |
|--|------------------|------|------|
| | 0 | 5 | 10 |
| 0 | 1.82 | 1.55 | 1.32 |
| 0.31 | 2.20 | 1.82 | 1.55 |
| 0.57 | n.a. | 2.20 | 1.82 |

were illuminated from below and above using fiber-optics. This provided adequate light for the video camera. Subsequent frame by frame replay on a linked personal computer allowed 3 points to be digitized in each frame: 1) tip of snout, 2) point on top of the head directly between the eyes (center of gravity; see Hunt von Herbing and Boutilier 1996) and 3) the tip of the tail. Only larvae that a) swam in a straight path at an apparently constant speed (judged by eye), b) executed at least 2 full tail-beats in view, and c) did not touch the surface of the solution, were included in the analysis.

Results

Results are presented for each of the four age/size classes of haddock larvae tested in order to clearly illustrate how temperature and viscosity affected swimming speeds. We assume that our observations of larval swimming in seawater at the different temperatures resulted in values for swimming speeds that are likely to occur in nature and, therefore, provide a reasonable basis for comparing swimming behavior when viscosity has been manipulated independently.

The mean total length (TL) for each larval haddock age/size class was obtained from 10 larvae per size group and were as follows: for larvae 5 days post-hatch (dph), TL=5.09 \pm 0.43 mm, for 12 dph larvae, TL= 6.07 \pm 0.61 mm, for 19 dph larvae, TL= 6.81 \pm 0.47 mm and for 25 dph larvae, TL = 8.06 \pm 0.80 mm.

Temperature effects on larval swimming speed in sea water. A three-factor ANOVA was used to determine the effects of the independent variables; temperature, viscosity and larval fish size, on the dependent variable (swimming speed). In this analysis all factors (age/size, temperature and viscosity) were fixed, but the analysis was unbalanced because there were some temperature-viscosity combinations that were not tested. For each age/size group, there were significant differences in swimming speed among temperatures (Table 2; Fig. 2a). The change from both temperature and viscosity affecting swimming speed in small larvae to viscosity having a negligible effect for large larvae appears in the interaction term of larval size x viscosity (Table 2). A significant interaction of the three factors; size x viscosity x temperature, means that in order to evaluate the effect of tem-

Table 2. Results for a three-factor ANOVA in an unbalanced design, of the effects of three factors: 1) fish size class, 2) viscosity, and 3) temperature on swimming speed in larval Atlantic haddock (Melanogrammus aeglefinus). The experiment was unbalanced, as not all combinations of temperature and viscosity were possible. Df=degrees of freedom, F= F-ratio, P= probability where $\alpha=0.05$

| Variable | Effect | df | F | P |
|---------------------|---------|----|--------|-------|
| SIZE CLASS | 0.13993 | 3 | 1.290 | 0.277 |
| VIS | 0.06123 | 2 | 1.355 | 0.259 |
| TEMP | 0.08893 | 2 | 12.640 | 0.000 |
| VIS* SIZE CLASS | . | 7 | 2.336 | 0.023 |
| TEMP*SIZE CLASS | 0.04241 | 5 | 1.663 | 0.141 |
| VIS*TEMP | 0.08181 | 3 | 3.426 | 0.017 |
| TEMP*VIS*SIZE CLASS | . | 9 | 3.604 | 0.000 |

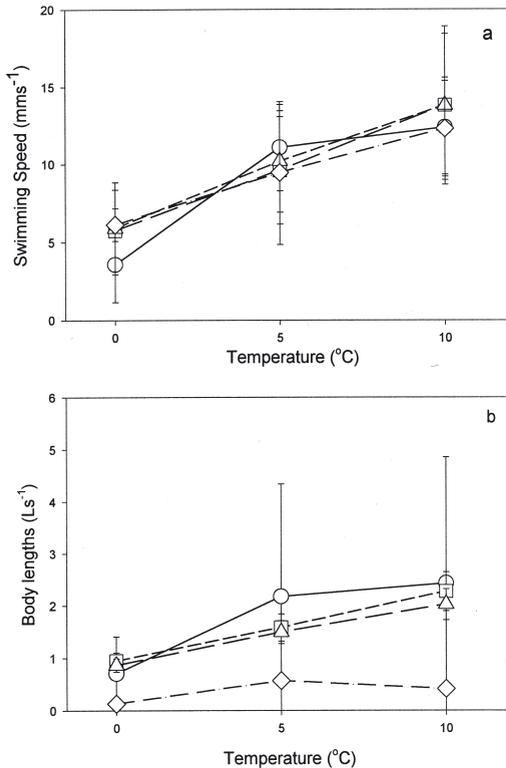


Figure 2. (a) Changes in mean absolute swimming speed (mms^{-1}) and, (b) mean swimming speed in body length per second (Ls^{-1}) (b) with temperature, at four ages/sizes in Atlantic haddock larvae in seawater. Symbols represent means (± 1 S.D.) for 5 fish swimming in seawater at three different temperatures (0 $^{\circ}$, 5 $^{\circ}$ and 10 $^{\circ}\text{C}$). (o=5 days post hatch(dph), 5.0mm TL (dph), \square = 12 dph, 6.0mm TL, Δ = 19 dph, 6.8mm TL; \diamond = 25 dph, 8mm TL)

change in viscosity. At 0 $^{\circ}\text{C}$, mean swimming speed did not decrease as viscosity increased to $2.2 \times 10^{-6} \text{ m}^2\text{s}^{-1}$, and was not different from sea water at 0 $^{\circ}\text{C}$ (kinematic viscosity= $1.82 \times 10^{-6} \text{ m}^2\text{s}^{-1}$).

There was a strong relationship between Re_L and kinematic viscosity (ν) for the youngest/smallest larvae ($\text{Re}_L = 101.23 - 43.39\nu$, $r^2 = 0.53$, $P < 0.0001$). For all conditions, most small larvae swam at Re_L between 15 and 50, with a median of 25 and extremes of 6 and 73. Larvae in the coldest, most viscous environments experienced a mean Re_L of 8 and with extremes of 4-18.

For the above results, only values from sequences of constantly swimming larvae were used in the analysis. However, several instances of burst swimming were also recorded and these events had significantly higher speeds than for routine or constant swimming (Paired t-test, $t = 3.54$,

temperature on larval swimming speed, viscosity and larval size must be specified, as all factors were interdependent (Table 2).

A linear relationship with temperature (T) provided a significant ($P < 0.0001$) fit for each age/size size group; for 5 dph ($U = 4.56 + 0.88T$; $r^2 = 0.59$); for 12 dph, ($U = 5.68 + 0.81T$; $r^2 = 0.45$), for 19 dph, ($U = 6.00 + 0.79T$; $r^2 = 0.42$) and for 25 dph, ($U = 6.20 + 0.62T$; $r^2 = 0.37$).

Relative mean swimming speeds reported as body lengths per second (Ls^{-1}) of the smallest age/size class of larvae (5 dph), was 71% higher at 10 $^{\circ}\text{C}$ than at 0 $^{\circ}\text{C}$, decreasing from 2.4 Ls^{-1} at 10 $^{\circ}\text{C}$ to 0.7 Ls^{-1} at 0 $^{\circ}\text{C}$ (Fig. 2b). In contrast, for older/larger larvae relative mean swimming speed increased to a lesser extent and values are as follows; for 12 dph, speed decreased 58% from 2.3 Ls^{-1} to 0.9 Ls^{-1} , for 19 dph, speed decreased 57% from 2.1 Ls^{-1} to 0.8 Ls^{-1} and for 25 dph, speed decreased 50% from 0.5 Ls^{-1} to 0.1 Ls^{-1} (Fig. 2b).

Viscosity effects on larval swimming speed in sea water. Young/small larvae. The effect of kinematic viscosity, independent of temperature, on the swimming speed of the youngest/smallest haddock larvae (5 dph; 5.09 mm) was very clear and swimming speed decreased as viscosity increased (Table 2; Fig. 3a). For the temperatures of 10 $^{\circ}$ and 5 $^{\circ}\text{C}$, mean swimming speeds declined over the viscosity range of $1.32 \times 10^{-6} \text{ m}^2\text{s}^{-1}$ to $1.82 \times 10^{-6} \text{ m}^2\text{s}^{-1}$ at similar rates of about 1.0-1.2 mms^{-1} for every $0.1 \times 10^{-6} \text{ m}^2\text{s}^{-1}$

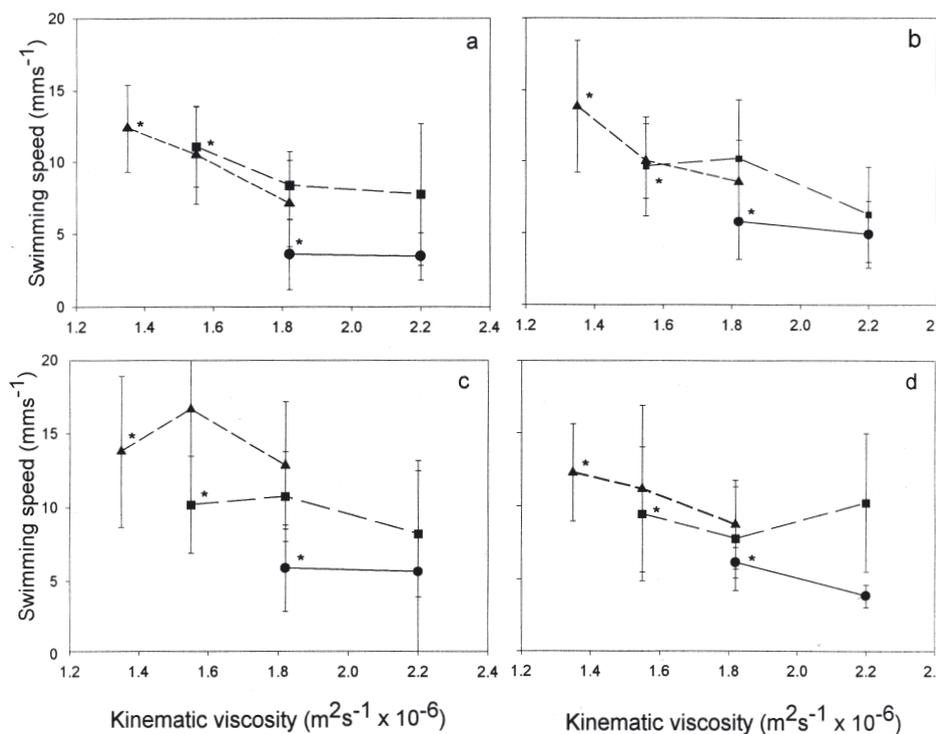


Figure 3. Changes in mean swimming speed (mms^{-1}) with viscosity at three temperatures in haddock larvae at; (a) 5 days post hatch (dph), 5.0mm TL (b) 12 dph, 6.0mm TL, (c) 19 dph, 6.8mm TL and (d) 25 dph, 8mm TL. Solid lines connect means for a common temperature. Symbols represent means (± 1 S.D.) for 5 fish swimming in seawater (symbols with an asterisk) and of methyl cellulose solutions (filled symbols with no asterisk). (triangles= 10°C , squares = 5°C , and circles = 0°C).

$P < 0.05$). For example: at 0°C and a viscosity of $2.22 \times 10^{-6} \text{ m}^2\text{s}^{-1}$, mean burst speed was $46.95 \pm 29.79 \text{ mms}^{-1}$ compared to a mean routine speed of $3.44 \pm 1.63 \text{ mms}^{-1}$ in the same temperature and viscosity conditions. Similarly at 5°C and $1.55 \times 10^{-6} \text{ m}^2\text{s}^{-1}$, mean burst swimming speed was $54.94 \pm 17.23 \text{ mms}^{-1}$ versus a mean routine speed of $11.66 \pm 2.77 \text{ mms}^{-1}$, and at 10°C and $1.82 \times 10^{-6} \text{ m}^2\text{s}^{-1}$, mean burst swimming speed was $40.33 \pm 20.99 \text{ mms}^{-1}$ versus a mean routine speed of $7.12 \pm 2.99 \text{ mms}^{-1}$. In general, burst speeds were 5 to 13 times faster than mean routine swimming speeds with the greatest difference occurring at the lowest temperature.

Older/larger larvae. Among the three older/larger size classes of haddock larvae (12-25 dph; 6-8 mm) increases in kinematic viscosity independent of temperature resulted in a decrease in mean swimming speed for 12 dph larvae only, and only at the lowest kinematic viscosities of $1.32 \times 10^{-6} \text{ m}^2\text{s}^{-1}$ and $1.55 \times 10^{-6} \text{ m}^2\text{s}^{-1}$ (Fig. 3b). Twelve day old larvae at 10°C experienced a 28% decrease in mean swimming speed as kinematic viscosity increased from $1.32 \times 10^{-6} \text{ m}^2\text{s}^{-1}$ to $1.55 \times 10^{-6} \text{ m}^2\text{s}^{-1}$, yet

speed remained relatively unchanged as the viscosity increased to $1.82 \times 10^{-6} \text{ m}^2\text{s}^{-1}$. Swimming speeds at the lower temperatures of 5° and 0°C did not appear to change with increases in viscosity. For the oldest/largest larvae (19 & 25 dph), swimming speed at the same temperature did not decrease with increasing viscosity even at the higher viscosity ranges (Figs. 3c-d).

Across all conditions, large larvae swam mostly at Re_L between 40 and 100 (interquartile range) with a median of 30-40. For 12 dph larvae, which experienced a decline in swimming speeds at 10°C as viscosity increased from $1.32 \times 10^{-6} \text{ m}^2\text{s}^{-1}$ to $1.55 \times 10^{-6} \text{ m}^2\text{s}^{-1}$, mean Re_L ranged from 62 to 39. In all fluids with the highest kinematic viscosities ($2.22 \times 10^{-6} \text{ m}^2\text{s}^{-1}$), mean Re_L was low and ranged between 15 and 28.

As with the small larvae, some burst swimming events occurred for larger larvae and speeds were higher than mean swimming speeds. For example, for 19 dph larvae at 5°C and a kinematic viscosity of $1.82 \times 10^{-6} \text{ m}^2\text{s}^{-1}$, mean burst speed was $23.87 \pm 7.59 \text{ mms}^{-1}$ versus a mean routine speed of $8.99 \pm 3.03 \text{ mms}^{-1}$ and for 25 dph at 10°C and $1.32 \times 10^{-6} \text{ m}^2\text{s}^{-1}$ mean burst was $27.25 \pm 4.26 \text{ mms}^{-1}$ versus a mean routine speed at $12.29 \pm 3.31 \text{ mms}^{-1}$.

Separating physical and physiological effects of temperature on swimming speed.

Young/small larvae. For all viscosity and temperature conditions, a multiple regression, of swimming speed on kinematic viscosity and temperature accounted for 45% of the total variance ($r^2=0.45$). Both viscosity and temperature were significant factors ($P<0.0001$) and values from the regression coefficients (-0.33 and 0.42) respectively, indicated that these factors were both important to larval swimming speed. A simple linear regression on all the data showed that kinematic viscosity alone could account for 34% of the variation in speed while temperature accounts for 39% of the variation in speed ($P<0.0001$). Within the natural viscosity range ($\leq 1.82 \times 10^{-6} \text{ mm}^2\text{s}^{-1}$), kinematic viscosity and temperature together accounted for 50% of the variation in speed.

Older/larger larvae. For all three groups of larvae, mean swimming speed decreased with decreasing temperatures (Figs. 3b-d). However, among the three groups, mean swimming speed of 12 dph larvae differed in response to changing temperature and viscosity conditions compared to 19 and 25 dph larvae. For 12 dph larvae, at 10°C , mean swimming speed decreased with decreasing temperature and increasing viscosity, as viscosity increased to $1.55 \times 10^{-6} \text{ m}^2\text{s}^{-1}$ (viscosity equivalent to seawater at 6°C). At 5° and 0°C , there was no measurable change in speed as viscosity increased to $2.2 \times 10^{-6} \text{ mm}^2\text{s}^{-1}$. Thus, the estimated 30% decrease in speed between 10° and 6°C can be attributed to both a physical and physiological effect of increasing temperature and increasing viscosity. However, little of the 40% decrease in speed between 5° and 0°C could be attributed to the physical effect of increasing viscosity. For both 19 and 25 dph larvae, little of the estimated 50-57% decrease in swimming speed could be attributable to increasing physical effects due to increasing viscosity.

A multiple regression of swimming speed on temperature and kinematic viscosity for 12 day old larvae showed that both temperature and kinematic viscosity accounted for 40% of the total variation of swimming speed ($P<0.0001$). The regression coefficients for kinematic viscosity and temperature were -0.40 and 0.30, respectively.

A multiple regression of swimming speed on temperature and kinematic viscosity for 19 and 25 day old larvae considered together, $r^2=0.41$ and $r^2=0.27$; $P<0.001$ and could account for 41% and 27% of the speed respectively. For 19 dph larvae, considered alone, the regression coefficients were -0.11 and 0.57 , but viscosity was not a significant factor ($P=0.17$). Similarly for 25 dph larvae, analyzed alone, physical effects did not have a significant effect on swimming speed ($P=0.07$) and regression coefficients were -0.15 and 0.42 . A multiple regression applied only within the natural viscosity range ($\leq 1.82 \times 10^{-6} \text{ m}^2\text{s}^{-1}$) showed that both viscosity and temperature explained the variation in swimming speed for larvae greater than 6 mm in length ($P<0.001$), but the magnitude of the effect of physical factors compared to physiological factors on speed decreased with increasing age and size. For example, for 12 dph larvae, 6 mm haddock larvae, 33% of the variation of speed was explained by changes in both viscosity and temperature ($r^2=0.33$), and regression coefficients (-0.38 and 0.26) indicated that physical effects were still important and viscosity alone explained 30% of the variation in swimming speed ($P=0.01$). For 19 dph larvae, approximately 7 mm, a multiple regression within the natural viscosity range ($\leq 1.82 \times 10^{-6} \text{ m}^2\text{s}^{-1}$) showed that although both viscosity and temperature explained 30% of the variation in swimming speed, viscosity was not a significant factor ($P=0.79$).

Discussion

Changes in water viscosity independent of temperature had a significant impact on the routine/voluntary swimming performance in small haddock larvae. For the smallest and youngest haddock larvae, increases in kinematic viscosity resulted in a decrease in swimming speed, while effects of temperature were negligible in comparison. As larvae increased in size the effects of both temperature and water viscosity on swimming performance became equally important. At the largest larval fish sizes, temperature became the dominant factor in influencing swimming speed and changes in viscosity became negligible.

In a similar study to the present one, Fuiman and Batty (1997) found that routine swimming in herring larvae was strongly influenced by changes in viscosity and temperature. Small, 9.6 mm herring larvae were strongly affected by both viscosity and temperature, which explained 44% of the total variance in swimming speed. In small herring larvae, when viscosity was increased by the addition of methyl cellulose, independent of temperature, swimming speed decreased dramatically suggesting that at temperatures of 7°, 10° and 14°C, physical factors had more of an effect than physiological factors on swimming (Fuiman and Batty 1997). For large, 18.2 mm herring larvae, both temperature and viscosity affected swimming speed and together explained 44% of the variation of swimming speed (the same as for small larvae). However, between 7°-13°C, temperature was the dominant factor explaining 54% of the increase in swimming speed and the effects of viscosity were negligible.

Interestingly, small herring larvae (9.6 mm) in the study by Fuiman and Batty (1997) were almost double the size of small haddock larvae (5 mm) in the present study, yet viscosity explained most of the variation in swimming speed for both species (43% for herring and 45% for haddock). Viscosity was the most important factor for swimming in both species at these small sizes, however,

effects of temperature were not significant for herring, while they were for haddock. Results for large herring larvae (18.2 mm) also agreed with the results for larger haddock larvae (7-8 mm), which found that temperature explained most of the variation in swimming speed (44% in herring and 30-40% in haddock). Temperature was the most important factor for both species at larger larval sizes; however, effects of viscosity were significant for herring while they were not for haddock.

For large herring larvae, viscosity effects were still present at Re_L values between 300 to 450 (Fuiman and Batty 1997). These Re_L values are much higher than was expected for the transitional or intermediate hydrodynamic regime, which is characterized by both viscous and inertial forces and extends from Re_L values of 20 to 200 (Weihs 1980; Webb and Weihs 1996). Larvae at $Re_L < 200$ were thought to be in a viscous regime, while those at $Re_L > 200$ were in an inertial regime. Fuiman and Batty (1997) found that for herring larvae at 7°- 14°C, viscous factors still play a role in influencing swimming performance at Re_L values between 300-400. This was also supported by studies of burst swimming in guppies (22 mm body length) in which speeds were reduced with increasing viscosity at constant temperature (Johnson et al. 1998). These results suggest that temperature-induced viscosity effects continue to be important for both these species at the test temperatures, up to around an Re of 5000. This led Fuiman and Batty (2002) to suggest that although the extent that viscosity affects burst swimming performance decreases with increasing fish size, viscous effects do not entirely disappear until much higher values of Re_L 8800 (guppies; Johnson et al. 1998).

In the present study, haddock larvae of all sizes swam at $Re_L < 200$. Over the natural viscosity range for haddock larvae ($\leq 1.82 \times 10^{-6} \text{ m}^2\text{s}^{-1}$) up to a mean size of 6.0 mm, physical factors effects were still important and viscosity alone could explain 30% of the variation in swimming speed. For haddock larvae greater than 6 mm in size and swimming at $Re_L < 100$, at temperatures from 0° to 10°C, both temperature and viscosity explained 30% of the variation in swimming speed, but viscosity was not a significant factor for larvae greater than ~7 mm. Thus for haddock larvae, changes in temperature becomes more important to swimming performance at smaller body sizes than in herring larvae. Differences among species may be related to differences in the range of temperatures used in the experiments and/or due to species-specific size related differences in the development of locomotory structures and swimming capacities.

The minimum temperatures used in the present study were generally colder (0°, 5° and 10°) than those used in the herring study (6-7°, 9-10°, and 13-14°). At colder temperatures a small change in temperature results in a larger change in kinematic viscosity compared to warmer temperatures (Fig.1).

For example, a decrease in temperature from 5° to 0°C results in a change in kinematic viscosity of $0.44 \times 10^{-6} \text{ m}^2\text{s}^{-1}$ per degree change in temperature, while a decrease in temperature from 15° to 10°C results in an increase in viscosity of $0.3 \times 10^{-6} \text{ m}^2\text{s}^{-1}$ per degree change in temperature. Previous studies separating the effects of temperature on the physics and physiology of swimming have been conducted at higher temperatures (e.g. Linley 1986 (6°-26°C); Podolsky and Emlet (12°-22°C); Kauffman and Wieser (15°-20°C); Fuiman and Batty 1997 (7°-14°C)) compared to the present study (0°-10°C). Yet, the physical relationship between temperature and kinematic viscosity has not been considered when comparing results among these studies in order to develop a general conclusion as to the effects of temperature change on swimming performance in rapidly developing and growing fishes.

In short, for haddock larvae that are 5-7 mm in length, physical factors play a major role in determining the swimming performances and temperature plays a minor role, particularly at the smallest sizes of 5 mm and over their natural temperature (0°-10°C) and viscosity ranges ($1.32 \times 10^{-6} \text{ m}^2\text{s}^{-1} - 1.82 \times 10^{-6} \text{ m}^2\text{s}^{-1}$). At 7 mm, both temperature and viscosity are equally important in explaining most of the variation in swimming speed, at least under natural conditions. At larval sizes greater than 7 mm, changes in viscosity or physical factors do not seem to affect swimming speed between temperatures of 0°-10°C, and temperature or physiological factors explained 30% of the variation in swimming performance. Thus, physiological factors begin to have a much greater effect on swimming speed than physical effects at much smaller sizes in haddock larvae than in herring larvae at similar temperatures.

At the highest viscosity ranges ($>1.82 \times 10^{-6} \text{ m}^2\text{s}^{-1}$) which are outside the natural viscosities encountered by either haddock or herring larvae, small larvae were limited by the ability to maneuver through a very viscous medium. Small larvae may have reached a terminal speed, which did not decrease even with increasing viscosity, but increased slightly at higher temperatures. That swimming speed did not change as a function of viscosity at higher viscosity ranges may be partly due to the relatively primitive state of development of locomotor structures and muscles in larvae (Galloway et al. 1998). Both these species in their early larval stages lack fully developed fins and swimming is more metabolically costly in larvae than for juveniles and adult fish (Dabrowski 1986, Kauffman 1990). In environments in which viscosity is the same and temperatures change, higher swimming speed may be due to a lower metabolic cost of transport in warmer water. Differences in the metabolic cost of transport of larval fish at different temperatures is supported by studies for three different species; cod (*Gadus morhua*), herring (*Clupea harengus*) and Danube bleak (*Chalcalburnus chalcoides*) (Hunt von Herbing and Boutilier 1996; Yin et al. 1995; Kauffman and Wieser 1998). For cod larvae, metabolic cost of transport at 5°C was 1.4 times that at 10°C, for herring larvae metabolic cost at 7°C was 1.5 times that at 13°C and for Danube bleak larvae metabolic cost at 15°C was 1.1 times that of 5°C. Thus, the relative change in magnitude in metabolic costs of swimming at higher temperatures (15°-20°C) versus that at lower temperatures (5°-10°C) is smaller suggesting that viscosity had less of an effect on swimming performance at higher temperatures than at lower temperatures. These studies provide some interesting insights into the interactions of temperature on physical and physiological factors of swimming in larval fishes (see also Hunt von Herbing 2002). However, in order to understand how temperature affects different aspects of the swimming performances in the transitional hydrodynamic regime that most fish larvae occupy, further studies must include quantification of swimming kinematics at different temperatures, viscosities and at different fish sizes. This will determine the relative importance of physical factors and physiological factors on swimming performance as larvae develop locomotory structures and change their swimming patterns and mechanics throughout ontogeny and will be the subject of a forthcoming paper.

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Effects of temperature and parental background on the embryonic survival and metabolic rate of newly hatched Arctic charr

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Abstract

Arctic charr (*Salvelinus alpinus* (L.)) is an endangered species in Lake Saimaa, south-eastern Finland. As a part of a stock enhancement programme, several year classes of cultivated brood stocks have been founded. Genetic diversity of Lake Saimaa Arctic charr has recently been studied and it has proved to be low, probably due to population decrease and subsequent inbreeding. To study possible interactions between the genotypes and environment during early ontogenetic development, we created family groups by crossing randomly selected single parents from cultivated brood fish. The eggs of these Arctic charr families were incubated at 2 °C and 7 °C, and embryonic survival was determined at the eyed stage. After hatching, oxygen consumption of alevins was measured at the incubation temperatures. Temperature was found to have effects on both embryonic survival and standard metabolic rate of Arctic charr while parental background affected only survival. Variability in the embryonic survival could be attributed to the female at both temperatures whereas the male only had an effect at the lower temperature. Low incubation temperature was advantageous for Arctic charr due to higher utilization of yolk reserves and lower mortality which indicates adaptation of Arctic charr to cold water temperatures.

Introduction

Arctic charr (*Salvelinus alpinus* (L.)) is an endangered species in Lake Saimaa, south-eastern Finland. Genetic diversity of Lake Saimaa Arctic charr has proved to be low (Primmer et al. 1999), probably due to population decrease and subsequent inbreeding. As a part of a stock enhancement programme, several year classes of cultivated brood stocks have been founded, but they have suffered from increased embryo and alevin mortality as well as disease susceptibility. Poor survival of Arctic charr is not restricted to the Lake Saimaa population alone, since de March (1995) reported high mortality during egg incubation also in Labrador and Norwegian strains.

The distribution of Arctic charr covers alpine and Arctic regions, and it can be considered the most cold adapted of all the salmonids (Johnson 1980). Lake Saimaa Arctic charr spawn during October–November, at the time when lakes begin to freeze in this area so that the egg incubation takes place under the ice where temperatures are well below 5 °C. Under experimental conditions, Lake Saimaa Arctic charr preferred cobbles to finer material as spawning substrate (J. Piironen, pers. obs.). Females dug a redd before laying their eggs and covered it by tail beats after fertilization. According to Pavlov et al. (1994), Arctic charr females in the Lakes Onega and Ladoga, Russia, did not bury their eggs after spawning but the eggs were freely spread among the rocks and gravel.

Organisms tend to be more stenothermal during early embryogenesis than in later developmental stages (Cossins and Bowler 1987). According to Gruber and Wieser (1983), embryos and fry of Arctic charr survive well at temperatures between 4 and 8 °C but obviously embryos are viable at lower and higher temperatures as well (e.g. de March 1995, Bebak et al. 2000). Increase in temperature results in accelerated development but it may also elevate the mortality and the percentage of abnormal embryos which are known to increase towards both thermal limits of a species (Cossins and Bowler 1987). De March (1995) observed greater hatching success of Arctic charr eggs at 3 °C than at 6 °C.

The aim of this study was to find out whether embryonic survival and metabolic rate of newly hatched Arctic charr are affected by parental background or temperature. The role of parentage in embryonic and larval survival has been studied in numerous fish species (reviewed by Kamler 1992), but information about parental effects on fish metabolism is scarce. Since metabolic rate represents a measure of an animal's physiological processes, combining information on survival and metabolism could give new insight into the factors behind poor survival of Lake Saimaa Arctic charr. If mortality is parentally affected, it may have serious consequences for stock conservation, since some families (genotypes) can be selected against thus affecting the genetic diversity of the brood fish. To study this, family groups of Arctic charr were created by crossing randomly selected parents from cultivated brood fish following the North Carolina Design II (Lynch and Walsh 1998).

Materials and Methods

Parental fish (mean length 68.3 cm; $sd \pm 5.2$ cm and mean weight 3.6 kg; $sd \pm 1.0$ kg) originated from 1991–1994 year-classes of cultivated brood fish (first hatchery generation) founded from wild spawners. A total of 15 blocks of randomly selected two female x two male factorial fertilizations, i.e. a total of 60 families, were carried out at the Saimaa Fisheries Research and Aquaculture

in south-eastern Finland during 15-26 November 2001. After the fertilization, the eggs were left under the flowing water overnight before removing the dead, unfertilized eggs. Then the eggs from each family were divided into eight groups of 80-90 eggs. Four groups of eggs (four replicates of 80-90 eggs) from each family were incubated at 2 ± 0.2 °C and 7 ± 0.1 °C.

The experimental design is known as a North Carolina II design and it allows the total phenotypic variance in the offspring traits to be partitioned into female and male effects and, in addition, observable variance components can be expressed in terms of hypothetical underlying causal factors i.e. female effects can be divided into maternal and genetic effects (Lynch and Walsh 1998). Due to practical limitations, however, only 12 families were used in oxygen consumption measurements and hence we did not include quantitative genetics in the statistical analysis. As a result, we were able to separate the effects of female and male and their interaction.

Three four-family blocks of Arctic charr randomly selected from the experimental blocks were fertilized on the 15th (block 1: females and males 1 and 2), 20th (block 2: 3 and 4) and 26th (block 3: 5 and 6) of November, 2001. The incubation took place in floating plastic cylinders with a net bottom (mesh size ca. 2 mm, bottom area ca. 78.5 cm²) kept in 1.1 m² circular rearing tanks (water depth ca. 30 cm). Water flow into the tanks during incubation was about 18 l min⁻¹. Dead eggs (i.e. those turning totally or partly opaque) were counted and removed from the incubators for the first time about one week after fertilization, and at about two week intervals thereafter. At the eyed stage of embryos (2-15 January, about 343-355 degree days at 7 °C, 6-20 March, about 222-224 degree days at 2 °C), the final embryonic survival was determined after 'chocking' the rest embryos by pouring them at least twice from incubators into a plastic cup. By this treatment the unfertilized eggs turned opaque and they could be removed before counting the remaining live embryos. The embryonic survival was then expressed as a percentage of live, eyed embryos from the total amount of eggs at the beginning of incubation.

After hatching (in February at 7 °C, in May at 2 °C), oxygen consumption of the alevins was measured at 2 ± 0.1 °C and 7 ± 0.1 °C by two automated intermittent-flow respirometers equipped with YSI 5750 polarographic oxygen sensors (Forstner 1983). The term alevin applies here to hatched, endogenously feeding fish (e.g. Kamler 1992). The number of fish in each family varied from six to ten (Table 1) and the measurements of the family blocks were made in the same order as the fertilizations. At 7 °C block 1 was measured on 83-92 days post fertilization (dpf), block 2 on 92-100 dpf, and block 3 on 99-107 dpf. Measurement periods at 2 °C were 176-182 dpf in block 1, 177-186 dpf in block 2, and 182-187 dpf in block 3.

Both respirometer systems included three parallel acrylic chambers (volumes 148-167 ml) and the flow rate was about 200 ml min⁻¹. The oxygen consumption in each chamber was recorded for 30 minutes every second hour and the average rate during this period was extrapolated to an hourly value. The signals from the polarographic oxygen sensor were fed on-line into the computer and integrated each minute. Microbial oxygen consumption in the respirometer was measured at the beginning and end of the experiments and it was subtracted from the total decline of oxygen. Each experiment lasted for 18-22 hours (overnight) and they took place in the dark. After the experiments the fish were anaesthetized with tricaine, weighed (fresh mass, FM) and measured for total length (TL). Length (l) and height (h) of yolk were also measured and yolk volume (V) was calculated by the equation for a prolate spheroid: $V = 0.5236 * l * h^2$.

Table 1. Size (FM = fresh mass, TL = total length), yolk volume (V) and number (n) of Arctic charr alevins used in oxygen consumption measurements. Values indicate mean \pm se. Within-family statistical differences between the sizes of newly-hatched alevins incubated at different temperatures are denoted as follows: ^{ns} $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (tested by Student's *t*-test).

| Family (♀x♂) | 7 °C | | | | 2 °C | | | |
|-----------------|------|-----------------|----------------|----------------------|------|-------------------------------|-------------------|----------------------|
| | n | FM (mg) | TL (mm) | V (mm ³) | n | FM (mg) | TL (mm) | V (mm ³) |
| 1 x 1 | 10 | 103.6 \pm 2.4 | 23.6 \pm 0.3 | 54.4 \pm 4.3 | 10 | 114.0 \pm 1.7** | 26.2 \pm 0.1*** | 35.7 \pm 2.9 |
| 1 x 2 | 10 | 100.5 \pm 2.7 | 23.9 \pm 0.3 | 48.3 \pm 5.0 | 10 | 114.9 \pm 1.7*** | 26.0 \pm 0.2*** | 36.0 \pm 2.6 |
| 2 x 1 | 10 | 111.7 \pm 5.3 | 25.0 \pm 0.4 | 57.6 \pm 5.5 | 10 | 133.0 \pm 3.5** | 28.0 \pm 0.2*** | 38.7 \pm 2.6 |
| 2 x 2 | 8 | 110.2 \pm 4.8 | 25.3 \pm 0.6 | 49.5 \pm 3.6 | 10 | 134.0 \pm 2.6*** | 28.5 \pm 0.1** | 35.3 \pm 1.9 |
| 3 x 3 | 9 | 128.0 \pm 7.0 | 26.0 \pm 0.3 | 67.2 \pm 8.0 | 9 | 154.0 \pm 6.9* | 28.9 \pm 0.3*** | 47.5 \pm 6.2 |
| 3 x 4 | 9 | 130.6 \pm 4.5 | 26.2 \pm 0.3 | 85.1 \pm 2.1 | 10 | 140.3 \pm 5.5 ^{ns} | 28.7 \pm 0.2*** | 41.5 \pm 5.9 |
| 4 x 3 | 10 | 126.9 \pm 3.1 | 27.3 \pm 0.3 | 60.2 \pm 4.7 | 10 | 146.8 \pm 2.6*** | 30.0 \pm 0.2*** | 33.6 \pm 2.2 |
| 4 x 4 | 10 | 127.1 \pm 3.2 | 27.5 \pm 0.5 | 64.1 \pm 7.4 | 9 | 144.2 \pm 2.2*** | 29.9 \pm 0.1*** | 28.0 \pm 2.6 |
| 5 x 5 | 10 | 121.1 \pm 3.3 | 27.4 \pm 0.3 | 43.8 \pm 2.7 | 9 | 148.5 \pm 0.9*** | 30.5 \pm 0.1*** | 14.6 \pm 1.4 |
| 5 x 6 | 9 | 121.5 \pm 3.4 | 27.3 \pm 0.2 | 43.6 \pm 3.4 | 9 | 145.2 \pm 1.2*** | 30.3 \pm 0.1*** | 15.6 \pm 0.9 |
| 6 x 5 | 6 | 131.3 \pm 1.9 | 27.6 \pm 0.4 | 50.3 \pm 1.8 | 10 | 142.4 \pm 0.9*** | 30.2 \pm 0.1** | 14.3 \pm 0.9 |
| 6 x 6 | 7 | 129.7 \pm 2.0 | 27.1 \pm 0.6 | 50.3 \pm 6.6 | 10 | 141.2 \pm 1.6*** | 30.1 \pm 0.2** | 14.5 \pm 1.7 |

Standard metabolic rate (SMR) represents a measure of the minimum rate of metabolism in the absence of muscular activity, food consumption and its subsequent processing (e.g. Jobling 1994) and here it was defined as the mean of the two lowest oxygen consumption values. Due to low flow rate and general activity pattern of Arctic charr alevins (quiescent in the absence of external stimuli), we consider our estimate of SMR valid although the activity of the fish was not directly recorded. On the basis of mean SMRs of each family at the two temperatures, family-specific Q_{10} values were calculated: $Q_{10} = (\text{SMR}_7/\text{SMR}_2)^{10/(7-2)}$ where SMR_7 and SMR_2 are the SMRs at 7° and 2 °C, respectively. The Q_{10} value indicates the increase of the metabolic rate resulting from a 10 °C increase in temperature (e.g. Cossins and Bowler 1987).

Parental and temperature effects on the survival and SMR were tested by random effects ANOVA. Alevin size and yolk volume were also analysed with degree days as a covariate. Pearson's correlation coefficients were calculated for pairwise comparisons between different variables.

Results

In all except one family, embryonic survival was higher at low incubation temperature (Fig. 1). Mean survival of all families was 60.6% at 2 °C while at 7 °C it was 46.0%. At both temperatures there was large variation in survival between the families. The female affected survival at both temperatures as well as in the combined data whereas the male had a significant effect only at 2 °C (Table 2). Interestingly, the female x male interaction was only statistically significant at 2 °C (Table 2).

Standard metabolic rate of alevins was higher at 7 °C than at 2 °C (Fig. 2). Parental background did not affect SMR at either temperatures (Table 2) and neither were SMR_2 and SMR_7 correlated

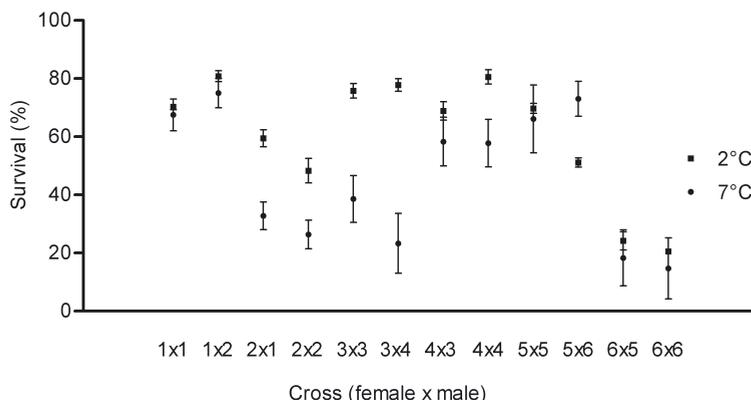


Figure 1. Embryonic survival (mean \pm se) of Arctic charr eggs incubated until the eyed stage at two different temperatures.

($r=0.049$, $p=0.880$); families with high or low SMR_2 did not have high or low SMR_7 , respectively. Hence, there were large differences in Q_{10} value between the families (Fig. 2). There was a statistically significant positive correlation between embryonic survival and SMR at 2 °C (but not at 7 °C) and a negative correlation between embryonic survival and Q_{10} (Fig. 3) i.e. the families with low SMR_2 and strong temperature response had suffered from high mortality during egg incubation.

In addition to effects on survival and SMR , temperature affected the size of newly hatched alevins. Fish hatched from eggs incubated at low temperature were markedly larger than their siblings at high temperature (Table 1). Female effects on alevin size and yolk volume were statistically significant in the combined data whereas the male did not have an effect (Table 2). Correlations between alevin weight, egg weight and female weight were all positive but, probably due to the small number, statistically non-significant. Alevin size did not have an effect on SMR although there were statistically significant differences between the families at both temperatures (one-way ANOVA, $p<0.001$). This is most likely due to isometric scaling of SMR during the early post-hatch stage (Giguère et al. 1988), and it justifies the use of mass-specific values of SMR .

Discussion

Variability in the embryonic survival of Arctic charr could be attributed to the female at both temperatures whereas the male effect was apparent only at the lower incubation temperature. Nagler et al. (2000) observed that in rainbow trout (*Oncorhynchus mykiss*) the female affected embryonic survival but the male did not. Paternal effects, however, have been reported in tench (*Tinca tinca*) and vendace (*Coregonus albula*), and they were revealed earlier in ontogenesis than the maternal effects (reviewed by Kamler 1992). Effects of egg quality on offspring survival have been studied extensively. Apart from the fact that larger eggs generally produce larger hatchlings with higher survival, other egg quality parameters can have important consequences for the offspring vitality

Table 2. ANOVA tables for family and temperature effects on embryonic survival, alevin size, yolk volume and standard metabolic rate of Arctic charr.

| Source | Df | MS | F | p |
|--|-----|----------|--------|--------|
| Embryonic survival | | | | |
| Incubation temperature | 1 | 0.702 | 33.497 | <0.001 |
| Female | 3 | 1.091 | 52.007 | <0.001 |
| Male | 3 | 0.014 | 0.688 | 0.562 |
| Female x Male | 3 | 0.041 | 1.980 | 0.125 |
| Temperature x Female | 3 | 0.140 | 6.676 | <0.001 |
| Temperature x Male | 3 | 0.027 | 1.299 | 0.281 |
| Error | 72 | 0.021 | | |
| Embryonic survival at 7 °C | | | | |
| Female | 3 | 0.943 | 24.249 | <0.001 |
| Male | 3 | 0.015 | 0.39 | 0.761 |
| Female x Male | 3 | 0.034 | 0.885 | 0.458 |
| Error | 36 | 0.039 | | |
| Embryonic survival at 2 °C | | | | |
| Female | 3 | 0.287 | 94.357 | <0.001 |
| Male | 3 | 0.027 | 8.704 | <0.001 |
| Female x Male | 3 | 0.031 | 10.033 | <0.001 |
| Error | 36 | 0.003 | | |
| Alevin total length | | | | |
| Temperature | 1 | 6.512 | 8.374 | 0.004 |
| Female | 3 | 29.682 | 38.168 | <0.001 |
| Male | 3 | 0.706 | 0.908 | 0.438 |
| Female x Male | 3 | 0.276 | 0.355 | 0.786 |
| Error | 200 | 0.778 | | |
| Alevin fresh mass | | | | |
| Temperature | 1 | 170.041 | 1.434 | 0.233 |
| Female | 3 | 1319.726 | 11.127 | <0.001 |
| Male | 3 | 85.961 | 0.725 | 0.538 |
| Female x Male | 3 | 31.369 | 0.264 | 0.851 |
| Error | 200 | 118.608 | | |
| Alevin yolk volume | | | | |
| Temperature | 1 | 3037.729 | 19.048 | <0.001 |
| Female | 3 | 1280.881 | 8.032 | <0.001 |
| Male | 3 | 158.465 | 0.994 | 0.397 |
| Female x Male | 3 | 84.607 | 0.531 | 0.662 |
| Error | 197 | 159.481 | | |
| Standard metabolic rate at 7 °C | | | | |
| Female | 3 | 0.493 | 2.365 | 0.249 |
| Male | 3 | 0.123 | 0.590 | 0.662 |
| Female x Male | 3 | 0.208 | 0.436 | 0.728 |
| Error | 58 | 0.478 | | |
| Standard metabolic rate at 2 °C | | | | |
| Female | 3 | 0.206 | 4.141 | 0.137 |
| Male | 3 | 0.146 | 2.933 | 0.200 |
| Female x Male | 3 | 0.050 | 0.100 | 0.959 |
| Error | 30 | 0.495 | | |

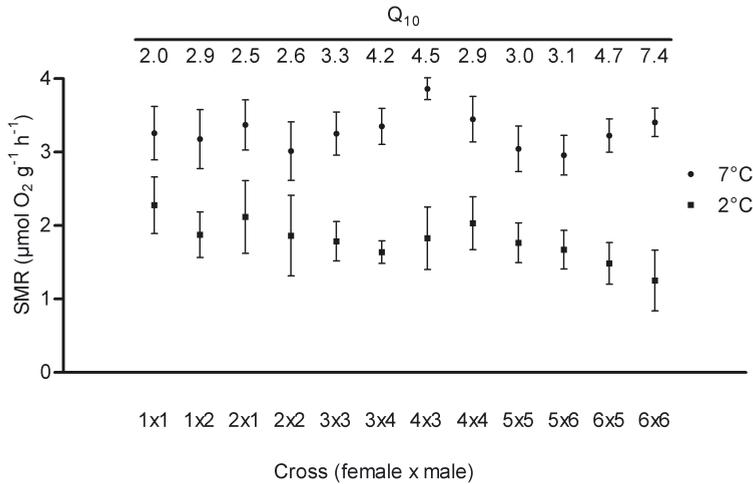
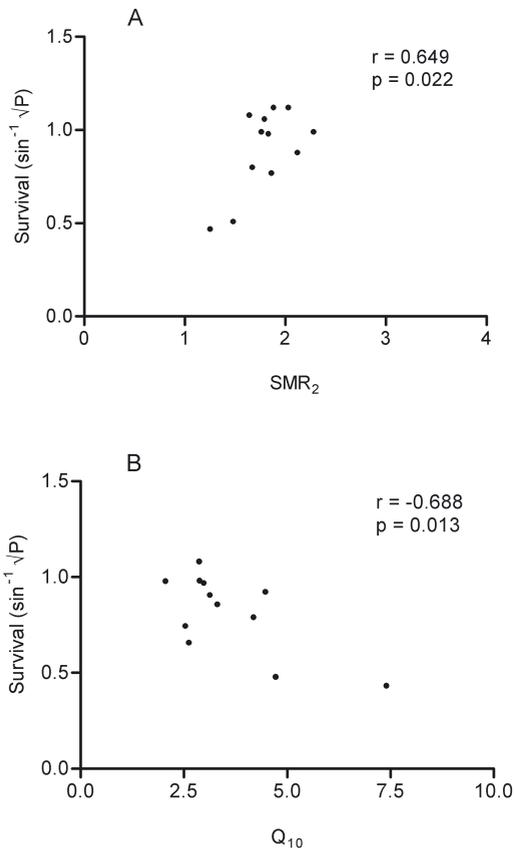


Figure 2. Standard metabolic rate (mean \pm se) of Arctic charr alevins at two temperatures. The increase of the metabolic rate resulting from a 10 °C increase in temperature (Q_{10}) is also given.



as well (Kamler 1992). Factors such as water, protein, lipid and energy contents have been shown to be able to affect the survival of embryos and larvae of different fish species (Kamler 1992). In Atlantic salmon (*Salmo salar*) measures of size and proximate composition attributes were highly correlated suggesting that size measures are reasonable surrogates for composition (Berg et al. 2001).

Lower incubation temperature resulted in larger hatchlings, both in terms of length and weight. This indicates, assuming similar water contents at both temperatures, that the conversion efficiency of yolk into body tissues was higher at lower temperature. Kamler (1992) reviewed effects of incubation temperature on hatchling size, and reported different tempera-

Figure 3. Correlations between A) arcsin and square root-transformed embryonic survival and SMR_2 at 2°C, and B) arcsin and square root-transformed embryonic survival (mean survival at 2 and 7°C) and Q_{10} value of different Arctic charr families.

ture responses in different species. Some species responded similar to the Arctic charr in this study while in others the response was the opposite, or size was at its maximum at intermediate temperatures (Kamler 1992). The most probable explanation for this discrepancy is different temperature coefficients of developmental and metabolic rates in different species and temperature conditions which together determine the partition of yolk energy between tissue production and respiration (Kamler 1992). In the case of Arctic charr, low incubation temperature is obviously advantageous since larger juveniles of salmonids have increased survival and higher overall fitness compared to their smaller conspecifics (e.g. Berg et al. 2001 and references therein). This is emphasized when the higher embryonic mortality at higher incubation temperature is taken into account.

Temperature had a significant effect on SMR of Arctic charr. The mean Q_{10} value of all measured fish was 3.4 but there was substantial variation between the families with Q_{10} ranging from 2.0 to 7.4. Oxygen consumption of Arctic charr embryos and alevins had previously been measured by Gruber and Wieser (1983) who reported Q_{10} to be 4.9 between 4 and 8 °C in embryos before hatching. They did not calculate Q_{10} for alevins since their method of measuring oxygen consumption inevitably included activity costs, which increased with temperature thus artificially elevating the Q_{10} value. Calculated from the equation given by Lyytikäinen and Jobling (1998) Q_{10} value of underyearling Lake Inari Arctic charr was 2.9 over the temperature range 11-17.7 °C. In stenothermic species such as Arctic charr, large temperature coefficients have been considered to be typical (Gruber and Wieser 1983), and it is common that the Q_{10} value is high at low temperatures and it decreases with increasing temperature (Cossins and Bowler 1987, Jobling 1994). Since Q_{10} and SMR_2 were in the present study negatively correlated ($r=-0.862$, $p=0.000$) and there was no statistically significant positive correlation between Q_{10} and SMR_7 ($r=0.370$, $p=0.236$), high Q_{10} values were largely determined by low SMR at 2 °C.

Closer examination of the results revealed that the two families (same female) with weakest survival had also lowest SMR_2 s and highest Q_{10} values. In fact these two families determined the whole correlations between survival and SMR_2 as well as survival and Q_{10} , since excluding them resulted in non-significant correlations. Whether low SMR_2 is a cause or consequence of problems during egg incubation can only be speculated. For instance, it is not certain whether SMR of newly hatched fish reflects the SMR during embryonic period; although Cutts et al. (2001) demonstrated that in juvenile Arctic charr SMR is a consistent trait i.e. relative SMRs of individual fish were maintained over a period of six months. Cutts et al. (2001) also observed that SMR, aggression and competitive ability were positively associated, which may implicate further elimination of low-SMR fish also in their juvenile period. In externally feeding fish, SMR is known to decline under unfavourable conditions (e.g. under nutrition, Jobling 1994), but virtually nothing is known about regulation of metabolic rate in endogenously feeding embryos. The survival of these two families was also weak at 7 °C where their SMRs were at the mid-level of all families, thus no clear connection could be found between the viability and maintenance metabolism at the two different temperatures.

Parental background did not affect SMR of Arctic charr. It is common that individual variation in SMR of fish is large. This may result in situations where statistical testing shows no significant differences between families or even populations, especially if the number of measured fish is relatively small. Of course, it is possible that such differences do not really exist but since individual variation in physiological traits may have important consequences for offspring viability and

fitness in variable environments, one could reasonably expect to find them. Indeed, Lahti et al. (2002) reported population-specific differences in SMR of Finnish brown trout (*Salmo trutta*), and it could well be that increasing the number of families or individuals within a family in the present study would have revealed some family effects.

In conclusion, temperature was found to have effects on both embryonic survival and standard metabolic rate of Arctic charr while parental background affected only survival. Low incubation temperature was advantageous for Arctic charr due to better utilization of yolk reserves and lower mortality. This indicates adaptation of Arctic charr to cold water temperatures.

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Determining the optimal temperature range for Atlantic cod (*Gadus morhua*) during early life

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Key words: *growth, survival, temperature optima, fish, embryo, larva, gadus*

Abstract

Embryonic development and mortality, and larval growth, yolk-absorption, feeding incidence and mortality were analyzed in relation to temperature in an attempt to ascertain the optimal temperature and define the thermal response of cod during early life histories. Three batches of Atlantic cod (*Gadus morhua*) eggs from Newfoundland stock were incubated at temperatures of 2, 4, 8 and 12 °C. Observations during the non-feeding period were made every 4 degree-days for the 4, 8 and 12 °C treatments, and every day for the 2 °C treatment. Once feeding was initiated, observations were made every 12 degree days for all treatments. Data were collected from two synchronized experiments. For experiment 1 the development stage of eggs, yolk-sac size (of yolk-sac larvae), and the standard length, feeding incidence and condition of larvae were recorded. For experiment 2, the effect of temperature on the mortality of eggs and larvae was evaluated. The mean time for eggs to reach the exogenous feeding stage (stage duration) and the initial feeding incidence of larvae, the mean daily increase in larval length (growth), mean yolk reduction, and the mean survival times for eggs and larvae were calculated. The growth-temperature relationship was evaluated using a log-normal model, from which two temperature values were calculated. First, where growth rate per degree was maximized ($G_{TMAX} = 4.2$ °C), and second, where growth per day was maximized ($G_{MAX} = 7.9$ °C). Increasing temperature resulted in a linear increase in ln (yolk reduction), an exponential decrease in stage duration, and a dome-shaped relationship with feeding incidence. The mean survival time during the egg stage showed an exponential decrease with temperature while, for larvae, survival time was modeled using a log-normal equation. We present an optimal temperature range from the results of the study that uses the temperature of maximum growth rate per degree, as opposed to the temperature of maximum growth rate, to define the optimal temperature for young cod.

Introduction

The complex relationship among environmental variability, basic biological processes and recruitment of fish stocks are poorly understood despite a significant investment in research directed towards environment-recruitment relationships (Sundby 2000). An equal increase of both instantaneous growth (G) and mortality (Z) rates with temperature (Houde and Zastrow 1993), suggests that increases in temperature result in a proportionate increase in G and Z and no change in total fish production. However, the ability of a species, population or individual, to persist over a range of temperatures is limited by thermal adaptation of biochemical, cellular and organismal processes (see Hochachka and Somero 2002); as opposed to relationships based on cross taxa meta-analyses of Pepin (1991), Houde (1989a) and Houde and Zastrow (1993). As a result, species or population-specific responses to temperature are those responsible for defining the geographic ranges of stocks and variation in year to year survival. Within species, a dome-shaped relationship between growth rate and temperature has been described (see Jobling 1997). Changing relative metabolic cost at different temperatures results in the maximum ingestion rate being seen at higher temperatures and maximum conversion efficiency (growth per unit of available energy) at lower temperature than that of maximum growth (Jobling 1997). That is, the most efficient growth occurs at a lower temperature than the maximum growth rate. Although this broad understanding of temperature effects on fish exists there has been little done to define temperature responses for individual species in a quantitative manner.

Jobling (1997) provides a framework on which to base the shape of a species response to temperature. The next step is to create some limits and move towards a cod-specific conceptual model. Although cod are found in the North Atlantic in temperatures from below 0 °C to 20 °C, spawning sites are located between 70° N off Norway and the 10 °C surface isotherm (Brander 1997) in temperatures from -1.5 °C to 9.2 °C (Galloway et al. 1998). Sundby (2000) observed that cod stocks from the colder part of the species range tend to have increased recruitment in warmer than average years, while those from warmer parts of the range have increased recruitment in cooler than average years. This suggests that there is a narrower range of temperatures that promotes strong recruitment, or cumulative survival, than would be inferred from the geographic extent of the species, and that temperatures can be either too warm (Flierl and Wroblewski 1984) or cold (Ouellet et al. 1997; Galloway et al. 1998) for good survival. On Georges Bank, a dome-shaped relationship between larval growth and water temperatures was observed during 1984-85 (Campana and Hurley 1989) and 1992-94 (Larry J. Buckley, Graduate School of Oceanography, University of Rhode Island, Narragansett, RI, 02882, United States, Personal Communication) with the maximum growth rate found at 5.9 °C and 7 °C in each study, respectively. These observations suggest a dome-shaped species level response in growth and recruitment to temperature within the range of temperatures between -1.5 °C and 9.2 °C.

Laboratory studies can serve to simplify some of the interactions in inherently complex natural systems by removing factors that confound temperature effects, such as food limitation. In laboratory experiments, cod have been incubated and hatched at temperatures from -1.5 °C to 12 °C. LT_{50} studies have demonstrated that thermal tolerance range of cod embryos is -1.8 °C to 12.0 °C (Johansen and Krogh 1914; Valerio et al. 1992) with a midpoint of 6 °C to 6.5 °C (given in

Rombough 1997). Once larvae hatch, the temperature range over which they can persist increases significantly and culturing is commonly accomplished at temperatures between 10 °C and 15 °C (Personal Observation). By altering the thermal regime after hatch, researchers have observed increasing growth rates up to temperatures of 14 °C -16 °C for larvae from Norwegian and Arctic stocks (Otterlei et al. 1999). Finn et al. (2002) observed growth rates of 23% per day⁻¹ and 37% per day⁻¹ for larvae of 53-60 and 60-66 days post hatch, respectively, in temperatures between 14 °C and 17 °C.

The midpoint of the zone of thermal tolerance, the median temperature experienced in spawning areas, the tendency for recruitment to be maximized at intermediate temperatures and the temperatures supporting maximum growth on Georges Bank all suggest that the optimal temperature for cod during early life would be around 5 °C - 7 °C. However, laboratory growth information using larvae indicates that cod have a high growth capacity at temperatures over 15 °C. This provides an interesting question: why is there a discrepancy in the results of field and laboratory studies?

Sundby (2000) advocates a link among copepod biomass, sea surface temperatures associated with the North Atlantic Subpolar Gyre and increased larval survival. Galloway et al. (1998) also propose a dependence on the interaction between zooplankton abundance and temperature as a possible cause of lower survival in Norwegian cod. An increase in the metabolic maintenance requirements of ectotherms with increasing temperature means that a reduction in food supply will reduce the temperature at which growth performance is maximized. As a result, the temperature of maximum growth rate decreases with decreasing ration size (Jobling 1997); and there will be different temperatures of maximum growth at different prey densities. Maximum growth at 5.9 °C (Campana and Hurley 1989) and 7.2 °C - 7.4 °C (Larry J. Buckley, Graduate School of Oceanography, University of Rhode Island, Narragansett, RI, 02882, United States, Personal Communication) on Georges Bank has been attributed to food limitation lowering the temperature of maximum growth rate from laboratory values of closer to 15 °C. However, there is danger in assuming that food limitation is the major explanation for discrepancies between field and laboratory results. In the productive Georges Bank ecosystem, for example, local prey abundance and temperature profiles are the result of complicated and dynamic oceanography. Prey densities on Georges Bank vary greatly (Lynch et al. 2001), making the assumption that prey densities are consistent enough to result in a *specific* temperature of maximum growth due to food limitation difficult to accept. Furthermore, all ectothermic organisms (phytoplankton and zooplankton) in the environment are affected by temperature, not just larval fish. Therefore the temperature of the environment will also adjust the production of prey species, such that increases in temperature will increase larval feeding *and* production from lower trophic levels. Despite the initial plausibility of the food limitation hypothesis, there is no direct evidence to support the concept that temperature induced increases in larval production exceeds increases in the reproduction and production of phytoplankton and zooplankton (Sundby 2000).

Temperature change strongly influences rate processes of ectothermic organisms and has been shown to be particularly influential during the more sensitive embryonic and larval periods of fish (Rombough 1997; Pepin 1991; Houde 1989a; Houde and Zastrow 1993) when mortality can range between 5-30%•day⁻¹ (Batty and Blaxter 1992). The effects of temperature on vital rates during the egg and larval period of Atlantic cod (*Gadus morhua*) will be presented with a particular inter-

est in defining the shape and limits that the thermal environment imposes on life processes. The effects of temperature during early life can be evaluated through the study of: (1) differences in state variables - either in meristic (eg: vertebral counts) or morphometric characters (eg: size at hatch), and (2) observation of rate processes over time (eg: growth and mortality rates). This study employed the second approach, investigating the effects of temperature on rates of development, growth and mortality. Cod eggs and larvae were cultured in the laboratory at four different temperatures (~ 2, 4, 8 and 12 °C). Two experiments were conducted, one on development and growth of embryos and larvae, respectively, and the other on mortality of the embryos and larvae at different temperatures. This research is meant to complement work on differences in morphology (Jordaan 2002) by comparing the effects of temperature on the vital rates of cod embryos (development, mortality) and larvae (growth, yolk-sac absorption, mortality) and relating them to some observed recruitment relationships.

Materials and Methods

Experiment 1: Development and Growth. Three batches of naturally spawned eggs were collected from the captive broodstock captured from Trinity Bay, Newfoundland, and held at the Ocean Sciences Center (OSC), Logy Bay, Newfoundland, Canada. The broodstock were maintained at 5 °C - 6.5 °C year-round. Two batches of eggs in 1999 and one batch in 2000 were shipped to the Aquaculture Research Center (ARC) on the campus at the University of Maine in Orono, Maine, United States. The transport water temperature and salinity upon arrival at the ARC were 7.7 °C and 31.4‰ in 1999, and 6.1 °C and 31.6‰ in 2000. There was no detectable ammonia in the transport seawater in either year. Once at the ARC laboratory, each batch of eggs was disinfected in 400 ppm gluteraldehyde solution for 10 min. The eggs were then placed in 4 l plastic bags (n=8), with slight aeration, filled with seawater equal to the temperature upon arrival. Half of the egg-filled bags (n=4) were placed in the 4 °C and 8 °C treatments, and allowed to acclimatize for four hours. After the acclimatization period, half of the bags in the 4 °C system were moved to the 2 °C system, and half the bags in the 8 °C system were moved to the 12 °C system, and again all the bags were left for four hours. One tank in each system was stocked with 35 ml of eggs for batch 1, 40 ml for batch 2 and 35 ml for batch 3. The remaining eggs were used to determine mortality described below in experiment 2. Only buoyant eggs were used.

Four recirculating seawater systems, each containing four separate 75 l tanks, were used to rear eggs and larvae. The systems were randomly assigned temperatures approximating 2, 4, 8 and 12 °C. The temperature was maintained in each system using Honeywell™ T775 temperature controllers, connected to alarms that modulated the flow of chilled glycol (-12 °C) through heat exchangers. Temperature was recorded every 5 minutes using a temperature logger (Onset™ Stow-Away tidbit +23° F to +99° F model). Salinity was also checked on a daily basis and maintained between 33-35‰. Photoperiod was set on a 16:8 hr light:dark cycle. To approximate natural light conditions the lights were set to reach a maximum of 350 lux at mid-day and approximated a sine curve, with no light during the dark period of the cycle. Water samples were taken on a regular basis for water quality (ammonia and nitrite).

Feeding. Prey densities (8-10 ml⁻¹) were well above the 4 ml⁻¹ required to guarantee growth and survival throughout development at 8 °C (Puvanendran and Brown 1999). Feedings were done during the light period, 6 times per day for batch 1 and batch 2 and every hour for batch 3. Rotifers (*Brachionus* spp.) were supplied from the day following 100% hatch until larvae attained 7mm standard length. From then until 10mm standard length, the larvae were offered a mix of rotifers and *Artemia* nauplii, thereafter receiving solely *Artemia* nauplii. Rotifers and *Artemia* were enriched with DHA Selco™ and Algamac 2000™ for 12 hours prior to feeding for batch 1 and 2. For batch 3, a combination of Algamac 2000™ and Innovative Aquaculture™ Algae Enrichment Formula enriched for 12 hours was used.

Sampling procedure. Sampling times were set according to a 4 degree-day period (mean temperature × time in days), except the 2 °C treatment. This meant that the larvae were sampled at 06:00, 14:00 and 22:00 for the 12 degree treatments, 10:00 and 22:00 for 8 degree treatment and 22:00 for the 2 and 4 degree treatments. This sampling regime was maintained through incubation to 50% feeding for batches 1 and 2 and from hatch to 50% feeding for batch 3. After this, the sampling was continued on a 12 degree day basis for all batches.

During the embryonic period, all tanks were checked on a daily basis at the above sampling times. A flashlight was used to quickly scan the tanks. The presence of hatching fish and the distribution of the eggs/larvae in the tank were noted. When hatching commenced, larvae were randomly taken from the tank until 20 eggs/larvae had been removed. If there were at least 9 larvae, then it was considered 50% hatch. Larvae were then removed until there were 20 larvae total sampled.

Subsequent samples were continued on the above schedule, collecting 20 larvae each sample period. The time marking 100% hatch was when there were no eggs remaining. When there were 10 or more fish in the sample with food in their gut, it was considered the time of 50% feeding. Sampling was continued on the “4 degree-day” schedule until 50% feeding. Thereafter, sampling was done only at 22:00 to allow for comparison of gut fullness after a day of feeding. These samples were taken once per day for the 12 degree and 8 degree treatment, once every 2 days for the 4 degree treatment and once every 4 days for the 2 degree treatment.

Once sampled, the 20 eggs/larvae were gently placed by pipette on a 1 ml well slide, with 1 mm² grid, and anesthetized with tricane methanesulfonate (MS-222). Photographic images of the sampled larvae were taken with a digital camera paired with a dissecting microscope. The image was saved to the computer using a Flash Point 128™ frame grabber (Integral Technologies).

Measurements. The time required for all larvae to reach exogenous feeding, for which there were three batches worth of data, was used as development time and will be referred to as the stage duration (D). Exogenous feeding was used to define the end of the embryonic stage, rather than time to hatch, because hatching has been shown to occur at different stages of development (Jordaan 2002). Methods outlined by Jordaan (2002) for staging of larvae were employed to identify larvae that had reached the point of first-feeding, even if physical deformities prohibited feeding. The staging table was based on Fridgeirsson (1978) and Hunt von Herbing et al. (1996).

Feeding condition was assigned by ranks based on ingestion of microparticulate diets, given by Baskerville-Bridges and Kling (1999), with 5 categories of fullness. Pooling all the feeding cate-

gories reduced the 5 categories to 2. Once a tank had no increase in the number of feeding larvae, three consecutive evening samples were pooled to calculate the mean incidence of feeding larvae ($= \text{number of feeding larvae} / (\text{number of feeding larvae} + \text{empty larvae})$). This was done for each batch and temperature.

The 1 mm grid served to calibrate measurements, and the calibration was checked on each image by measuring a 1 mm square to ensure accuracy in the calibration settings. The standard length of each larva was measured to the nearest 0.1 mm using Image Pro Plus™ V4.0 (Media Cybernetics). Standard length was measured from the tip of the snout to the end of the notochord. Yolk-sac area, calculated from the two-dimensional images, was also measured in Image Pro Plus™ by outlining each larval yolk-sac and using the area command. This assumes that changes in area are representative of changes in volume.

Experiment 2: Mortality. Egg period mortality. Mortality was monitored in 2.5 l tanks ($n=5$ for batches 1 and 2). The 2.5 l tanks were placed in the two empty 75 l tanks of each recirculating system and were held in place by a frame of PVC piping. Each 2.5 l tank contained a central screened outflow and air supply, as well as, water inflow delivered to the surface of the tank, which was scaled to mimic the conditions of the rearing tanks.

Each 2.5 l tank was stocked with 100 eggs at the same time that experiment 1 was initiated. Eggs and larvae, when alive, are buoyant. Mortality counts were completed by stopping the air supply, and allowing a few minutes for the eggs (and later larvae) to settle out. A siphon was established with a 5 mm diameter Nalgene™ tubing fitted with a solid plastic extension of same diameter. The solid plastic end was used to remove all the material that settled out, the dead eggs were counted and the airflow restored. The siphoned eggs were checked periodically to ensure that the embryos were dead. This revealed that in some cases extremely deformed embryos (no chance of hatching) sunk out of the water but that live embryos did not.

Mortality counts were done on a 4 degree day basis, except for the 2 °C treatment (daily) and were recorded as the number found dead. This results in mortality counts being taken every 8 hours for the 12 °C treatment, every 12 hours for the 8 °C treatment and every 24 hours for the 2 °C and 4 °C treatments. At the conclusion of the experiments, the tanks were cleaned out and the number of living embryos and the number of unviable eggs and dead embryos counted.

Larval period mortality. At 100% hatch, all the 2.5 l tanks were removed, cleaned and replaced. The tanks ($n=5$ for batches 1 and 2, $n=10$ for batch 3) were restocked with 100 larvae. Feedings were given at the same times and to maintain the same densities as in the rearing tanks. The same sampling methodology was used as embryo mortality. The sampling schedule followed the rearing tank schedule, having a reduced sampling effort after 50% feeding.

Design and Statistical Analyses. In the development and growth experiments only one tank was used for each batch and the experiment was replicated over time using the three batches (4 temperature treatments x 3 replicate times), with time used as a blocking factor (Sokal and Rohlf 1995).

The growth rate, reported as a change in length (mm) instead of mass, was modeled by linear regression models to calculate an average increase in length per day for each batch at each tempera-

ture. Yolk-sac area was transformed to a linear measure by calculating the square root and regressed against time, returning a rate of yolk-reduction for each temperature and batch. The rate of yolk-sac absorption was ln transformed for the analysis against temperature. This was done to improve the performance of the data with regard to assumptions of normality and constant variance. All the above analyses resulted in one rate measure for each batch and temperature (n=12 total).

Mortality data did not meet the assumptions of constant variance or normality of residuals, even when the data was natural log-transformed and regressed against time. Therefore, the non-parametric Kaplan-Meier (K-M) or product limit estimator (Lee 1992) was used to generate a survivorship curve. Individual mortalities are recorded as deaths (failures) associated with the hour of removal. Hours were used instead of days because of the different sampling times in the temperature treatments (3 times per day for the 12 degree treatment). The surviving eggs/larvae at the conclusion of the experiment were right-censored, which identifies the larvae as alive at the time of sampling. The K-M also estimates a mean survival time in hours for each tank. This mean survival time was used to model the temperature effect on survival, which allows for good comparison with the development times.

The above data reductions resulted in four temperatures and three batches, n=12, used in the analyses of vital (mortality, growth, and development) rates. The only exception to this is for the egg mortality-temperature curve. High mortality during transport of batch 3 resulted in the decision to favor data collection during the larval phase, so no egg mortality experiment was run for that batch, resulting in only 8 data points for this analysis. The temperature of maximum slope (where the 1st derivative of an equation is maximized) and of the maximum value (where the 1st

Table 1: Results from regression analyses for all variables against temperature (T). The type of equation, estimated parameters (with standard error of the estimate in parentheses), the degrees of freedom (df), f-ratio (F), r², and p-values (P) are given for each.

| Variable | Equation type | Equation and estimated parameters | df | F | r ² | P |
|--------------------------------|---------------|--|----|--------|----------------|----------|
| Feeding incidence | Gaussian | $= 0.92 (0.09) - 0.5((T/6.75(0.5))/4.5(0.6))^2$ | 11 | 9.11 | 0.67 | 0.0069 |
| Feeding | Log-normal | $= 0.90(0.08) - 0.5(\ln((T/4.89(0.4))/0.87(0.1)))^2$ | 11 | 11.92 | 0.73 | 0.003 |
| Stage duration | Exponential | $= 1079.6(78.9) - 0.16(0.02)T$ | 11 | 115.73 | 0.92 | 0.0001 |
| Growth | Log-normal | $= 0.114(0.01) - 0.5(\ln(T/280.8(0.4))/0.013(0.014))^2$ | 11 | 15.26 | 0.77 | 0.0013 |
| ln(yolk absorption) | linear | $= -3.6(0.18) - 0.22(0.02)T$ | 11 | 82.54 | 0.89 | < 0.0001 |
| Mean survival time (embryonic) | Exponential | $= 472.16(41.2) - 11.97(0.02)^2$ | 7 | 62.7 | 0.91 | 0.0002 |
| Mean survival time (larval) | Log-normal | $= 514.26(22.5) - 0.5(\ln(T/276.1(0.7))/0.018(0.002))^2$ | 11 | 62.10 | 0.93 | < 0.0001 |

derivative is equal to 0) were calculated for a number of equations using the solver application in Microsoft® Excel. For a number of equations, one of the estimated parameters from the regression model was the temperature of maximum rate. These were cross-checked in Excel to ensure that solver was operating well.

Because there were slight differences between the temperature treatments of each batch, temperature was treated as a continuous variable and batches as replicates. The rate data (change in length, yolk-sac reduction) and the duration data (stage duration, mortality) were modeled over the range of temperatures, using regression analysis in Sigmaplot™ 2001 for Windows™ (ver 7.0). All linear and non-linear models were solved by means of the least-squares method. When more predictive, non-linear regression results are reported. Suitable non-linear regression models were determined by consulting Hoerl (1954). The normality of the residuals was tested using the Kolmogorov-Smirnov (Sokal and Rohlf 1995) test and homogeneity of the variance was tested using the Levene Median test (Brown and Forsythe 1974). Assumptions of normality of residuals and constant variance held in reported analyses and transformations of the data were only applied if the assumptions were initially violated. Degrees Kelvin was used instead of degrees Celcius for the log-normal modeling of growth and survival because values around 0 °C do not function well in many equations.

Results

Temperature affected egg development and mortality, larval growth, feeding incidence, yolk-absorption, and mortality (Table 1).

Experiment 1: Development and growth. There was a significant dome-shaped relationship between the incidence of first-feeding larvae and the incubation temperature (Figure 1, Table 1). The gut fullness of the larvae was dramatically altered by the temperature treatment. In the 2 °C and 12 °C treatments, the incidence of larvae at low and high feeding levels made up approximately the same proportion of larvae. That is, always about 50% of the larvae were barely or not feeding. In the intermediate temperatures, it is clear that feeding was initiated. Two different models, Gaussian and log-normal, were used in predicting a temperature of maximum incidence of first-feeding (Table 1). Both models were significant and there appeared to be no valid reason to choose one over the other. The temperature of maximum incidence of first-feeding was 6.6 °C predicted from the Gaussian model and 4.8 °C predicted from the log-normal model.

Stage duration (D), the time from acclimation to the time when all larvae were at the feeding stage, is plotted against temperature in Figure 2. Batch 2 had slightly shorter development times compared to batch 1 and 3 because it arrived later in development. This was not corrected for in any way because the overall relationship to temperature, and most importantly the relative differences among temperature treatments is conserved. The resulting regression equation of D in regards to temperature (T), with standard error of the estimates in parentheses is given in Table 1. Increasing temperature exponentially decreased the time it took to reach the point at which feeding is initiated.

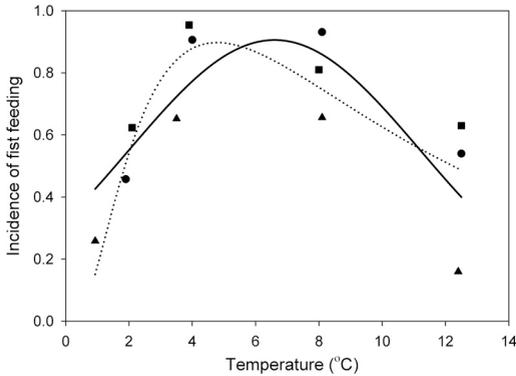


Figure 1. The incidence of first-feeding larvae across temperature for batch 1 (circles), batch 2 (squares) and batch 3 (triangles). Results of Gaussian (solid line) and log-normal (dashed line) non-linear regressions relating incidence of first feeding larvae to temperature are shown.

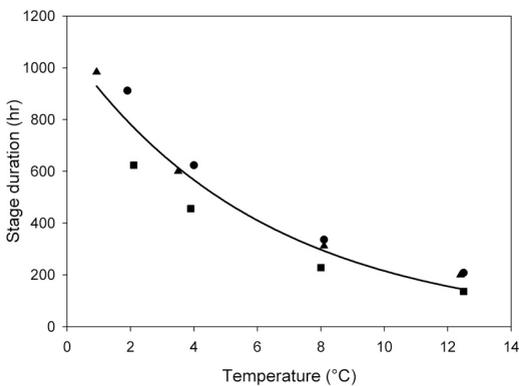


Figure 2. The stage duration (hr) of the non-feeding period of cod egg and yolk-sac larvae at different temperatures. The resulting relationship is an exponentially decreasing time spent during the egg stage with increasing temperature. Circles = batch 1, squares = batch 2, triangles = batch 3.

When the growth for all temperatures was modeled to a log-normal relationship, a strong significant relationship resulted (Figure 3A, Table 1). Only non-starving individuals were used in the analysis. The following points summarize the resulting growth-temperature relationship: (1) there is an inflection point in the growth-temperature relationship, seen as the maximum slope when the first-derivative of the growth-temperature curve is plotted, identified as G_{TMAX} ($\text{mm} \cdot \text{day}^{-1} \cdot ^\circ\text{C}$) (Figure 3B); (2) there is also a temperature where growth rate was maximum (G_{MAX}). The predicted temperature of G_{MAX} , where the slope of the growth-temperature relationship is 0, was 7.9°C , and the predicted temperature of maximum slope (G_{TMAX}) was 4.2°C .

The relationship between yolk-sac absorption and temperature is shown in Figure 4. The absolute value of the rate of yolk reduction was ln-transformed and is presented as the linear regression of $\ln(\text{yolk absorption rate})$ or $\ln(Y)$, with the standard error of the estimate, against temperature (T) is given in Table 1.

Experiment 2: Mortality. The Kaplan-Meier (K-M) estimated mean survival time during the egg stage was regressed against the environmental temperature using an exponential model (Figure 5A, Table 1).

The K-M estimated mean survival time was regressed against the environmental temperature

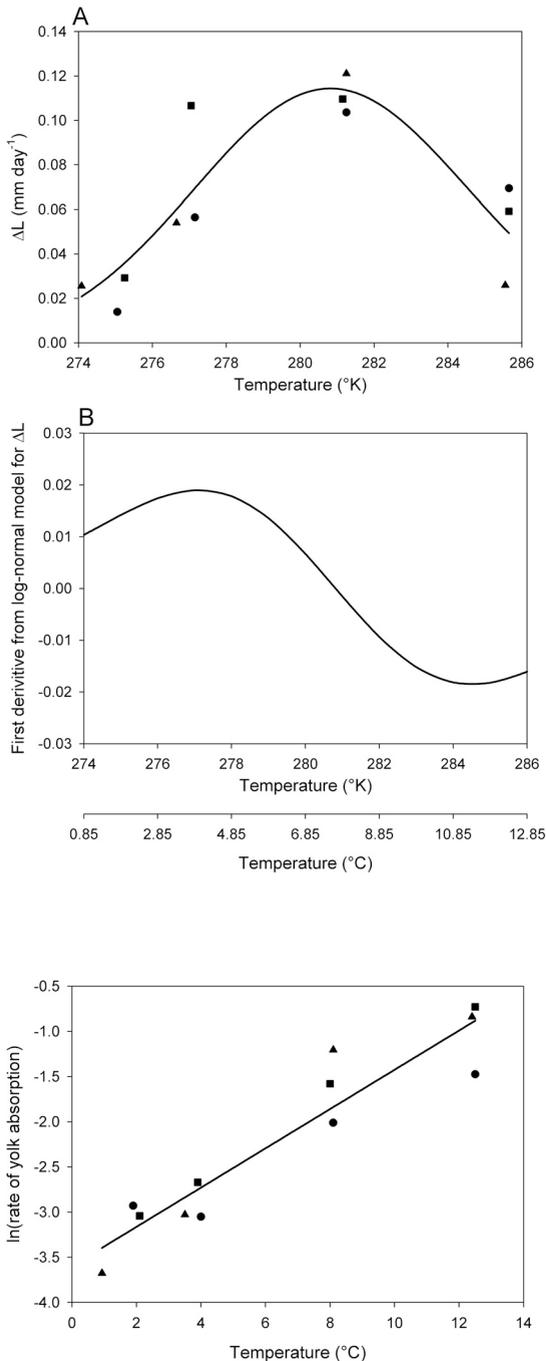


Figure 4. The results of the linear regression of $\ln(\text{rate of yolk-sac reduction})$ at the different temperatures. Circles = batch 1, squares = batch 2, triangles = batch 3.

Figure 3. The effect of temperature on the growth rate, in mm day^{-1} , of cod larvae using only data from post yolk-absorption. A) Log-normal modeled regression of growth rate at different temperatures. Circles = batch 1, squares = batch 2, triangles = batch 3. B) Plot showing the first derivative of log-normal modeled regression in A.

using a log-normal model for the larval (Figure 5B, Table 1). The resulting trend “flips” from an exponential fit, expected if mortality occurred at a rate equal to development, to a relationship with a peak value contained within the temperature range. The influence of temperature was more pronounced during the larval period than the egg period, seen by the move to a log-normal model for larvae from a more linear model for eggs.

Discussion

The regression of growth against temperature reflects an expected framework offered by Jobling (1993, 1997). The first derivative of the log-normal equation established where the slope was maximized (ie: where the greatest growth rate per unit temperature was realized ($G_{\text{TMAX}} = 4.2^\circ\text{C}$)). The maximum slope under ad libitum feeding corresponds to where the greatest potential for growth per degree occurs and below and above this temperature, larvae experienced reduced growth rate per unit of temperature change. In order to discuss the significance of G_{TMAX} , it is necessary to discuss the cause of the increase and decrease in growth with temperature. The enzymatic rate-temperature relationship is the primary force behind growth rate-temperature relationships (Blaxter 1992). The temperature-enzyme relationship is significant because enzymes are only marginally stable at the appropriate phys-

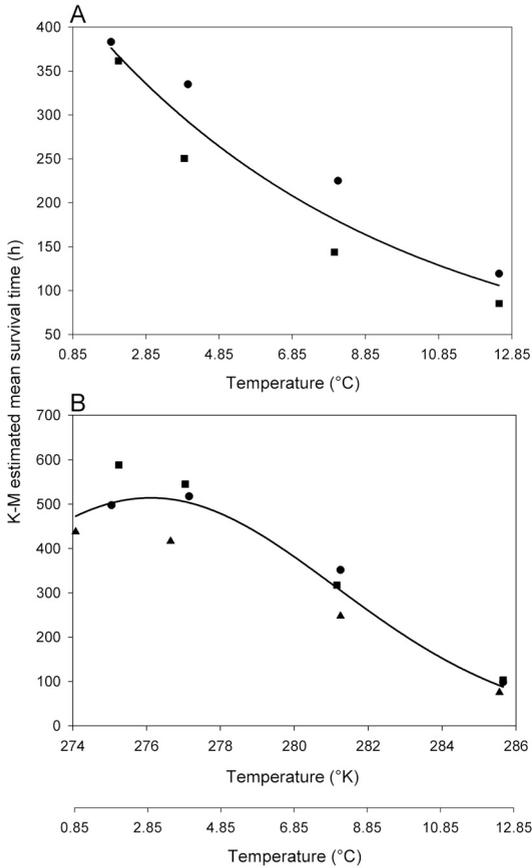


Figure 5. Kaplan-Meier estimated mean survival time (hr) at the different temperatures. A) The mean survival time (hr) during the embryonic period, with the estimated exponential decay function (solid line). B) The estimated mean survival time (hr) for the larval stage at the different temperatures, with the resulting log-normal regression equation (solid line). Circles = batch 1, squares = batch 2, triangles = batch 3.

iological temperature (Hochachka and Somero 2002). Increasing temperature results in an increase in enzyme reaction rates because the kinetic energy of reaction products increases (Eckert et al. 1988) and this translates into an accelerated rate of development and growth. Counteracting this “normal” temperature effect are “negative” effects which are largely due to changes in protein conformation that effect enzyme active binding sites (Hochachka and Somero 2002). Eventually, changes in the enzyme tertiary structure cause the enzymes to permanently unfold rendering enzyme systems inactive (Eckert et al. 1988; Hochachka and Somero 2002). The denaturation of enzymes is a lethal aspect of temperature for ectotherms and is responsible for setting the upper thermal lethal limit. Reduced protein function occurs at temperatures far below denaturation and loss of enzymatic activity (Somero et al. 1996). There are other possible negative temperature effects including: (1) differential effects on enzymes involved in determining protein turnover rates (Hoolihan et al. 1995), (2) developmental factors limiting prey capture, (3) behavioral factors involved in determining prey capture success, and (4) changes in water viscosity (See Hunt von Herbing and Keating, this publication). G_{TMAX} is seen as a better proxy for the optimal temperature because it is the estimate of where maximum growth per degree occurs, before negative temperature effects begin to minimize the positive influence of temperature of growth.

The maximum growth per day (G_{MAX}) occurs where the first-derivative of the growth-temperature curve is equal to 0. At G_{MAX} , found at 7.9 °C in the present study, the two processes (negative and normal temperature effects) are equal. At higher temperatures, the rate of growth begins to decline as “negative” temperature effects begin to dominate over “normal” temperature effects. The G_{MAX} calculated in this study is low compared to results of Otterlei et al. (1999), where G_{MAX} was calculated at 14.2 °C and 16.2 °C for two stocks of cod, and Steinarsson and Björnsson (1999) where G_{MAX} was calculated at 9.7 °C - 13.4 °C. In both those studies, however, eggs were incubated

at one temperature and the treatments were applied to the larval period, whereas in this study the temperature treatments were applied early in embryonic development. The effect of extreme temperatures on embryonic development is viewed as the most likely contribution of the lower G_{MAX} values in the present study. The consequences of environmental perturbations last beyond the time of exposure because of deformities and inefficiencies that can reduce feeding and growth (Brown and Núñez 1998). In the present experiment, the poor growth performance of larvae held at 12 °C may be due to subtle embryonic abnormalities passed on from the embryonic stage. Greater numbers of temperature treatments would have allowed increased confidence in a precise calculated G_{MAX} value. The poor performance of the 12 °C treatment, however, suggests that there is not much room for an increase in G_{MAX} . Nonetheless, it is believed that if there were more temperature treatments and a more complete model, the temperature of G_{MAX} and maximum slope could be slightly higher.

Although the efficiency of growth is difficult to measure in larvae, weight-at-length calculations given by Otterlei et al. (1999) suggest that larvae in the fastest growing temperatures were lighter at a given size. The weight-at-length at 14 °C for two stocks was lower than at 10 °C, despite faster growth at 14 °C (Otterlei et al. 1999). Lankford et al. (2001), using Atlantic silversides (*Menidia menidia*), provide experimental evidence suggesting that maximization of the rates of ingestion and growth may incur fitness costs in the form of increased predation risk. This suggests that the temperature for maximum growth per day (Otterlei et al. 1999; Steinarsson and Björnsson 1999) may not be the optimal temperature for survival. From this we suggest that, although the temperature of maximum growth is informative regarding the shape of the temperature relationship, it is not a good proxy for the optimal temperature of growth/survival in the wild. Although the difference between the temperatures where growth rate is maximum (G_{MAX}) and where growth rate per degree is maximized (G_{TMAX}) may appear subtle, the conceptual and practical differences regarding the designation of optimal conditions for growth and survival, and hence recruitment, is significant.

If we are to argue that temperature estimates for optimal growth differ from maximum growth, there should be a clear rationale for the influence on larval survival. Optimal foraging theory suggests that energy gain should be optimized (Hughes 1997). Inefficient growth imposes two parallel deleterious possibilities: (1) individuals will have slowed growth while ingesting equal quantities of prey or volume of endogenous supply used, or (2) individuals will have to ingest more prey per unit of growth. In the first case, individuals will tend to have a longer stage duration and incur higher risks (e.g. predation) because of an extended period during stages of life experiencing high mortality (Houde 1997). In the second case, larvae will have to search and capture more prey, thus exposing themselves to higher potential for predation mortality (Skiftesvik 1992) through both increased encounter rates with predators and reduced energy available for predator evasion. Note that in each case larvae are exposed to higher risk of predation, which is viewed as the most important source of mortality during early life (Bailey and Houde 1989). The two possibilities are the result of increasing metabolic costs and both result in increased risks, but the responses operate on different temporal scales. Deviations in temperature away from G_{TMAX} may be more important than G_{MAX} because efficiency in growth, rather than maximization of growth, will have significant influence of mortality. Larvae must balance the costs of additional

growth in sub-optimal conditions with the costs of longer development period before metamorphosis and settlement.

Maximum feeding incidence was found within range of temperatures from 4.8 °C to 6.6 °C, indicating that optimal conditions are restricted to this temperature range. The initiation of feeding was a serious bottleneck to survival in the extreme temperature treatments. The maximum feeding incidence and maximum slope of the growth-temperature predicted from log-normal equations were very similar at 4.8 °C and 4.2 °C, respectively.

The result of the development-temperature regression analysis, a standard exponential curve, reflects a typical time to hatch-temperature relationship (Blaxter 1992). The effect of temperature on development is best appreciated by comparing the exponential curve to a tangent to the curve within the optimal temperature range. The exponential curve shows increased development times at the extreme temperatures (near 2 °C and 12 °C), compared to the tangent line. This leads to the question: is there increased mortality as a result of increased time required for development during the egg stage. Future work considering the effects of increased relative development times, particularly in contrast to co-occurring species, and the thermal regime may help explain the significance of varying thermal regimes on development and how this is related to survival. The development-temperature relationship could also be viewed as an expected mortality curve. For example, larvae with significant morphological flaws will experience mortality as the population transitions from endogenous to exogenous feeding. The timing of this mortality is dependent on the development rate or duration of the embryonic stage, and mortalities would accumulate at a rate reflecting this. Pepin (1991) concluded that the point-of-no-return (PNR), when even if feeding is first initiated the larvae will die, is strongly related to temperature. This can be appreciated by the strong temperature effect on both development of embryos and the absorption of the yolk stores in the present study.

There are two processes involved in shaping the mortality-temperature relationships in this study. The first process, as it was for the growth-temperature relationship, is the “normal” temperature effect. Changes in rate processes due to temperature (Hochachka and Somero 2002) result in varying development times with temperature in ectothermic organisms such as fish larvae (Blaxter 1992). If the rate of death is the result of the rate of development, the mortality-temperature relationship would be expected to approximate the development-temperature relationship. Pepin (1991) calculated that larval mortality rates are significantly affected by temperature ($P < 0.001$), while cumulative mortality is not, demonstrating that the normal temperature effect influences rates and does not affect cumulative mortality in specific life stages (stage-specific mortality). It is important to note that Pepin (1991) uses a meta-analysis. Otherwise there is an apparent contradiction in saying that cumulative mortality is not influenced by temperature while asserting that temperature can influence the recruitment of fish. It is important to differentiate conclusions that are based across species, where general trends in temperature effects are exposed, and conclusions based on one species, where specific limits on the ability of the species to persist over temperature ranges can be calculated.

The second process involved in altering the mean survival time is the cumulative mortality or the negative temperature effect. Increasing numbers of dying larvae will result in increasing mortality rates. Larvae from the 2 °C and 12 °C treatments in this study had poor feeding levels com-

pared to the 4 °C and 8 °C treatments. The mean time of survival reported in the mortality-temperature relationships will reflect this as a decrease in the mean survival time. As temperature becomes deleterious to developing cod, the negative temperature effect operates in addition to the normal temperature effect. During the egg stage, the relationship did not move strongly away from the development-temperature pattern. The relationship was more linear, caused by higher levels of mortality particularly in the period just subsequent to transfer into the systems, and at the time of hatching.

The influence of the second process is clearer during the larval period at the lower temperatures where the mean survival time was lower at 2 °C versus 4 °C. For larvae, the curves had a much more dome-shaped appearance compared to the development-temperature relationship. Within a range of temperatures near the center of the tolerance range (4 °C - 8 °C), there is little effect of cumulative mortality. As temperatures away from the optimal range are approached, the influence of temperature on rate processes, the normal temperature effect, is confounded by additional cumulative mortality (mortality independent of rate processes). Larval mortality was strongly influenced by temperature because sub-lethal effects became lethal once the larvae became active swimmers and where prey capture was necessary for survival. Prey capture requires active foraging and this constitutes a bottleneck to early larval survival at the temperature extremes, seen by higher numbers of non-feeding larvae in the extreme temperature treatments. Furthermore, intrinsic (genetic) factors may dominate prior to yolk-absorption moving to extrinsic (environmental) factors as the fish begin to feed (Hunt von Herbing et al. 1996), supporting the idea that larval mortality is more influenced by temperature than the egg stage.

Conclusions and Implications

Sundby (2000) observed that recruitment tends to be better in warmer years in the cold part of the geographic range of cod and in cooler years in the warmer part of the geographic range. It appears, therefore, that although populations can exist over a fairly broad range of temperatures, highest cumulative survival occurs over a smaller range of temperatures somewhere in the middle of the species thermal range. Without a quantitative genetic study it is not possible to determine the contribution of environment and genetics in shaping these responses. The relative contribution (genetic versus environmental) to any variation in performance of larvae will be necessary information for predicting effects of climate change on stocks of fish. But simple experiments relating the most efficient growth to environmental factors should help researchers understand mechanisms that result in short and long-term fluctuations in survival. Figure 6 shows the optimal temperature range calculated by combining information given by the regression equations. The results of this study are limited to the following conditions: (1) eggs from inshore Newfoundland stock, (2) movement of eggs from one temperature to experimental temperature very early in development, and, (3) feeding was ad libitum. Deviations from these conditions will change the influence temperature has on the vital rates of cod as determined in this study.

From the present study, it appears that relatively large changes in temperature (+/- ~4 °C) could affect survival through disruption of normal morphological development. Changes in tem-

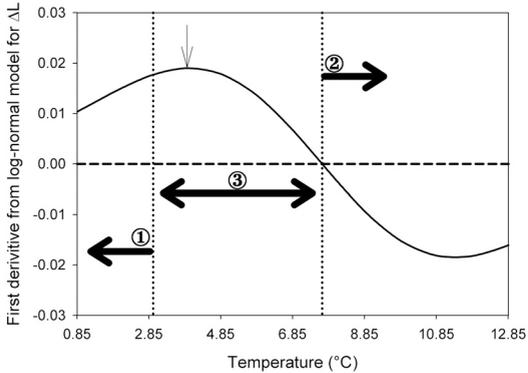


Figure 6. The theoretical thermal limits of developing cod embryos and larvae if temperature treatment is applied early in development. Three zones are identified. (1) Where calculations from the larval mean survival time indicate that no increase was associated with decreasing temperature treatment (2.96 °C; Figure 5B) although larvae were growing at a slower rate (Figure 3A). (2) Where decreasing growth rates occur with increasing temperature past the point of G_{MAX} (7.67 °C; Figure 3B). (3) The optimal thermal zone, where cod larvae will exercise a trade-off between efficient growth and fast growth. Food limitation or strong competition will tend to drive the optimal temperature for growth towards G_{TMAX} (vertical arrow). Ad libitum prey conditions devoid of pressure from predators, as found in laboratory studies, will allow the optimal temperature for growth to move toward G_{MAX} , where the benefits and costs of temperature on growth are equal.

Much smaller changes in temperature (± 1 °C) affect the growth of larvae (Figure 6), which is considered a good proxy for fitness (Schluter 1994; Hughes 1997). The lower threshold for temperature effects on growth makes it the most likely source of temperature induced cumulative mortality, and the most likely source of year-to-year differences in cumulative mortality. Houde (1989b) suggests that episodic events, such as the massive advective losses and failed egg production, can have a significant but not necessarily catastrophic impact on the recruitment for a year. Houde (1989b) suggests that chronic losses of individuals over time can constitute a more serious impact on recruitment through orders of magnitude influences on cumulative mortality. Reduced efficiency of growth over long time periods could be a mechanism by which temperature could result in a significant mortality of a population.

Because growth at maximum rates is not necessarily optimal, those involved in the culture of fish should weigh the benefits of a more efficient use of feed with the slower growth rates that accompany a lower temperature. Furthermore, because of negative temperature effects near the tem-

perature in the wild are not usually enough to cause mortality through disruption of development (direct mortality) since sea-surface temperature anomalies typically are on the order of 4 °C (Pepin 1991). However, on Georges Bank, gulf-stream origin warm core rings are capable of entraining larvae (Flierl and Wroblewski 1985) and bring a substantially higher possible temperature anomaly (>15 °C). As seen from the results of this study, the effects of a greater than 8 °C change in temperature would cause serious problems for the youngest stages of cod. Moreover, large scale fluxes of cold (< 2 °C), fresher water (30‰) from the Scotian shelf also cross-over the Northeast channel and are incorporated into the Georges Bank circulation pattern (Bisagni and Smith 1998). The Scotian shelf water contains large numbers of gadid eggs (Bisagni and Smith 1998) suggesting that eggs and larvae from Browns Bank could be incorporated into Georges Bank, if the changes in the thermal environment do not act as a barrier to dispersal. Bisagni and Smith (1998) also suggest an interaction between Gulf Stream warm core rings and Scotian shelf water fluxes, which means that temperature effects may be compounded due to the presence of both extremes within a relatively small temporal and spatial window.

perature of maximum growth, larvae may appear to grow well while being under stressed conditions (reduced immunocompetence, etc). There is a complete lack of information on ration size (prey concentrations) and their interaction with temperature for larval stages, information that would be very useful in understanding the complex relationship between prey availability and temperature. We suggest that when attempting to consider the optimal temperature of a population using laboratory results, that the temperature resulting in the fastest growth per day per unit temperature (maximum slope of the growth rate-temperature relationship) is a better proxy for optimal survival in the wild than the temperature resulting in maximum growth per day.

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Growth and survival of haddock (*Melanogrammus aeglefinus*) larvae at different salinities

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Key words: *Haddock larvae, salinity, growth and survival*

Abstract

A feeding experiment was conducted with haddock (*Melanogrammus aeglefinus*) larvae at four salinities (15, 25, 30 and 35 ppt), with five replicates per treatment. Larvae were fed rotifers from 4 days post hatch until the end of the experiment (25 days post hatch, DPH). No larvae survived beyond 18 DPH at 15 ppt. Larval growth, measured as mean dry weight, was highest in the group raised at 25 ppt, 1.08 mg compared to 0.76 mg at 35 ppt at 25 DPH. Highest larval survival was recorded at 30 ppt (31.7 %) and lowest at 35 ppt (1.1 %) at 25 DPH. The results from this experiment indicate that growth and survival of haddock larvae can be increased by adjusting the salinity in the start feeding tanks.

Introduction

Salinity is an important factor in the survival, metabolism, and distribution of marine fish larvae (Olla and Davis 1992). The effect of salinity on larvae may be a result of several factors, such as, the effects of the total osmotic concentration, the incidence and concentration of particular ions, and the availability of oxygen due to the inverse correlation between them (Lasker and Theilaker 1962). At hatching, the skin of teleost larvae is a thin two-layered epithelium, they have no gill filaments and the kidney is present only as a pronephric glomerulus (Houde and Schekter 1983; Morrison 1993), thus, larvae have no osmoregulatory mechanisms as in adults. Osmotic and ionic movement takes place across this surface to an extent that depends upon the salinity of the water. In nature, most larvae from cold-water species begin feeding in the upper water column where the salinity is often lower than that in deeper waters. There is very little information about the natural

ecology of haddock larvae, outside the Norwegian coast. However, the salinity in the area outside Austevoll Aquaculture Research Station where the broodstock of haddock were collected, varies between 25–30 ppt in spring season, when the larvae start feeding. In intensive aquaculture systems at Austevoll Aquaculture Research Station, the water supplied to feeding tanks is typically of deep-water origin, and is high in salinity (35 ppt). Mortality during start feeding of cold-water species such as halibut and cod is typically high in such systems (Raae et al 1988; Hamre et al 2002).

Haddock is a promising new species for aquaculture, but very little information on start feeding is available for this species. Downing and Litvak (1999) have investigated different light intensity and tank colour during start feeding. Hamlin and Kling (2001) have tested at what age haddock can be weaned to dry diet. The purpose of this experiment was to investigate if salinity influences the growth and survival of haddock larvae during the start feeding.

Materials and Methods

Experimental tanks and system design. The experiment was carried out at the Institute of Marine Research, Austevoll Aquaculture Research Station, Storebø, located 60° 05' N and 5° 16' E on the west coast of Norway. The haddock broodstock were collected from the area outside Austevoll Aquaculture Research Station and kept in 7000 l tanks, where the haddock spawned naturally. A single batch of haddock eggs was collected from the spawning tank. The eggs were incubated in black 70 l flow through tanks at about 8 °C. Two days after hatching, 1000 larvae were transferred to each of 20 black 50 l polyethylene tanks. The water was supplied at a flow rate of 400 ml min⁻¹ at the surface of each tanks from a recirculating system and exited through a cylinder in the middle of the tank, covered with 350 µm mesh. Four different salinity treatments (15, 25, 30 and 35 ppt), with five replicates, were chosen. The temperature in the egg incubators was 8 °C, while that in the tanks was initially 8.5 °C. Temperature was increased 1 °C per day until it reached 12 °C, at which it was maintained for the rest of the feeding period. The light intensity was 400 µW cm², with 12L:12D photoperiod. The larvae were fed rotifers (*Brachionus plicatilis*) day 4 after hatching. Rotifers were enriched with Rotimac (BioMarine AquaFauna Inc., USA) and were fed to larvae three times a day, 1 million day⁻¹ tank⁻¹ the first day, 3 million the second day, 4 million after 8 days and from day 15 onward 6 million day⁻¹ tank⁻¹. Every fourth day the bottom of the tanks were cleaned.

Measurements and analysis. Temperature, salinity and oxygen were measured daily. Ammonium level was measured once a week. Feeding incidence was measured 2 hours after feed was introduced, 30 larvae in each tank were examined under a light microscope (Leica MS 5). The experiment lasted until 25 DPH, except for the 15 ppt group; due to high mortality in this group it was ended 18 DPH. Survival was measured at the end of the experiment. Growth was measured as dry weight on day 4, 11, 18 and 25 after hatching, 24 larvae were sampled each time from each tank. The larvae were anaesthetised with MS 222 (Argent Chemical) washed in distilled water, and frozen for later weight analysis. The larvae were freeze-dried before weight determination with an electro balance (Metler Toledo, UMX 2).

Statistics. The weight data on day 18 failed the normality test. Thus, the non-parametric Kruskal-Wallis one-way analysis of variance on ranks was used to compare mean weights between treatments. On day 25 the normality test passed and a pairwise multiple comparison, Tukey test, was used to contrast treatments. Survival was compared between treatments using Dunn's test. Statistical software used was SigmaStat. Differences and effects were considered significant at $P < 0.05$.

Results

The level of ammonium was less than $6 \mu\text{M NH}_4^+$ in all tanks during the experiment and oxygen saturation varied between 93 - 100 %. The feeding incidences were 100 % in all groups 2 hours after feed was introduced.

Table 1. Percent survival (\pm sd) at the end of the experiment in the different salinity treatments.

| 25ppt (25 DPH) | 30ppt (25 DPH) | 35ppt (25 DPH) |
|----------------|----------------|----------------|
| 2.8 ± 0.5 | 31.7 ± 5.6 | 1.1 ± 0.4 |

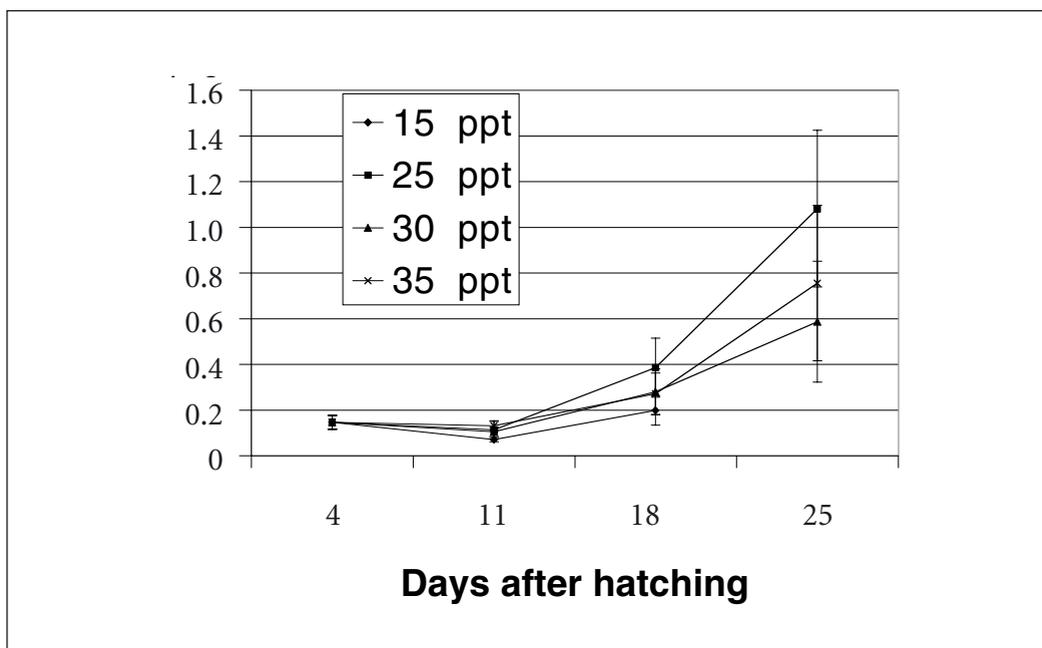


Figure 1. Mean dry weight \pm sd (mg) of haddock larvae reared in the four salinity treatments.

Survival. The highest survival rate, 31.7 %, was in the group with 30 ppt, and was significantly higher compared to 1.1 % in the group with 35 ppt (Table 1). ($P < 0,05$, Dunn'Methods). In the 15 ppt group, the mortality was high and this group was ended 18 DPH.

Growth. On day 18, larvae raised in salinity of 25 ppt had significantly ($p < 0.001$) higher mean weight (0.385 mg) compared to larvae raised (0.198 mg) in the 15 ppt group (Figure 1). The larvae raised in 30 and 35 ppt had similar mean weights (0.279 and 0.270 mg). On 25 DPH, the highest mean larval weight was still in the 25 ppt group, 1.081 mg and significant higher compared to 30 ppt group 0.590 mg ($p < 0.001$) and the 35 ppt group 0.755 mg ($p < 0.05$).

Discussion

The results from this experiment indicate that survival and growth of haddock larvae can be increased by lowering the salinity relative to the salinity of the deep water (34-35 ppt) used in the start feeding tanks at Austevoll Aquaculture Research Station. Morgan and Iwama (1991) proposed that energetic costs are lower in an iso-osmotic environment compared to hyper- and hypo-osmotic environment and that the energy savings are sufficient to permit increased growth. However, optimal salinity for growth and survival seems to differ among species, life stages and also seasons (Lambert et al. 1994).

Several authors have reported about better growth and survival with marine species by lowering the salinity. Johnson and Katavic (1986) found that survival and growth of sea bass (*Dicentrarchus labrax*) larvae were increased when ambient salinity (38 ppt) was reduced, and an intermediate salinity of 26 ppt produced the highest growth. Mouto et al. (2002) also report better growth rates in *Sparus aurata* at an intermediate salinity of 20 ppt, compared to 33 ppt. At the end of the experiment, the activity of total acid proteinases in the stomach and intestine extracts as well as the activity of the total alkaline proteinases, trypsin and alkaline phosphatase in the intestine were determined. Major protease activity was strongly influenced by salinity. The total protease activity and trypsin activity in the stomach were significantly higher in fish reared in low salinity. However, in the intestine the alkaline and acid protease activity was significant higher in fish reared in high salinity. The reasons for better growth of larvae raised in lower salinity might be due to improved digestibility and absorption efficiency, which could improve food conversion efficiency. Lambert et al. (1994) fed cod larvae on two different food regimes, and three different salinities (7, 14 and 28 ppt) and found no differences in food intake. However, larval cod had higher growth rates at lower salinities, probably due to an increase in food conversion efficiency. They reported best growth in 14 ppt compared to 28 ppt and 7 ppt. Holliday (1969) explained that the advantage of iso-osmotic salinity for teleost larvae may be reduced metabolic activity (to regulate the body fluids in order to restore the level of osmotic pressure to near normal) and, thereby, increased growth rate. Kinne (1960) has shown that young *Cypinodon macularius* exhibited differences in food intake at different salinities; for example, at 30 °C most food was eaten in 35 ppt, less in 15 ppt and 55 ppt, and least in freshwater. Growth was similarly affected. However, conversion efficiency of the food was a maximum in 15 ppt, less in 35 ppt, and less still in freshwater.

Survival in different salinities is affected by a combination of tissue tolerance and osmotic regulation shown by Weisbart (1968). He found that the alevins of *Oncorhynchus tshawytscha* survived longer in seawater than alevins of *O.kisutch* and *O.nerka* by virtue of a high tissue tolerance but that *O.gorbusha* and *O.keta* survived longer because of a higher ability to regulate serum sodium and chloride concentrations, and blood osmotic pressure. Johnson and Katavic (1986) report of increased larval survival on sea bass larvae with reduced salinity (below ambient levels). Holliday and Jones (1965) and Houde (1973) also observed increased survival of herring larvae at lower salinities; they associated these results with reduced activity and reduced metabolic demand.

Marine fish eggs and larvae are hypo osmotic to seawater (Holliday and Jones 1965). Yolk-sac larvae of *Clupea harengus* maintain a tissue ionic concentration that is approximately equivalent to about 12 ppt in seawater of salinity of 34 ppt (Holliday and Blaxter 1960). This is about one third the strength of seawater, even though the organs involved in osmoregulation of juveniles and adults are not developed at this stage (Morrison 1993). Regulation is presumably based on the movement of inorganic ions by cells of the ectoderm, and to osmoregulate the larvae need to use energy (Holliday and Jones 1965). During this experiment we used live feed. The water content in live feed is around 80 %, compared to dry diet for which the water content is less than 10 %. Great effort is put into developing dry diet for marine larvae. Feeding larvae with dry diet in 35 ppt water will probably exacerbate osmoregulatory problems for the larvae. In a weaning experiment, Hamlin and Kling (2001) found a similar survival rate (37.9 %) in a feeding experiment with haddock, start fed with rotifers at salinity 32 ppt. However, in the dry diet groups the survival rate was low (2.5 - 6.3 %) in early weaning groups. One reason to low survival during weaning might be problems with osmoregulation.

The results from this feeding experiment show different optima for survival (30 ppt) and growth (25 ppt), there might be several explanations for this. One might be that the optimum salinity was not found in this experiment and that it might lie somewhere between 25 and 30 ppt. Another reason might be that the larvae in the group with the highest survival did not get enough energy from the feed. However, theoretically, there should have been enough food items to supply the energy demands of the larvae (Houde and Schekter 1983; van der Meeren 1995). A third explanation might be size dependent mortality. Otterå (1992) reported higher mortality among smaller cod in a feeding experiment testing size selective mortality and growth. In the groups with highest mortality, the smallest larvae may have died. The lowest survival in this experiment was in the 35 ppt group 25 DPH, however, this group did not have the highest mean weight. The survival was higher in the 30 ppt group than that reported by Downing and Litvak (1999), who found a survival rate of 2 % with larvae raised in 32 ppt. Hamlin and Kling (2001) also used 32 ppt salinity and they found a survival rate on 37.9 %, which is similar to the highest survival rate from this experiment. The weight increment found in this experiment is similar compared to the weight increment found by Hamlin and Kling (2001). However, it is difficult to compare these results since the initial larval weight was different in these two studies. Further investigation is required in order to clarify optimum salinity for start feeding haddock larvae.

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Behaviour of Atlantic cod (*Gadus morhua*) larvae: an attempt to link maternal condition with larval quality

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Abstract

Egg quality has been linked to maternal condition in several species of fish. Such inter-female and/or inter-batch differences in egg quality should be visible in the larvae that hatch from them. In an attempt to characterize possible links between the condition of Atlantic cod (*Gadus morhua*) females and that of their progeny, we tested the hypotheses that female nutritional status, size and/or incubation temperature are related to larval quality (growth rate and behaviour). Fertilized eggs were obtained from discrete spawning couples that had been maintained under controlled feeding and temperature regimes. Eggs and larvae were handled so as to maintain those from any given male-female cross and spawning event in isolation. Temperature was held at 6 °C and photoperiod was 14 h L:10 h D. The treatments applied to cod females were maternal ration x maternal temperature history (in 1995) and, in a second experiment, maternal ration x maternal size (in 1996). Three of the five or six male-female pairs from each of the four ration-temperature treatment groups, or ration-size treatment groups were targeted as sources of fertilized eggs. Larvae from these groups were subsampled at various intervals and their size and dry mass measured. Specific growth rate (SGR) was calculated from these data. Silhouette (shadow) video photography (SVP) was used to record the behaviour of cod larvae originating from these females-egg groups. SVP observations of cod swim paths were analysed frame-by-frame to extract the three-dimensional swim path coordinates of each larva. These swim paths were decomposed and analysed for swim speeds, durations and distances, “pause” (non-active displacement) duration, and vertical and horizontal changes of direction. The significant relationships between larval SGR and the swim path variables extracted from our behavioural observations support their use as indicators of

larval performance-quality. While the incubation temperature of cod females was not related to our behavioural proxies of larval performance, there was at least an indication of a link with maternal size and ration, although the behavioural responses were inconsistent and, therefore, difficult to interpret. We conclude that, at least under non-extreme conditions (i.e., very low or very high temperatures and/or nutritional condition and/or size), the condition of cod females does not strongly affect the behavioural performance of the larvae that they produce. This conclusion must be qualified by saying that the behaviour of cod larvae from even a single male-female cross and egg batch is highly variable and that this confounds (and possibly masks) differences in the overall performance of larval groups.

Introduction

The concept of spawning stock biomass (SSB) – a fundament of fish population assessments – has recently been refined to include the observation that females do not all contribute equally to the fecundity of a population (Hislop 1988; Chambers et al. 1989; Solemdal 1997; Ouellet et al. 1997; Trippel et al. 1997; Dutil et al. 1998, 1999; Marshall et al. 1998; Marteinsdottir and Steinarsson 1998; Trippel 1999; Marteinsdottir and Begg 2002; Koster et al. 2003; Rätz and Lloret 2003). To succeed with this refinement of SSB, the “quality” of eggs and larvae (*sensu* Brooks et al. 1997), and not only the quantity, must be measurable, and strongly and consistently correlated with some index of spawner condition. Even in the simplest case of a species that releases all (or most) of its gametes at one time, this is a challenging assignment. It is even more difficult for species such as Atlantic cod (*Gadus morhua*), in which females are highly fecund determinate spawners that release their eggs in batches (Kjesbu 1989). Despite this, there have been several attempts to characterize the relationships between the condition of cod females and the number and quality of eggs that they produce (Kjesbu 1989; Kjesbu et al. 1991; Kjesbu 1994; Kjesbu and Holm 1994; Solemdal et al. 1995; Kjesbu et al. 1996; Chambers and Waiwood 1996; Marteinsdottir and Steinarsson 1998; Trippel 1998; Ouellet et al. 2001). Although the results of these studies have been inconsistent (reviewed by Ouellet et al. 2001, and see Discussion), it appears that, at least for females in good condition, (1) 17–19 egg batches are spawned per female over a period of four to six weeks, (2) the number of eggs liberated in each batch is variable, but is usually highest for the groups near the middle of the female’s spawning cycle, (3) egg size and dry mass decrease from the first to the last batch, (4) egg diameter of the first batch is positively correlated with female length and (5) there is an inverse relationship between egg diameter and egg mortality. Further, cod females with high condition factors produce more previtellogenic oocytes, and use a larger fraction of them during vitellogenesis, than females with low condition factors (Kjesbu et al. 1991). Overall fecundity is also related to female condition; females with low condition factors produce fewer eggs than those with high condition factors (Kjesbu et al. 1991).

Inter-female and/or inter-batch differences in egg quality should be visible in the larvae that hatch from them. In yellow perch (*Perca flavescens*), older-larger females produced larvae that were shorter, but with larger yolk sacs and higher energy reserves than larvae from younger-smaller females (Heyer et al. 2001). These larval characteristics translated into differential survivorship,

although not differential growth (Heyer and Miller 2004). Female sea bass (*Dicentrarchus labrax*) in poor nutritional condition produced a much higher percentage of non-viable larvae than females in good condition (Cerda et al. 1994). This was also the case for Nile tilapia (*Oreochromis niloticus*) (Gunasekera et al. 1996). In the Japanese flounder (*Paralichthys olivaceus*), the percentage of normal larvae, survival of larvae at 3 days post hatching (DPH), and an index of starvation tolerance, were all positively correlated with levels of n-3 highly unsaturated fatty acids, and of arachidonic acid, in the diets of females (Furuita et al. 2000, 2003).

Female condition was positively correlated with feeding success of cod larvae, and larvae from older-larger females exhibited higher specific growth rates (at 15 DPH) (Marteinsdottir and Steinarsson 1998; Marteinsdottir and Begg 2002). Eggs liberated towards the end of a given female's spawning cycle (i.e. those from the last few egg batches) appear to produce larvae whose overall activity is less than that for larvae from earlier egg batches (Fig. 11 in Solemdal 1997, drawn from the unpublished results of A.B. Skiftesvik). Because activity and feeding rates translate into rates of growth and survivorship, these observations imply that cod larvae hatching from different egg batches, and/or from different females, may be more-or-less viable. In an attempt to further characterize possible links between the condition of cod females and that of their progeny, we tested the hypotheses that female nutritional status, size and/or incubation temperature are related to larval quality (e.g. growth rate, and as defined by Brooks et al. 1997). To succeed, we first had to establish that behavioural observations (overall activity, swim speed, swim path length, etc.) could be used as indicators of larval quality – observations that poor and/or sick groups of larvae are moribund, and behave differently from good and/or healthy groups (e.g. Skiftesvik et al. 1993), established that this was feasible. The work reported upon here further establishes a link between the behaviour of cod larvae and their quality and, with this as a basis, tests the hypothesis that female condition is related to the quality of the larvae that they produce.

The relationships between female cod and their larvae were evaluated in laboratory experiments conducted in 1995 and 1996. Material from these same experiments was used for complementary studies on female reproductive physiology-fecundity, and on egg quality (Lambert and Dutil 2000; Ouellet et al. 2001). The results on larvae, reported here, completes the life history stages that were evaluated in this project's attempt to trace maternal effects from pre-spawned gametes through to larvae.

Materials and Methods

Brood stock. Adult Atlantic cod used as brood stock in these experiments were captured by trawl from the St. Lawrence Estuary in June 1994 and 1995. After capture, and prior to experiments, the fish were maintained under natural photoperiod and temperature in 7.5 m³ tanks at the Maurice Lamontagne Institute, Mont-Joli, Québec, Canada. During this period, animals were fed to satiation three times weekly on a diet of frozen capelin (*Mallotus villosus*).

Each year, 400 cod were divided into four groups of 100 fish that were transferred to separate 7.5 m³ tanks in September. During the first experiment (referred to hereafter as the 1995 experiment), brood stock was further separated into two groups which were maintained under two temperature

regimes. In the first, cod were acclimated to 10 °C and the temperature was then gradually decreased (from September 1994 through January 1995) to a minimum of 6 °C. Afterwards, temperature was kept constant throughout spawning, which began in April 1995. In the second temperature regime, cod were acclimated to 6 °C and temperature was then decreased to 2 °C. Afterwards, temperature was kept constant throughout spawning. The temperature regimes applied are realistic of those to which cod from this geographic region would be exposed during the period preceding the reproductive season (i.e. gonad maturation) (Green and Wroblewski 2000). During the second experiment (referred to hereafter as the 1996 experiment), temperature was maintained at 4–5 °C throughout.

In both the 1995 and 1996 experiments, brood stock were fed upon different dietary regimes (details presented in Dutil and Lambert 2000) in an attempt to generate females in a range of pre-spawning nutritional condition. In the 1995 experiment, cod females were randomly assigned to the different feeding regimes, irrespective of their size. However, in the 1996 experiment, cod females were partitioned into two size classes (<55 cm: 51.2 ± 1.19 cm and >55 cm: 60.9 ± 2.99 cm). For each of these size classes, one group of females was fed on a low ration, and another on a high ration (Lambert and Dutil 2000). The condition factor (Fulton's K), expressed as the ratio between total weight (g) and length³ (fork length in cm) multiplied by 100, was used as an index of condition for pre-spawning females. In cod, Fulton's K is a strong indicator of nutritional condition and energy reserves (Lambert and Dutil 1997a, b). Females were sacrificed 15 d after the production of their last egg batch. Fork length, total weight, and gutted liver and gonad weight were measured.

Spawning, egg collection and egg characteristics. During each experiment, females were paired with males in 1.2 m³ circular tanks. This allowed for egg production by each female to be monitored throughout their spawning cycle, and for every batch of eggs released by females to be collected individually. A complete description of the holding conditions and methods of egg collection and sampling is presented in Lambert and Dutil (2000) and in Ouellet et al. (2001).

The spawning activity of 17 females in the 1995 experiment, and 21 females in the 1996 experiment, was characterized. For each spawning event, the volume (ml) and number of eggs released in each and every batch was measured. Mean egg diameter, dry mass and energy content for each batch were measured (Table 1 in Ouellet et al. 2001). In the 1995 experiment, total lipid and total yolk protein content were also measured for each batch of eggs. Weighted mean egg diameter and dry mass were calculated for each female by normalizing the means for each batch by the number of eggs in that batch. Details of the analytical techniques used to obtain these data are presented in Ouellet (1997) and Ouellet et al. (2001).

Source of animals and experiments on larval behaviour. Three of the male-female pairs – from each of the four ration-temperature treatment groups (in 1995), or ration-size treatment groups (in 1996) – were targeted as sources of larvae. Two egg batches from each of these females were collected: the second or third (typically of relatively poor quality) and the fifth or sixth (typically of relatively high quality). As much as possible, these were the same spawning pairs and egg groups studied by Ouellet et al. (2001). A total of 21 groups of larvae were studied (Table 1).

Table 1. Summary data on Atlantic cod (*Gadus morhua*) females, egg batches, and larvae which were the source of material for the growth and behavioural experiments reported upon here. Designation = female number-egg batch number; Treatment t_1 1 = high ration; Treatment t_1 2 = low ration; Treatment t_2 1 = 6 °C; Treatment t_2 2 = 2 °C; Treatment t_2 3 = large female; Treatment t_2 4 = small female; Condition Initial = pre-spawning condition factor (Fulton's K); Condition Final = post-spawning condition factor (Fulton's K); Incubation time = time (in days) between spawning and 50% hatching of eggs; Fecundity = % fertilized eggs (from visual estimate of a sub-sample); Initial volume = volume of eggs produced; SGR of larvae, BL = body length; BW = body weight. (-) denotes no data available. Data on adults are drawn from Lambert and Dutil (1999), and on eggs from Ouellet et al. (2001).

| Designation | Year | Treatments | | Adult females | | | Eggs | | | | Larvae | | | |
|-------------|------|------------|-------|---------------|-----------------|---------------------|---------------|---------------------|-------------------------------------|-----------------------------------|----------------------------|-----------------------|-----------------------------------|-------|
| | | t_1 | t_2 | Initial | Condition Final | Incubation time (d) | Fecundity (%) | Initial volume (ml) | Fresh weight egg ⁻¹ (mg) | Dry weight egg ⁻¹ (µg) | Energy J egg ⁻¹ | Egg diameter ±SD (mm) | SGR (% BL or BW d ⁻¹) | (mm) |
| B08-02 | 95 | 1 | 2 | 1.02 | 0.77 | 13 | 95 | 127.0 | 1.9 | 96.9 | 1.73 | 1.43 ± 0.04 | 1.60 | 11.68 |
| B04-06 | 95 | 2 | 2 | 1.11 | 0.82 | 13 | 95 | 156.0 | 1.9 | 94.4 | 1.55 | 1.38 ± 0.03 | (-) | (-) |
| B04-09 | 95 | 2 | 2 | 1.11 | 0.82 | 12 | 60 | 50.0 | 1.6 | 70.6 | 1.26 | 1.31 ± 0.04 | 1.09 | 5.50 |
| B15-05 | 95 | 2 | 1 | 1.13 | 0.78 | 13 | 80 | 169.0 | 1.3 | 64.9 | 1.15 | 1.27 ± 0.04 | 0.39 | 0.85 |
| B15-01 | 95 | 2 | 1 | 1.13 | 0.78 | 12 | 80 | 58.0 | 1.7 | 70.3 | 1.26 | 1.37 ± 0.03 | (-) | (-) |
| B01-01 | 95 | 2 | 2 | 0.88 | 0.64 | 12 | 95 | 52.0 | 2.3 | 116.2 | 1.97 | 1.44 ± 0.06 | 1.52 | 6.99 |
| B01-03 | 95 | 2 | 2 | 0.88 | 0.64 | 13 | 100 | 138.0 | (-) | (-) | (-) | 1.50 ± 0.03 | 1.98 | 10.87 |
| B01-07 | 95 | 2 | 2 | 0.88 | 0.64 | 14 | 70 | 175.0 | 1.9 | 87.4 | 1.66 | 1.38 ± 0.07 | 0.65 | 3.65 |
| B13-02 | 95 | 1 | 1 | 1.18 | 0.80 | 15 | 80 | 119.0 | 2.3 | 95.8 | 1.79 | 1.46 ± 0.06 | 2.01 | 6.317 |
| B13-05 | 95 | 1 | 1 | 1.18 | 0.80 | (-) | (-) | (-) | 1.8 | 82.7 | 1.46 | 1.36 ± 0.03 | (-) | (-) |
| B19-05 | 95 | 1 | 1 | 1.05 | 0.77 | 13 | 90 | 100.0 | 1.7 | 90.7 | 1.50 | 1.35 ± 0.04 | 1.89 | 5.07 |
| B19-09 | 95 | 1 | 1 | 1.05 | 0.77 | 15 | 10 | (-) | 1.2 | 56.3 | 1.03 | 1.22 ± 0.04 | 0.91 | 3.17 |
| B07-03 | 95 | 1 | 2 | 1.08 | 0.70 | 14 | 50 | 61.0 | 2.3 | 103.1 | 1.74 | 1.46 ± 0.03 | (-) | (-) |
| B07-06 | 95 | 1 | 2 | 1.08 | 0.70 | 15 | 95 | 110.0 | 1.8 | 74.9 | 1.35 | 1.37 ± 0.03 | 0.88 | 4.11 |
| B03-03 | 96 | 2 | 4 | 0.84 | 0.60 | 14 | 100 | 44.0 | 2.1 | 130.4 | 2.34 | 1.48 ± 0.02 | (-) | (-) |
| B03-07 | 96 | 2 | 4 | 0.84 | 0.60 | 12 | 99 | 25.8 | 1.7 | 110.1 | 2.02 | 1.43 ± 0.03 | 2.80 | 16.56 |
| B05-04 | 96 | 1 | 3 | 1.29 | 0.75 | 12 | 95 | 228.0 | 2.3 | 147.4 | 2.96 | 1.61 ± 0.04 | 2.04 | 10.13 |
| B05-06 | 96 | 1 | 3 | 1.29 | 0.75 | 14 | 80 | 216.0 | 2.2 | 143.5 | 2.83 | 1.54 ± 0.03 | 1.88 | 0.09 |
| B06-06 | 96 | 1 | 3 | 1.29 | 0.78 | 12 | 95 | 567.0 | (-) | (-) | (-) | (-) | 2.24 | 9.14 |
| B08-03 | 96 | 2 | 4 | 0.82 | 0.61 | 15 | 90 | 19.4 | 1.7 | 95.4 | 1.67 | 1.40 ± 0.13 | 1.79 | 8.26 |
| B13-03 | 96 | 1 | 4 | 1.05 | 0.68 | 13 | 99 | 96.6 | 2.0 | 120.0 | 2.35 | 1.45 ± 0.03 | 2.32 | 13.64 |
| B16-03 | 96 | 2 | 4 | 1.01 | 0.66 | 11 | 99 | 139.0 | 2.0 | 117.9 | 2.28 | 1.47 ± 0.03 | 2.59 | 14.86 |
| B17-03 | 96 | 1 | 4 | 1.19 | 0.82 | 14 | 90 | 86.2 | 2.4 | 152.0 | 2.72 | 1.48 ± 0.04 | 1.92 | 9.23 |
| B20-03 | 96 | 1 | 4 | 0.88 | 0.62 | 14 | 90 | 267.0 | 2.5 | 122.7 | 1.85 | 1.72 ± 0.20 | 2.29 | 10.29 |
| B20-07 | 96 | 2 | 3 | 0.88 | 0.62 | 15 | 99 | 67.5 | 1.6 | 95.6 | 1.78 | 1.37 ± 0.06 | 2.06 | 11.09 |
| B21-06 | 96 | 1 | 3 | 1.17 | 0.72 | 13 | 100 | 104.0 | 2.3 | 135.3 | 2.56 | 1.54 ± 0.05 | 3.08 | 21.35 |

Eggs were incubated in black 60 l round-bottom tanks (at 6 ± 1.0 °C). Larvae were transferred to fresh 60 l black tanks at 3 DPH (just prior to first exogenous feeding). The rearing basins were stocked with algae (*Nannochloropsis sp.*) (green water technique), and larvae were fed nutritionally enriched (INVE Aquaculture Super Selco®) rotifers (*Brachionus sp.*) at 7 ml^{-1} . Eggs and larvae were handled so as to maintain those from any given female and batch in isolation. Larvae were cultured at 6 °C on a 14 h L:10 h D photoperiod at a crepuscular-level light intensity of $1.20 \mu\text{E sec}^{-1} \text{ m}^{-2}$ (diffuse light from overhead fluorescent lamps).

Measurements made on larvae. Larvae from all targeted groups were sampled at various intervals for length and weight. Larval size (standard length) was determined from measurements on live larvae examined (on ice) under a microscope. Immediately after the standard length measurements, specimens were dried at 50 °C for 24 h and then weighed on an electrobalance to the nearest 0.001 mg. Drying was continued until there was no further change in mass. For each egg batch, 10 to 12 larvae were collected from the rearing basins beginning at approximately 6 DPH. A minimum of three such measurements were obtained between 6 and 20 DPH. The mean length and dry mass measurements were used to calculate the specific growth rate for mass (SGR_w), and for standard length (SGR_l), as follows (Ricker 1979):

$$\text{SGR}_w = \frac{BM_f - BM_i}{BM_i(T_f - T_i)} \times 100 \quad \text{and} \quad \text{SGR}_l = \frac{BL_f - BL_i}{BL_i(T_f - T_i)} \times 100$$

where, BM_f and BL_f are mean body mass (mg) and body length (mm) for the last day of measurements, BM_i and BL_i are for the first day of measurements and T_f and T_i are the ages of the larvae on the first and last day on which they were measured.

Imaging system for behavioural observations. Silhouette (shadow) video photography (SVP) (Arnold and Nutall-Smith 1974; Edgerton 1977; Browman et al. 1989; Browman and O'Brien 1992a,b; Browman et al. 1994) was used to observe the behaviour of cod larvae. SVP is superior to standard cinematographic or video imaging techniques in various ways. First, it allows filming of events in a large depth of field (in this case, approximately 20 cm) with a relatively large field of view (limited only by the size of the collimating lenses, here 15 cm). Second, magnification is independent of distance from the cameras and the resolution is very good; objects as small as 0.2 mm can be resolved. Third, image quality is unaffected by ambient light levels and the silhouette effect is attained without the use of intense light sources. As such, the behaviour of cod larvae can be observed under relatively natural conditions.

Our SVP observation system consists of two orthogonally-oriented optical rails, with the observation aquarium (20 x 20 x 20 cm) placed at their intersection (Fig. 1). The imaging optics on each rail consist of a far-red light emitting diode (LED) placed at the focal point of a 15 cm diameter bi-convex collimating lens whose output passes through the aquarium. The LED's output is less than $10^{-6} \text{ W cm}^{-2}$ – an intensity below the perceptual threshold for the red photoreceptor channel of fishes. The use of a collimated beam prevents perspective distortion and projects clear, sharp shadows of any organism (even a small and virtually transparent one) in the beam's path. Shadow

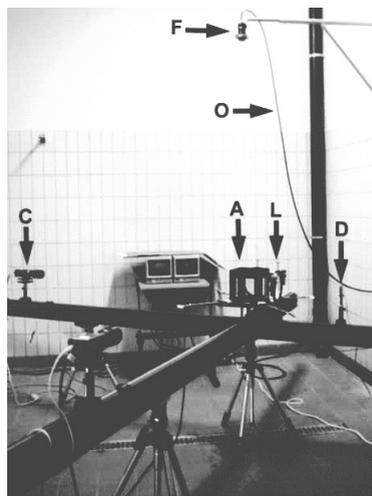


Figure 1. Silhouette video photography system used to make behavioural observations of Atlantic cod (*Gadus morhua*) larvae. Light from a 1-kW Xenon arc lamp (not shown) is focussed onto the aperture of a UV-visible liquid light guide (O) and passes through various optical components in a filter holder (F) before reaching the test aquarium (A) in which the animals are freely swimming in a 20 cm aquarium. In the experiments reported upon here, standard fluorescent fixtures (in the ceiling above the test tank) were used as the light source. The aquarium is located at the intersection of two 3 m long optical rails. Each rail supports a far-red light emitting diode (D) placed at the focal point of a 14.5 cm diameter biconvex collimating lens (L), and a video camera (C) to image the shadows projected by the collimated beam that passes through the aquarium. Also shown are the lasers (Ar) that are used to align all of the optical components on the rails prior to a given set of experiments. The red spot at the far end of one of the rails is the light emitted by the diode. (Reprinted from: *Journal of Experimental Biology* 203: 1649-1657).

images are collected by a single lens reflex lens (Tamron 70-210 mm zoom) attached to a 0.5 inch CCD sensor video camera (Panasonic WV-BL730) and recorded using a S-VHS video tape recorder (Panasonic AG-6730). The optical components on each rail are aligned using helium-neon lasers, which also allow the vertical viewing heights and orthogonal orientation of the two rails to be established precisely. The synchronously-recorded orthogonal views allow exact determination of the three-dimensional positions of targets (larvae and/or their prey) which appear in both fields of view simultaneously. The outermost 5 cm of the aquarium walls were covered with black plastic (matte-surface) contact paper. This restricted the field of view to the central 15 cm volume of water and ensured that the behaviours observed were not influenced by surface or edge effects; only cod larvae freely swimming in the water column were imaged, and their displacements analyzed.

Overview of analytical software. The custom computer programs TRAKFISH and ANAPATHS (Racca Scientific Consulting and JASCO Research Ltd., Victoria, British Columbia, Canada) were used to provide end-to-end processing of the video records. Their principles of operation will be described here to a sufficient extent to allow full understanding of the process through which larval motion statistics are obtained, including a description of the various software configuration settings and how they affect the analysis.

TRAKFISH has its roots in a software application developed for the study of flow patterns in experimental fluid dynamics by automatic tracking of particle tracers (Racca 1985; Racca and Dewey 1988). In the original approach, the test volume of a flow channel was photographed by a high-speed cine camera through a mirror arrangement that recorded simultaneous views through perpendicular windows; the film frames were then scanned to digital images that the software analysed. In TRAKFISH, the video digitization capability is built into the software along with video tape recorder step control functions, although the program can also process pre-digitized sequences from a real-time direct-to-disk video acquisition system (Tempus-PC, Kinetic Imaging Ltd). The latter also provides the option of recording the video sequences as individual non-interlaced fields

instead of interlaced frames, which effectively doubles the time resolution of the recordings and subsequent analysis to 50 images per second for the European video standard.

TRAKFISH performs simultaneous tracking of all identifiable targets directly from dual video sequences, each showing a view of the test aquarium from a perpendicular direction through the SVP set-up. Tracking is carried out for an arbitrary number of steps specified by the user at the start of a run; the increment between processing steps is also user specifiable and can be one or more video frames (or fields) – a larger increment may be appropriate for slow moving targets with steady trajectories. To identify the locations of the targets in the field of view, the digitized video images are subjected to binarization (selection of individual pixels as belonging either to a target or to background) based on either a brightness threshold or an edge strength threshold as selected by the user. This leaves clusters of connected target pixels commonly known as blobs; due to thresholding imperfections in real life images these may contain breaks or spurious bridges between adjacent targets. Blob processing is then performed to clean up these anomalies and to exclude targets whose size (area) is outside user specified limits, after which the centroids of the accepted objects are located. In an alternative operating mode used for larger subjects, TRAKFISH can instead locate the endpoints (head, tail) of elongated targets regardless of their bending or curling. At this stage a video frame (in either view) has been reduced to a collection of identified locations expressed in pixel coordinates. These are converted to real-world two-dimensional coordinates in the frame of reference of the aquarium through scaling formulae obtained from a calibration procedure that is performed when the geometry of the SVP apparatus, or the magnification of the zoom lenses, are modified. To calibrate TRAKFISH, a transparent plastic overlay with four fiduciary marks in a rectangular configuration is placed against each front window of the aquarium; the user then identifies the fiduciary marks by mouse-clicking them in the digitized views presented by the software. The rectangles defined by the fiduciary marks can be regarded as the projection onto the two image planes of a parallelepipedal “reference volume” located inside the aquarium; a scaled coordinate system is established by entering into the software the real-world dimensions of this reference volume and its offset from the bottom corner of the aquarium at the common edge of the front windows, which is taken as the origin. The dimensions of the entire water volume in the aquarium are also specified for the purpose of displaying in the main software window a projective outline, or “virtual aquarium”, within which the paths of tracked targets are plotted during a run.

The next step in the motion analysis by TRAKFISH is the matching of positional data in the two simultaneous perpendicular views to yield the three-dimensional spatial coordinates of targets. We shall refer to the projections in the two view planes of a particular target location in space as an *orthogonal pair*, after the term *stereo pair* used in photogrammetry to denote the corresponding views of a given feature in stereoscopic images. The correct identification of orthogonal pairs must be based solely on geometrical considerations, since visual cues such as object shape—which can be helpful in the matching of stereo pairs—provide little or no assistance in orthogonal views due to the potentially different aspect of an object seen from perpendicular directions. In the absence of parallax and misalignments the projection coordinates of orthogonal pairs would have identical values in the direction common to both views, which in the optical arrangement used here is the vertical or z axis. Thus, for projected coordinates (x_0, z_0) in one view, and (y_0, z_0) in the

other, the spatial location of the target would be at (x_0, y_0, z_0) . Racca (1985) derived mathematical relations to reconstruct the spatial position of a target point from its orthogonal projections fully accounting for parallax and, thereby, provided a criterion to verify whether two projected points constituted an orthogonal pair. In the SVP optical system used in this work, however, images of objects within the visible volume are projected onto the perpendicular view planes with no significant parallax thanks to the collimated back lighting; the z coordinates of orthogonal pairs can, therefore, be assumed to be identical. In practice, small discrepancies will always exist because of the effect of slight optical aberrations and misalignments and the variability in the automated placement of a target's centroid depending upon the shape and contrast of the object in each view. TRAKFISH does, therefore, allow the user to specify a matching tolerance (in mm; 3.0 mm in the analyses reported here) that represents the maximum discrepancy between z values of projected points that would still be accepted as forming an orthogonal pair. Because of this finite matching tolerance, at high densities of targets it is possible that there will be more than one match candidate for a point in one of the views. In this case, TRAKFISH ranks the candidates based upon the difference in z values (smaller differences yielding a higher rank) and accepts up to n highest ranked matches, n being a user specified parameter denoted as the multiple match allowance. It stands to reason that n in principle should be unity, as only one pair of projected points can arise from a target. The concept of multiple match allowance was introduced to allow for cases where two projected points in one view coalesce into a single centroid position, thus requiring a one-to-many matching, or where the correct pairing is indeed not the one with the least z difference because of the positioning variability mentioned above. Allowing multiple matches has the serious drawback of potentially creating ghost targets, or incorrectly reconstructed spatial positions that would be tracked along with real targets. The rationale for allowing this to occur is that the conditions resulting in multiple matches for any given point in a view are unlikely to persist for more than a few frames as the relative positions of targets shift, and it would be far more damaging to the motion analysis to lose a track because of an incorrect match than to have occasional, short lived spurious tracks from ghost points.

The outcome of the matching step is a collection of (x, y, z) triplets representing the spatial location of all identified targets (and possibly some ghost targets) for the current frame. The process of tracking targets consists primarily of finding, among the spatial locations in the current frame, the most appropriate continuations to any paths that have already been followed through previous time steps, and secondarily, of attempting to start new paths out of those locations that are not assigned to existing ones. The algorithm for following a target is based upon the assumption that from one time step to the next its velocity vector does not undergo extreme changes but rather evolves gradually in both magnitude and direction. Under this assumption, the target displacement during one time step can be linearly extrapolated as an approximation of the path in the next step. Having thus set a predicted position, the algorithm searches within a surrounding sphere of radius r_{old} (user specified; 6.0 mm in the analyses reported here) for available target locations. If any are found, the existing path is extended to the one closest to the predicted position and that target location is made unavailable to other trajectories. If no successor can be found within the search sphere, the path is terminated at the previous step. The process is repeated for all established paths.

A target location that does not become assigned to an existing path is tentatively considered a

new object for which a path can be started. The beginning of a path must be handled in a different way, since a predicted trajectory step cannot be constructed. In this case the algorithm uses a larger search sphere of radius r_{new} (also user specified; 12.0 mm in the analyses reported here) surrounding the starting position; r_{new} must be made as large as the maximum expected displacement for the subjects under study in one time step, unlike r_{old} that only needs be large enough to allow for the expected deviation from a steady path. To create the first path step the algorithm looks at available target locations in the next frame that are within the search radius r_{new} ; any such location could potentially be a successor. The location that is nearest to the original position is considered first. Based on this provisional step a position is extrapolated one step further as previously explained, and a second successor is sought within a radius r_{old} from it. If one is found, the two-step trajectory is accepted and added to the collection of active paths. Otherwise, the process goes back one frame and attempts to start a path using the next nearest tracer to the original position still within a radius r_{new} . The process continues until either a trajectory has been started or all available successors in r_{new} have been exhausted, in which case the new target is abandoned.

Because tracking is performed in a parallel fashion on all targets identified as video frames are processed, a handling hierarchy must be assigned to control the order in which paths are extended or started. TRAKFISH follows a hierarchy based on the current lifespan of trajectories, meaning that targets that have been followed through the largest number of steps are analysed first. Since a successor, once assigned, is made permanently unavailable to other trajectories, this scheme ensures that well-established paths will not be cut off by a new target appearing for the first time. To manage the overhead of this intricate process TRAKFISH maintains three arrays of target positions, corresponding to frames $F - 1$, F and $F + 1$ (with the understanding that “frames” here denotes images in an evenly spaced sequence, and not necessarily consecutive video frames or fields). On the first iteration, all targets in $F - 1$ are new and, therefore, undergo the two-stage start-up tracking through frames F and $F + 1$. The old $F - 1$ is then lost, F becomes $F - 1$, and a new $F + 1$ is created by processing a new pair of video frames (one for each view). On all following iterations, paths are handled in order of seniority, with newly appearing targets being considered last. Target locations in $F - 1$ that were assigned to paths have already a successor in F from the previous iteration, so their next position can be predicted and a successor sought in $F + 1$. New targets in $F - 1$ undergo two-stage tracking as usual. The frames are again shifted back one position and the process continues until $F + 1$ is the last frame in the sequence. As the analysis progresses the coordinates of tracked targets and the identifiers of the paths to which they belong are output to an ASCII file; the evolving trajectories are also displayed graphically within the outline of the “virtual aquarium”.

The software ANAPATHS was developed to perform various types of analysis on the path records generated by TRAKFISH. This program includes visualization, sorting, and editing functions to facilitate certain preliminary operations such as the selection of the longest or most enduring paths and the splicing of disjoint segments of the same path (which can occur due to tracking anomalies) into a continuous trajectory. The latter function is aided by a “wizard” (automated assistant) process that identifies and presents to the user all likely splicing candidates based on the spatial and temporal nearness of their end-points, allowing the selective approval and immediate implementation of individual splices.

The analytical functions available in ANAPATHS include the computation of the fractal dimensions of paths in single planes or in three-dimensional space to provide a measurement of the morphological complexity of swim patterns. The most important feature of the software in the context of the present study, however, is the pause/movement statistical analysis of swim trajectories. This component of ANAPATHS is based on research by Collins et al. (1995) on the reliable identification of stops in search pathways, and the code for ANAPATHS incorporates an extended version (Racca Scientific Consulting) of the computer program STOPGAP (©1991-1995 by R.D. Collins and M.K. Tourtellot, used by permission) introduced in that work. STOPGAP accepts one or more trajectories, being sequences of two- or three-dimensional coordinates with a constant time interval between consecutive points, and for each trajectory it identifies stops and periods of movement. A stop is defined to occur when the subject fails to move a specified minimum distance (length criterion) over a given period of time (duration criterion); both criteria are selectable by the user (0.3 mm and 0.3 s, respectively, in the analyses reported here) and are tuned to the movement pattern of the organisms being observed. The first stage in stop identification is to tentatively classify as being part of stops those trajectory steps in which the distance covered is less than the length criterion, and the remaining ones as being part of moves. The next stage is to iteratively evaluate stop duration. With each iteration the validation interval is increased by one time step; moves lasting less than that interval are redefined to be stops, and stops lasting less than that interval are redefined to be moves. The process continues until the validation interval reaches the duration criterion. Having identified and validated the stops and the intervening move periods for a trajectory, the original STOPGAP program computes statistics (mean, standard deviation, minimum and maximum) for stop duration, distance between points during stops, move duration, and distance between points during moves. The extended version that was developed for use in ANAPATHS computes three additional quantities from the analysis of stops and moves: the path length for each move period, the change in direction after a stop (turn angle) in a horizontal plane, and the same in a vertical plane. For the purpose of measuring the turn angles, horizontal and vertical motion vectors are computed based on the overall displacement of the subject during a move period; this approach is justified if it is assumed that no significant changes in direction of swimming occur during moves. Turn angles are recorded as unsigned values, that is, the direction of turn (its possible asymmetry, or handedness) is not taken into account in the statistics. For each path analysed, the program generates statistics for these additional parameters as well as the ones originally in STOPGAP. It also computes the overall activity level, defined as the percent of time spent moving over the lifetime of the trajectory. Furthermore, the stop/move analysis code in ANAPATHS generates discrete frequency distributions for stop duration, move duration, horizontal turn angle, vertical turn angle, move length and move speed. The upper limit of the binning range for each parameter (the lower limit being always zero), and the number of binning intervals used to compute the frequency distributions, are user specifiable. The results for an analysis run (which may contain statistical results for each of several paths selected) are output to a report file in plain text form. ANAPATHS provides a function to pool the results from one or more report files into a single set of statistics for all of the paths therein. The frequency distributions in these pooled statistics are normalized to a total of unity, unlike those for individual paths that contain raw counts of moves or stops per bin. ANAPATHS also outputs the raw values used to generate the frequency distributions.

Behavioural observations and their analysis. Larvae were tested at 3 and 6 DPH. Three DPH was just before the initiation of exogenous feeding; 6 DPH was after. Comparing and contrasting observations made on these two days was meant to provide a baseline with respect to differences in behaviour associated with the onset of feeding (*sensu* Skiftesvik 1992). On each test day, fifty larvae from any given group were placed into the test aquarium and video taped for 30 min. This was repeated three times, with 50 new larvae observed each time (and, thus, three replicates). Due to the nature of the imaging system, the behavioural observations cannot be attributed to any single larva – they swim in and out of the field of view and, therefore, it is impossible to tell one larva from another. Further, observations on any one larva in a tank containing 50 are not independent (in a statistical sense). Thus, for statistical analysis (see below), all observations made on the 50 larvae in any given tank collapse down to a sample size of one. To minimize the possibility of subsampling artefacts, swim paths of cod larvae were extracted using TRAKFISH from two to three 5 min. segments drawn from different times during the 30 min. of observation. Thus, for any given test group of larvae, there were three replicate 30 min. observations periods, each of which was then subsampled for 10-15 min.

Swim paths extracted by TRAKFISH were analysed using ANAPATHS. Path analysis followed principles (thoroughly described in Bell 1990, pp: 281-300; O'Brien et al. 1989; 1990) that are widely applied to study the movement patterns of species in many taxonomic groups (e.g. Anderson et al. 1997; Kramer and McLaughlin 2001). For each of the 5 min. subsamples, the longest 20 tracks were identified and combined (recall that, for statistical analysis, all of these paths must be collapsed into one since they are not independent). Thus, the 40-60 longest paths for each replicate 30 min. observation period – representing a mean displacement per replicate of 2.23 ± 0.178 m in 1995 and 0.734 ± 0.10 m in 1996 – were used as the basis for the analysis. A total of 215 m of cod larval swim paths were analysed.

ANAPATHS extracted the following variables from the swim paths: durations of stops (non-active displacement, as defined and operationalized by Collins et al. 1995), lengths and durations of moves, and turn angles (decomposed into vertical and horizontal components). For each of these variables, the software produced a mean (\pm standard deviation, SD) for the 120-180 longest paths from all three replicates. Average overall activity level (percent time actively swimming) and swim speed were also computed. Examples of the swim path and frequency distribution data extracted by TRAKFISH and ANAPATHS are presented in Figures 2 and 3.

Statistical analysis. This was a three-replicate experimental design. In 1995, the treatments were ration and incubation (of the spawning pairs) temperature. In 1996, the treatments were ration and female size. In evaluations of maternal influences, egg batch number was not considered as a separate treatment effect because there were no discernible differences in the behaviour of larvae from the different egg batches at 6 DPH (with the exception of swim speed, for which the difference was mildly significant by t-test, $P = 0.03$).

A model I analysis of covariance was applied to length and weight-age regression curves to determine whether larval growth (from all groups combined) was different between experiments (1995 vs. 1996). To evaluate the relationship between egg characteristics and larval growth, data from both years were pooled and the Pearson product-moment correlation coefficient, r , was applied. The behaviour of larvae at 3 vs. 6 DPH was compared using a one-way ANOVA (performed on each

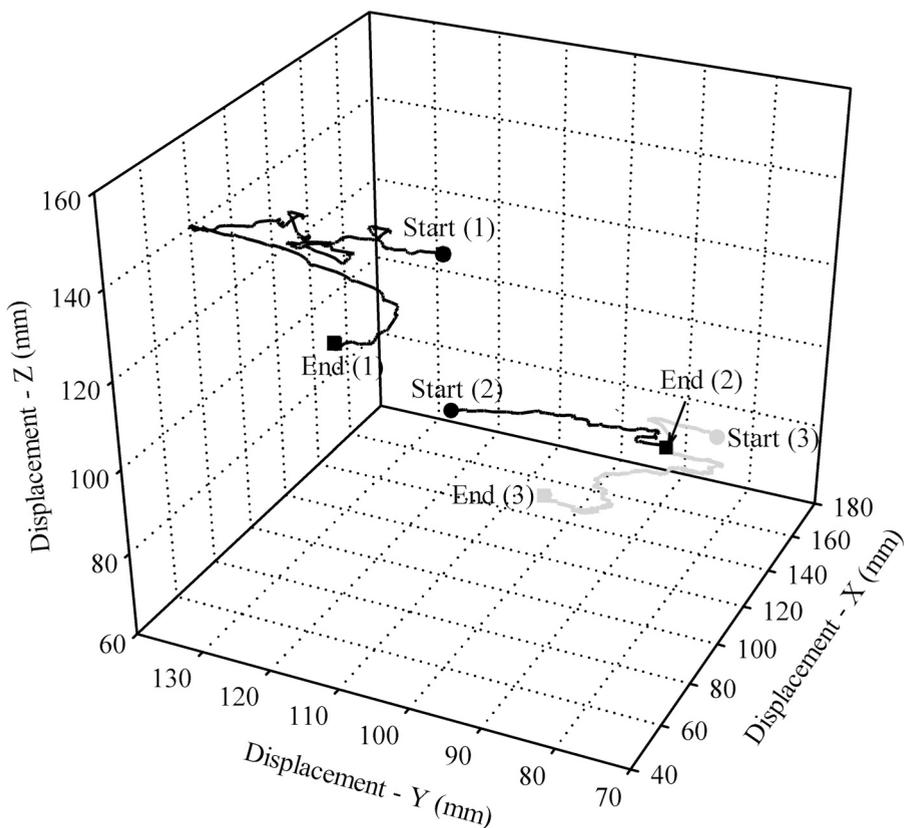


Figure 2. Typical swim paths for Atlantic cod (*Gadus morhua*) larvae extracted using the TRAKFISH software. Swim paths 1, 2 and 3 are 355, 104 and 190 mm long respectively.

behavioural variable). The same test was applied to compare larval behaviour (6 DPH only) in 1995 vs. 1996. Possible correlations between the SGR of cod larvae and the behavioural variables (6 DPH only) were evaluated using the Pearson product-moment correlation coefficient. Finally, the possible effects of female treatment (incubation temperature, ration, size) on larval behaviour (6 DPH) were examined by two-way ANOVA (including treatment interactions). For these latter tests, only behaviour at 6 DPH was tested because larvae were not yet feeding at 3 DPH.

Results

In order to establish the overall context for the work on larvae, the most salient of the previously published results on brood stock (Lambert and Dutil 2000), and on eggs (Ouellet et al. 2001), are summarized here. For complete details, readers are referred to these companion studies.

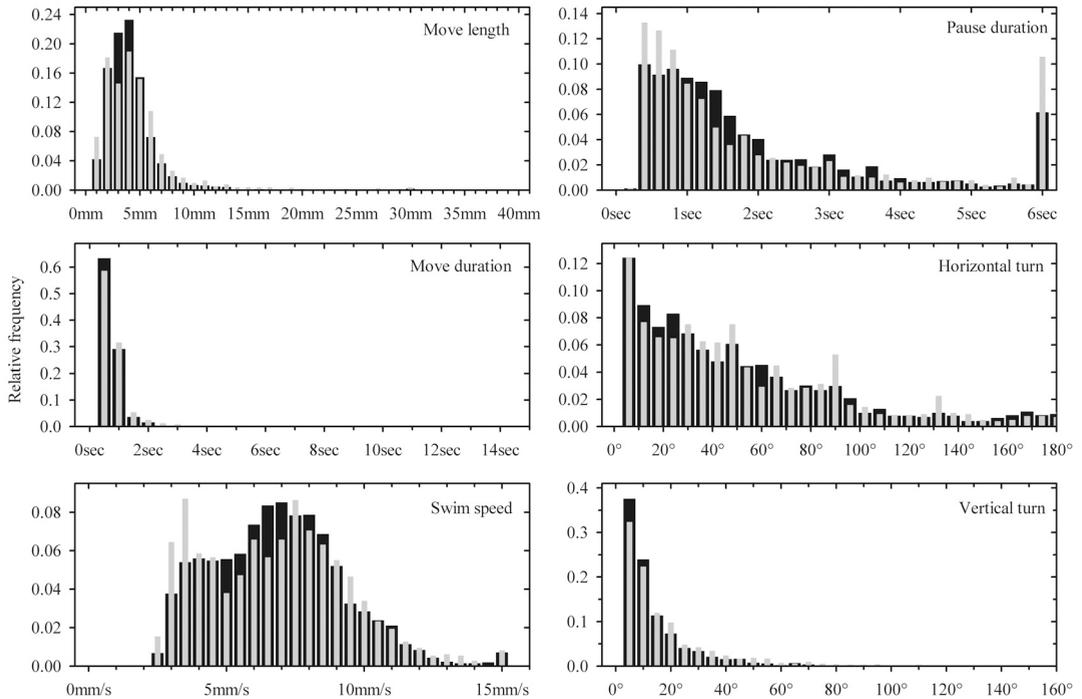


Figure 3. Frequency distributions for the behavioural variables extracted using TRAKFISH and ANAPATHS software on the same group of Atlantic cod (*Gadus morhua*) larvae at 3 and 6 days post hatch (DPH). Black bars = 3 DPH; grey bars = 6 DPH.

Female size and condition. Despite the differences in incubation temperature, and the partitioning of females into two size groups, the size of pre-spawning females was not discernibly different in the 1995 vs. the 1996 experiments. Female pre-spawning and post-spawning condition (Fulton's K) ranged from 0.66–1.24 in 1995, and 0.48–0.92 in 1996 (Table 1). Female length, and pre- or post-spawning condition were not correlated within either experiment. Post-spawning condition differed between years, with the mean being slightly lower in 1996 (0.66 ± 0.08) than in 1995 (0.76 ± 0.11) (Table 1). Smaller fish, and fish in lower condition, were better represented in the 1996 experiment (Table 1).

Relationships between female size and condition and egg production, viability and hatching success. The mean realized fecundity, mean number of egg batches, and number of days between batches all indicate that the fish were at least second time spawners, and their spawning performance was similar in both experiment years. The mean number of egg batches, realized fecundity, and the average interval between batches, were not discernibly different between years. Although there was a positive correlation between pre-spawning condition of females (maturing at 6 °C) and weighted mean egg diameter, dry mass, and energy content, this relationship was no longer statistically dis-

cernible when the only female with a low condition factor was excluded from the analysis. There was no relationship between female size and weighted mean egg diameter, weighted mean egg dry mass, nor energy content. While mean egg dry mass per batch declined over the spawning sequence of individual females, no definite trends in total egg dry mass per batch were observed. However, the batch number in the spawning sequence did have a significant negative effect on cod egg diameter and dry mass. The total lipid/protein ratios of eggs from different batches were not discernibly different, nor were they related to female pre-spawning condition. Total egg dry mass per batch of individual females during spawning was not related to post-spawning somatic, protein, or lipid reserves. Neither mean egg diameter nor dry mass were correlated with egg survival. Nor was there any relationship between egg survival to hatch and female pre-spawning condition. However, there was some indication that late embryos and larvae hatching from eggs produced by females in poor condition were less viable. Hatching success was independent of batch number and was not related to egg diameter or dry mass. Finally, female pre- and post-spawning condition and hatching success were not correlated in either experiment.

Larval growth. A total of 775 cod larvae were measured and weighed over the two experimental seasons. Larval groups from the 1996 experiment generally grew better than those from the 1995 experiment (analysis of covariance for body length, $P = 0.037$, and for body weight, $P = 0.007$) (Table 1, Figs. 4 and 5). Overall, the growth rates of larvae in these experiments (Table 1) were comparable to those reported for cod larvae in other laboratory-based work (e.g. Gamble and Houde 1984; Yin and Blaxter 1986; Bolz and Lough 1988; Otterå 1993). This supports the validity of the methods applied, and of analyses based upon the growth rate data.

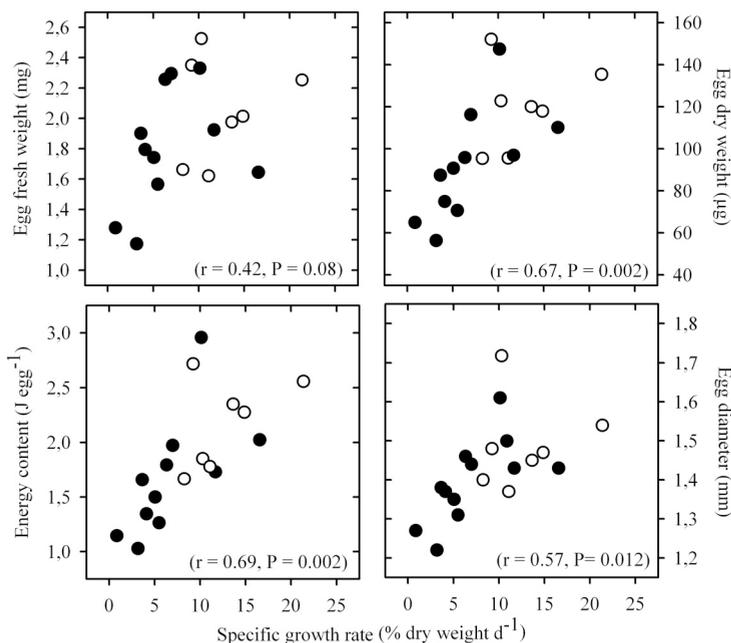


Figure 4. The relationship between the specific growth rate of Atlantic cod (*Gadus morhua*) larvae, as percent body length and percent body dry mass per day, and the characteristics of the eggs from which they hatched (1995 (filled circles) and 1996 (open circles) experiments combined). r is the Pearson-product moment correlation coefficient at probability P .

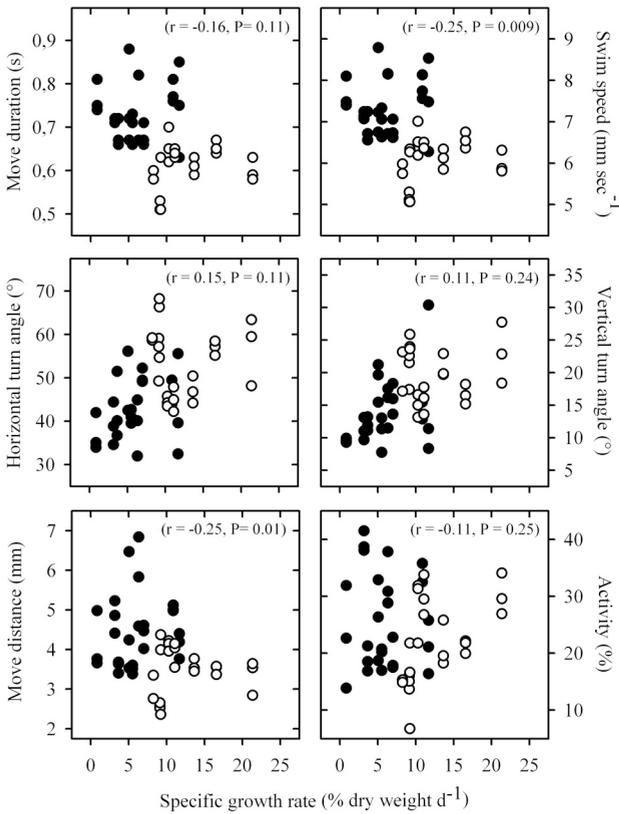


Figure 5. The relationship between the specific growth rate of Atlantic cod (*Gadus morhua*) larvae, as percent body length and percent body dry mass per day, and their behaviour (1995 (filled circles) and 1996 (open circles) experiments combined). r = the Pearson product-moment correlation coefficient at probability P .

Relationship between larval growth, egg characteristics and female treatments. There were statistically discernible (Pearson product-moment correlation coefficients) positive correlations between larval growth and egg diameter, dry weight and energy content (Fig. 4). A consistent relationship was exhibited for egg fresh weight, although it was not significant. There was no statistically discernible relationship between female pre- nor post-spawning condition factor (Fulton's K) and the growth of the larvae that they produced (Pearson product-moment correlation, $P > 0.05$). Nor was there any relationship between female ration, size, and/or incubation temperature and the growth of their larvae (one-way ANOVA, $df = 1$, $P > 0.05$ in all cases).

Behaviour of larvae at 3 vs. 6 DPH, and as related to growth rate. There were statistically discernible differences between the behavioural variables (except for move duration) on 3 vs. 6 DPH (Table 2, Fig. 3). Since the larvae were not feeding at 3 DPH, but were at 6 DPH, this demonstrates the validity of using these behavioural variables as indicators of changes in larval behaviour.

Swim speed, turn angles associated with repositioning movements, and move distances were all discernibly different in larvae from the 1995 vs. the 1996 experiment (Table 3). Since larvae from 1996 exhibited higher growth rates, this observation demonstrates a link between growth and at least some of the behavioural variables. The negative correlation between swim speed, move dis-

Table 2. Mean (\pm SD) values for each of the behavioural variables measured on all groups of Atlantic cod (*Gadus morhua*) larvae at 3 vs. 6 days post hatch (DPH) and the results of a one-way ANOVA ($df = 1$) testing for differences in these variables between the two days (both experiment-years combined).

| Behavioural variable | 3 DPH | 6 DPH | F | P |
|----------------------------------|-----------------|----------------|------|-------|
| Move distance (mm) | 3.5 \pm 1.0 | 3.9 \pm 1.0 | 5.20 | 0.024 |
| Move duration (s) | 0.6 \pm 0.1 | 0.6 \pm 0.1 | 1.26 | 0.264 |
| Swim speed (mm s ⁻¹) | 6.1 \pm 1.1 | 6.5 \pm 1.0 | 6.80 | 0.010 |
| Pause duration (s) | 2.7 \pm 1.2 | 2.1 \pm 1.0 | 9.68 | 0.002 |
| Horizontal turn angle (°) | 55.9 \pm 16.4 | 49.1 \pm 9.7 | 8.65 | 0.004 |
| Vertical turn angle (°) | 19.6 \pm 6.7 | 17.2 \pm 5.4 | 5.53 | 0.025 |
| Activity (%) | 19.7 \pm 9.7 | 24.3 \pm 9.2 | 8.12 | 0.005 |

Table 3. Mean (\pm SD) values for each of the behavioural variables measured on all groups of Atlantic cod (*Gadus morhua*) larvae in the 1995 vs. 1996 experiments (6 days post hatch) and the results of a one-way ANOVA ($df = 1$) testing for between-year differences in these variables (all maternal treatments combined).

| Behavioural variable | 1995 | 1996 (n=35) | F | P (n=32) |
|----------------------------------|-----------------|----------------|------|-------------|
| Move distance (mm) | 4.3 \pm 1.1 | 3.5 \pm 0.7 | 11.9 | <0.001 |
| Move duration (s) | 0.6 \pm 0.1 | 0.6 \pm 0.1 | 0.2 | 0.7 |
| Swim speed (mm s ⁻¹) | 7.1 \pm 0.9 | 5.9 \pm 0.6 | 34.5 | <0.001 |
| Pause duration (s) | 0.3 \pm 0.2 | 0.3 \pm 0.2 | 0.02 | 0.9 |
| Horizontal turn (°) | 44.0 \pm 7.1 | 54.7 \pm 9.1 | 29.0 | <0.001 |
| Vertical turn (°) | 14.5 \pm 4.7 | 20.2 \pm 4.6 | 25.0 | <0.001 |
| Activity (%) | 24.6 \pm 10.0 | 23.6 \pm 8.3 | 0.4 | 0.5 |

tance and growth rate (Fig. 5) is consistent with this, and further supports the validity of applying these behavioural variables as proxies of larval quality-performance.

Relationship between larval behaviour and maternal condition and incubation temperature. There were no statistically discernible relationships between female incubation temperature (i.e., in the 1995 experiment) and any of the behavioural variables measured, although the effect on swim speed was close to significance ($P = 0.06$) (Table 4). Further, there were no treatment interaction effects (female ration with incubation temperature) on larval behaviour in the 1995 experiment. Female ration had a significant impact upon larval activity in the 1995 experiment (Table 4), but not in the 1996 experiment (Table 5). Conversely, female ration had a significant impact upon larval swim speed in the 1996 experiment (Table 5), but not in the 1995 experiment (Table 5). Vertical turn angle (but not horizontal turn angle) was significantly affected by female ration in both years (Tables 4 and 5), while pause duration was only significantly affected in 1996 (Table 5). In the 1996 experiment, female size had a significant effect on larval activity, horizontal turn angle and pause duration (Table 5). Further, there was a significant treatment interaction effect (female ration with female size) on larval swim speed and horizontal turn angle (Table 5).

Table 4a. Mean (\pm SD) values for each of the behavioural variables measured on groups of Atlantic cod (*Gadus morhua*) larvae produced by brood stock reared on different rations and at different temperatures in the 1995 experiment-year. R = brood stock ration; T = brood stock incubation temperature.

| Behavioural variable | Treatment | | | |
|----------------------------------|------------------|-----------------|-------------------|-------------------|
| | R | | S | |
| | High (n = 14) | Low (n = 18) | Large (n = 18) | Small (n = 14) |
| Move distance (mm) | 4.6 \pm 0.3 | 3.9 \pm 0.3 | 4.6 \pm 0.3 | 4.0 \pm 0.3 |
| Move duration (s) | 0.6 \pm 0.02 | 0.6 \pm 0.03 | 0.6 \pm 0.03 | 0.6 \pm 0.02 |
| Swim speed (mm s ⁻¹) | 7.2 \pm 0.2 | 7.2 \pm 0.3 | 7.6 \pm 0.3 | 6.8 \pm 0.2 |
| Pause duration (s) | 1.7 \pm 0.3 | 2.3 \pm 0.4 | 1.7 \pm 0.4 | 2.3 \pm 0.3 |
| Horizontal turn angle (°) | 43.6 \pm 1.8 | 41.6 \pm 2.2 | 40.1 \pm 2.2 | 45.1 \pm 1.8 |
| Vertical turn angle (°) | 15.9 \pm 1.2 | 12.0 \pm 1.4 | 12.2 \pm 1.5 | 15.7 \pm 1.2 |
| Activity (%) | 29.0 \pm 2.3 | 21.1 \pm 2.7 | 27.4 \pm 2.8 | 22.7 \pm 2.2 |

Table 4b. Results of a two-way ANOVA ($df = 1$ for all comparisons) testing for between-treatment differences in the behavioural variables measured on all groups of Atlantic cod (*Gadus morhua*) larvae in the 1995 experiment-year. R = brood stock ration (testing high vs. low); T = broodstock incubation temperature (testing high vs. low); R x T = treatment interaction.

| Behavioural variable | Treatment effect tested | | | | | |
|----------------------------------|-------------------------|------|------|------|-------|------|
| | R | | S | | R x T | |
| | F | P | F | P | F | P |
| Move distance (mm) | 3.10 | 0.09 | 2.49 | 0.13 | 0.38 | 0.54 |
| Move duration (s) | 6.75 | 0.01 | 0.52 | 0.48 | 1.46 | 0.24 |
| Swim speed (mm s ⁻¹) | 0.01 | 0.93 | 3.93 | 0.06 | 0.49 | 0.49 |
| Pause duration (s) | 2.08 | 0.16 | 1.27 | 0.27 | 0.30 | 0.59 |
| Horizontal turn angle (°) | 0.53 | 0.47 | 3.00 | 0.09 | 2.12 | 0.16 |
| Vertical turn angle (°) | 4.37 | 0.05 | 3.54 | 0.07 | 0.55 | 0.46 |
| Activity (%) | 4.90 | 0.03 | 1.72 | 0.20 | 0.13 | 0.73 |

Table 5a. Mean (\pm SD) values for each of the behavioural variables measured on groups of Atlantic cod (*Gadus morhua*) larvae produced by brood stock differing in size and reared on different rations in the 1996 experiment-year. R = brood stock ration; S = female size.

| Behavioural variable | Treatment | | | |
|----------------------------------|------------------|-----------------|-------------------|-------------------|
| | R | | S | |
| | High (n = 14) | Low (n = 18) | Large (n = 18) | Small (n = 14) |
| Move distance (mm) | 3.5 \pm 0.2 | 3.7 \pm 0.2 | 3.7 \pm 0.2 | 3.5 \pm 0.2 |
| Move duration (s) | 0.6 \pm 0.0 | 0.6 \pm 0.0 | 0.6 \pm 0.0 | 0.6 \pm 0.0 |
| Swim speed (mm s ⁻¹) | 5.8 \pm 0.1 | 6.3 \pm 0.1 | 6.0 \pm 0.1 | 6.0 \pm 0.1 |
| Pause duration (s) | 2.4 \pm 0.2 | 1.8 \pm 0.2 | 1.7 \pm 0.2 | 2.4 \pm 0.2 |
| Horizontal turn angle (°) | 55.9 \pm 2.0 | 51.8 \pm 2.2 | 50.8 \pm 2.0 | 57.0 \pm 2.2 |
| Vertical turn angle (°) | 22.4 \pm 1.0 | 17.1 \pm 1.0 | 18.8 \pm 1.0 | 20.8 \pm 1.1 |
| Activity (%) | 21.7 \pm 1.9 | 25.0 \pm 2.1 | 27.2 \pm 2.0 | 19.5 \pm 2.1 |

Table 5b. Results of a two-way ANOVA ($df = 1$ for all comparisons) testing for between-treatment differences in the behavioural variables measured on all groups of Atlantic cod (*Gadus morhua*) larvae in the 1996 experiment-year. R = brood stock ration; S = female size.

| Behavioural variable | Treatment effect tested | | | | | |
|----------------------------------|-------------------------|--------|-------|------|-------|------|
| | R | | S | | R x S | |
| | F | P | F | P | F | P |
| Move distance (mm) | 0.53 | 0.48 | 0.92 | 0.35 | 3.01 | 0.09 |
| Move duration (s) | 0.12 | 0.73 | 1.39 | 0.25 | 0.66 | 0.43 |
| Swim speed (mm s ⁻¹) | 6.77 | 0.02 | 0.001 | 0.98 | 6.14 | 0.02 |
| Pause duration (s) | 4.44 | 0.04 | 6.49 | 0.02 | 0.33 | 0.57 |
| Horizontal turn angle (°) | 1.94 | 0.17 | 4.47 | 0.04 | 7.18 | 0.01 |
| Vertical turn angle (°) | 13.74 | <0.001 | 1.95 | 0.17 | 1.19 | 0.29 |
| Activity (%) | 1.39 | 0.25 | 7.27 | 0.01 | 0.04 | 0.84 |

Discussion

Relationships between female size and condition and egg production, viability and hatching success. Despite the strong influence of female size and nutritional condition on realized fecundity observed in these experiments (Lambert and Dutil 2000), there were only limited effects of maternal nutritional condition on egg survival and hatching success (Ouellet et al. 2001). Total fecundity (all egg batches) and egg dry weight were significantly lower in poor-condition females and female cod with high pre-spawning condition factors were in better post-spawning condition, and lost a lower proportion of their somatic mass and energy reserves, than did females in poor condition (Lambert and Dutil 2000). Although the total mass of eggs produced was positively correlated with the pre-spawning condition of females (in the 1996 experiment), there was no trend in the total egg dry mass per batch (Ouellet et al. 2001). Further, there was only a limited impact of egg characteristics (or batch number) on their viability and hatching success. Although this appears to contradict some of the previous work on cod, not all of that work is completely consistent (e.g. see the seemingly conflicting results in Solemdal et al. 1995 vs. 1998, and as reviewed by Ouellet et al. 2001). Overall, the results of our experiments are consistent with the findings of several analogous studies that investigated the relationships between egg characteristics and hatching success in fishes (as summarized in Table 3 of Ouellet et al. 2001).

Published observations on the relationships between the condition of female cod and the characteristics of their eggs are somewhat contradictory (Chambers and Waiwood 1996; Trippel 1998; Kjesbu et al. 1996; Marteinsdottir and Steinarsson 1998). These studies, which are thoroughly reviewed and discussed by Ouellet et al. (2001), indicate that it may be some combination (one or more) of female size, female condition, female age, and/or female spawning history that are correlated with the phenotypic characteristics of eggs. Just which of these is the most appropriate predictor remains unclear, and is possibly species- and/or stock-specific.

Relationship between larval growth and maternal condition, size and incubation temperature. The positive correlations reported here between cod larval growth rate and egg diameter are consistent with

the long-held and well-documented contention that differences in egg quality – whatever their origin – are transmitted to the larvae that hatch from them (e.g. Chambers and Leggett 1996; Marteinsdottir and Steinarsson 1998 and references cited therein). Since there is a relationship between female condition and egg quality (albeit sometimes weak and/or inconsistent), it follows that this relationship should extend to larvae. Nonetheless, direct evidence of maternal effects on the phenotypic characteristics of fish larvae (e.g. size at hatch, feeding rate, growth rate, buoyancy, DNA content) is limited, and results, at least in part, from the positive correlation between egg size and the size of larvae at hatching (Bengston et al. 1987; Pepin and Miller 1993; Heath and Blouw 1998; Marteinsdottir and Steinarsson 1998; Benoît and Pepin 1999; Heyer et al. 2001; Marteinsdottir and Begg 2002; Saborido-Rey et al. 2003; Heyer and Miller 2004). Despite these precedents, there was no relationship between female condition, size, and/or incubation temperature and larval growth in this study.

Relationship between larval behaviour and maternal condition and incubation temperature. Maternal effects may be defined as “...nongenetic influences derived from parental phenotypes or environments that have an impact on offspring phenotypes.” (Heath and Blouw 1998). Under this definition, maternal effects on the behaviour of their offspring are likely common, diverse and possibly adaptive (Bernardo 1996; Mousseau and Fox 1998a,b). To paraphrase Heath and Blouw (1998), this is so “...because behaviour is possibly the most immediate and malleable source of interaction between the phenotype of an organism and its environment.” Thus, since natural selection acts upon phenotypes, “...we expect adaptive evolution to be common at this interface.” When the connection between egg size and larval characteristics (discussed above) is superimposed upon this, maternal effects on the behaviour of larvae are to be expected.

Earlier work demonstrated quantifiable changes in behaviour associated with the transition from endogenous to exogenous feeding (Skiftesvik 1992), and also that the behaviour of sick larvae was different from those that were healthy (Skiftesvik and Bergh 1993). Thus, the variables that we measured should be sensitive enough to detect any maternal effects on the behaviour of larvae. With the exception of move duration, all of the behavioural variables were different at 3 vs. 6 DPH. Percent activity, swim speed, and move distance were all higher at 6 DPH than at 3 DPH, while turn angles and the duration of pauses were lower at 6 vs. 3 DPH. This is all consistent with the fact that larvae were not feeding at 3 DPH, but had begun to feed at 6 DPH: feeding larvae are more active and swim faster and longer than those that are not feeding (Browman and O'Brien 1992a,b; Skiftesvik 1992). Thus, the validity of using these variables as indicators of changes in larval behaviour is supported. Swim speed and move distances were higher in larvae from the 1995 vs. the 1996 experiment, and swim speed and move distance were negatively correlated with growth rate. Since, all else being equal, larvae that move more (and swim faster) will use more energy and, thus, grow slower, these observations further support the use of behavioural variables as indicators of larval quality.

There were no strong and consistent relationships between any indicator of female condition (size, ration, Fulton's K, incubation temperature) and the behavioural variables measured. This is consistent with the lack of a relationship between larval growth rate and female condition. Close examination of the behavioural data, including each and every swim path analysed, demonstrates a high level of variability (which is consistent with the observations of Ouellet et al. (2001) on

eggs). That, in-and-of-itself, makes resolving maternal effects all the more difficult. Spawning in batches over a relatively long (4–6 week) period is thought to increase the chances that the larvae of any given female will encounter feeding conditions adequate to support rapid growth and an increased probability of survival (this in the context of hypotheses such as Cushing's match-mismatch). In this scenario, perhaps the most adaptive strategy for females – at least those that release eggs into a pelagic and highly stochastic environment – is to produce eggs and larvae with a broad range of characteristics, in each an every batch of eggs that they release. That way, at least some individuals will possess characteristics that will enable them to survive, regardless of the conditions in which they find themselves. This high level of background variability serves to mask our ability to discern any possible maternal effects on the characteristics of eggs and larvae.

Conclusion. The sources of variability in the phenotypic characteristics (e.g. vital rates) expressed by fish eggs and larvae remain poorly known, although most ascribe at least some of it to maternal effects (reviewed in Heath and Blouw 1998). Several adaptive explanations for intrapopulation variation in egg and larval characteristics have been proposed (e.g. Parker and Begon 1986; Hendry et al. 2001; Einum and Fleming 1999); all of them assume that there is a maternal effect on the egg size-offspring fitness function. Using data drawn from the literature, Einum and Fleming (2002) demonstrated that maternal effects are more pronounced in species with demersal eggs and larvae than in species with pelagic eggs and larvae (such as cod). Thus, for pelagic batch spawners such as cod, the maternal effect on egg and larval phenotypes may be small. This is a possible reason for the lack of a more consistent maternal effect on the behaviour of cod larvae in these experiments. Other plausible explanations are that (i) the majority of egg groups followed were the progeny of females with relatively high pre-spawning condition factors; (ii) the relatively small range of pre-spawning condition factors for these females; (iii) the fact that the full complement of egg groups from each female could not be followed; (iv) high variability; and several other possibilities (taken up by Ouellet et al. 2001). On the other hand, and for all of these reasons, it is also possible that there simply is no consistent and clearly discernible relationship between maternal condition and larval quality in cod. To paraphrase Browman (1999), data which do not strongly support a research hypothesis should not be summarily dismissed as incorrect or irrelevant. Such results may provide more balance for a subject area thus far supported only (or primarily) by positive results; they may indicate that a subject area is not as mature or clearly defined as previously suspected; they may show that a particular line of research is not worth further efforts; or they may indicate that our current methodologies are inadequate for producing a definitive result. In the case of maternal effects on cod early life stages, all of the above should be considered.

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Life in green water: the effect of microalgae on the behaviour of Atlantic cod (*Gadus morhua*) larvae

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Key words: *Nannochloropsis* sp., green water, Atlantic cod, larvae, behaviour, swim paths, activity, first-feeding

Abstract

In the intensive culture of marine fish larvae, microalgae is often added to the water along with microzooplankton or microparticulate food (the “green water” technique). Although using green water generally improves the survival and growth rate of larvae, the mechanism(s) through which the microalgae act to generate this effect remains unclear. We tested the hypothesis that the presence of microalgae in the culture environment affects the behaviour of 5 day post hatch Atlantic cod (*Gadus morhua*) larvae. Using silhouette video photography, the behaviour of cod larvae was observed (i) with rotifers (*Brachionus* sp. at 7 ml⁻¹) but no algae, and (ii) with rotifers (7 ml⁻¹) and 100,000 *Nannochloropsis* sp. cells ml⁻¹. Light intensities (and spectra) in the experiments were similar to those used in culture systems and were not measurably different amongst the treatments. The following variables were extracted from the observations of cod swim paths: durations of pauses (non-active displacement), lengths and durations of moves, and turn angles (decomposed into vertical and horizontal components). These components of the swim pattern were compared to determine if the presence of algae affected the behaviour of cod larvae, and in what manner. There were no significant/consistent differences amongst treatments. The results indicate that the effect of green water on the behaviour of cod larvae is subtle and that any improvement in growth and/or survivorship associated with green water is most likely a result of the indirect effects that microalgae might have on the nutritional quality of their food, and/or on larval physiology.

Introduction

In the intensive culture of marine fish larvae, microalgae is often added to the water along with microzooplankton or microparticulate food (Reitan et al. 1997; Muller-Feuga 2000; Lee and Ostrowski 2001; Shields 2001). This is commonly referred to as the “green water” technique. Although using green water generally improves the survival and growth rate of larvae (e.g. Naas et al. 1992; Bengston et al. 1999), the mechanism(s) through which the microalgae act to generate this effect remains unclear. Evidence in support of several possibilities has been reported, including improvement of digestive functions (e.g. Cahu et al. 1998; Lazo et al. 2000), enhanced nutritional value of prey (because they feed on the microalgae before being eaten themselves)(e.g. Silva 1999), increased assimilation efficiency (e.g. Papandroulakis et al. 2002), and higher prey contrast (through the effect of the algae on the underwater light field) (e.g. Cobcroft et al. 2001).

Although the effect of microalgae on feeding incidence and intensity has been studied, the manner in which algal cells might alter the behaviour of marine fish larvae has not been thoroughly characterized. Thus, we tested the hypothesis that the presence of microalgae in the culture environment affects the behaviour of Atlantic cod (*Gadus morhua*) larvae.

Materials and Methods

Source of cod larvae. Larvae used in these experiments were obtained from a single spawning event between one male and one female cod (B08-03 in Table 1 of Browman et al. 2003). Females were paired with males in 1.2 m³ circular tanks, allowing for every batch of eggs released by females to be collected individually. Complete descriptions of the brood stock, holding conditions, and methods of egg collection and incubation, are presented in Lambert and Dutil (2000) and in Ouellet et al. (2001). Eggs were incubated in black 60 l round-bottom tanks (at 6 ± 1.0 °C). Larvae were transferred to fresh 60 l black tanks at 3 days post hatch (DPH) (just prior to first exogenous feeding). The rearing basins were stocked with algae (*Nannochloropsis* sp.) at 100 000 cells l⁻¹ (green water technique), and larvae were fed nutritionally enriched (INVE Aquaculture Super Selco®) rotifers (*Brachionus* sp.) at 7 ml⁻¹. Larvae were cultured at 6 °C on a 14 h L:10 h D photoperiod at a crepuscular-level light intensity of 1.20 $\mu\text{E sec}^{-1} \text{m}^{-2}$ (diffuse light from overhead fluorescent lamps). The same light intensity (and light source) was used in the behavioural experiments.

Experiments on larval behaviour. Silhouette (shadow) video photography (SVP) was used to observe the behaviour of cod larvae. The physical characteristics of the system, its advantages, and details of the software used to extract the data, are presented elsewhere in this book (Browman et al. 2003). Larvae were tested at 5 DPH, two days after the initiation of exogenous feeding. Fourty larvae were placed into a 20 x 20 x 20 cm test aquarium and video taped for 30 min. in water (taken from the culture tank from which they were sampled) which had been vacuum-filtered through a 5 μm mesh sieve and to which rotifers (at 7 ml⁻¹) had been added. Next, algae were added to the aquarium (at 100,000 cells ml⁻¹) and the larvae were observed for another 30 min. Upon completion of this

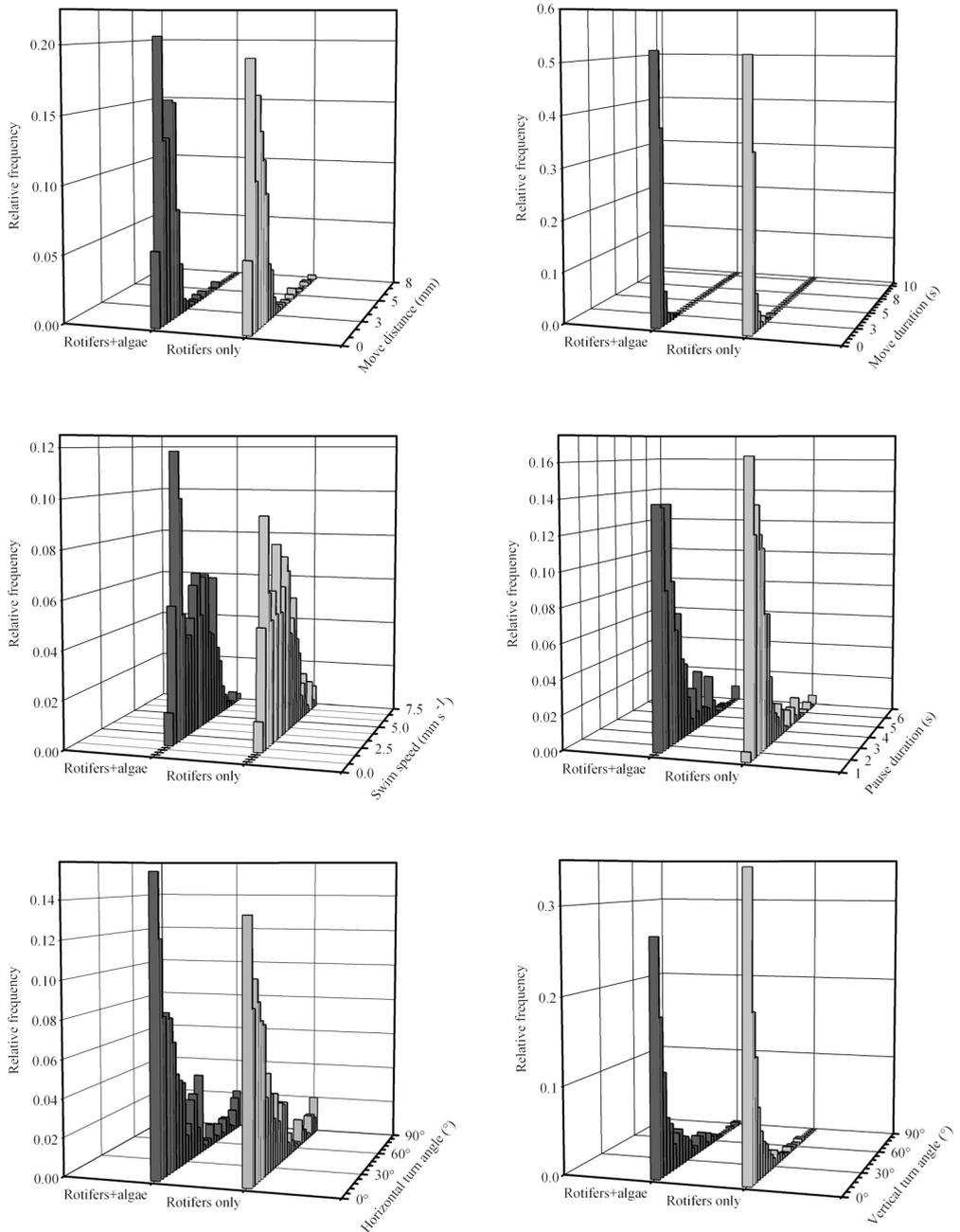


Figure 1. Frequency distributions of behavioural variables (both replicates combined) measured on 5 day post hatch Atlantic cod (*Gadus morhua*) larvae feeding in the presence of rotifers only (*Brachionus* sp. at 7 ml^{-1}), and with rotifers and algae (*Nannochloropsis* sp. at $100\,000 \text{ cells ml}^{-1}$).

Table 1a. Mean (\pm SE) values for each of the behavioural variables measured on 5 day post hatch Atlantic cod (*Gadus morhua*) larvae feeding in the presence of rotifers (*Brachionus* sp. at 7 ml^{-1}) only, and rotifers with algae (*Nannochloropsis* sp. at $100\ 000\text{ cells ml}^{-1}$). Values for the two replicates (Rep) are presented separately.

| Behavioural variable | Rep | N | Rotifers only | N | Rotifers+algae |
|--------------------------------------|-----|-----|----------------|-----|----------------|
| Move distance (mm) | 1 | 98 | 4.9 ± 0.2 | 298 | 3.8 ± 0.1 |
| | 2 | 122 | 3.7 ± 0.2 | 86 | 4.4 ± 0.3 |
| Move duration (s) | 1 | 98 | 0.6 ± 0.04 | 298 | 0.5 ± 0.02 |
| | 2 | 122 | 0.5 ± 0.02 | 86 | 0.5 ± 0.02 |
| Swim speed (mm s^{-1}) | 1 | 98 | 8.3 ± 0.3 | 298 | 7.3 ± 0.2 |
| | 2 | 122 | 7.3 ± 0.2 | 86 | 8.0 ± 0.3 |
| Horizontal turn angle ($^{\circ}$) | 1 | 87 | 41.1 ± 3.9 | 274 | 48.1 ± 2.6 |
| | 2 | 110 | 40.1 ± 3.6 | 76 | 38.3 ± 5.6 |
| Vertical turn angle ($^{\circ}$) | 1 | 87 | 14.4 ± 1.9 | 274 | 26.5 ± 1.8 |
| | 2 | 110 | 18.9 ± 2.1 | 76 | 19.2 ± 2.7 |
| Pause duration (s) | 1 | 91 | 1.2 ± 0.1 | 286 | 1.8 ± 0.1 |
| | 2 | 118 | 2.1 ± 0.2 | 79 | 1.8 ± 0.2 |

Table 1b. Results of Kolmogorov-Smirnov two-sample tests evaluating for between-treatment differences in the behavioural variables measured on 5 day post hatch Atlantic cod (*Gadus morhua*) larvae feeding in the presence of rotifers (*Brachionus* sp. at 7 ml^{-1}) only, and rotifers with algae (*Nannochloropsis* sp. At $100\ 000\text{ cells ml}^{-1}$).

| Behavioural variable tested | Kolmogorov-Smirnov statistical parameters | Rotifers only vs. rotifers and algae |
|-----------------------------|---|--------------------------------------|
| Move distance | D | 0.07 |
| | $K_{0.05}$ | 1.36 |
| | $D_{0.05}$ | 0.09 |
| | Decision | Same |
| Move duration | D | 0.05 |
| | $K_{0.05}$ | 1.36 |
| | $D_{0.05}$ | 0.09 |
| | Decision | Same |
| Swim speed | D | 0.07 |
| | $K_{0.05}$ | 1.36 |
| | $D_{0.05}$ | 0.09 |
| | Decision | Same |
| Horizontal turn angle | D | 0.05 |
| | $K_{0.05}$ | 1.36 |
| | $D_{0.05}$ | 0.11 |
| | Decision | Same |
| Vertical turn angle | D | 0.13 |
| | $K_{0.05}$ | 1.36 |
| | $D_{0.05}$ | 0.11 |
| | Decision | Different |
| Pause duration | D | 0.0848 |
| | $K_{0.05}$ | 1.3581 |
| | $D_{0.05}$ | 0.0937 |
| | Decision | Same |

sequence, a new group of 40 larvae were sampled (from the same culture tank), placed into a second test aquarium, and the same sequence was repeated. Thus, there were two replicates for each treatment.

The behaviour of cod larvae was evaluated by analysing a 5 min. segment of video images drawn from the middle of each treatment's 30 min. observation period (but never near the transition from one treatment to the next). Due to the nature of the imaging system, the behavioural observations cannot be attributed to any single larva - they swim in and out of the field of view and, therefore, it is impossible to tell one larva from another. Further, observations on any one larva in a tank containing 40 are not statistically independent and, therefore, all observations made on the 40 larvae in any given tank collapse down to a sample size of one. Thus, there were two replicate 30 min. observation periods for each treatment, each of which was subsampled for 5 min. The swim paths of cod larvae were extracted from the video taped images and analysed using custom software (see Browman et al. 2003). For each of the 5 min. subsamples, the longest 20 paths were identified and combined (recall that, for statistical analysis, all of these paths must be collapsed into one since they are not independent). Paths totalling approximately 2.6 m in length were used as the basis for the analysis. The following variables were extracted from the swim paths: durations of pauses (non-active displacement, as defined in Browman et al. 2003), lengths and durations of moves, and turn angles (decomposed into vertical and horizontal components). For each of these variables, the software produced a mean (\pm standard error, SE) for the various behavioural variables associated with the 20 longest paths from each of the two replicates. The frequency distributions of these variables (Fig. 1) were compared using Kolmogorov-Smirnov two-tailed tests to determine if the presence of microalgae affected larval behaviour, and in what manner.

Spectral irradiance ($\text{W m}^{-2} \text{nm}^{-1}$) was measured at 1 nm intervals (280 – 800 nm) inside the test aquarium - in filtered water and in water containing the same numbers of rotifers and/or microalgae as used in the experimental treatments - using a high-sensitivity/high resolution scanning spectroradiometer (OL-754, Optronic Laboratories Inc., Orlando, Florida, USA). Before measurements, the instrument was calibrated against a NIST-traceable 200 W tungsten-halogen standard lamp (OL 752-10) and its wavelength and gain accuracy were verified using a dual source calibration module (OL-752-150).

Results and Discussion

Earlier work demonstrated quantifiable changes in behaviour associated with the transition from endogenous to exogenous feeding (Skiftesvik 1992), that the behaviour of sick larvae was different from those that were healthy (Skiftesvik and Bergh 1993), and that larval growth rate is reflected in their behaviour (Browman et al. 2003). Thus, the variables that we measured should be sensitive enough to detect any change in behaviour associated with the presence of microalgae. Nonetheless, with the exception of vertical turn angle, there were no statistically discernible (Kolmogorov-Smirnov two-tailed comparisons, $P > 0.05$) between-treatment differences in the behavioural variables (Table 1, Fig. 1). This is possibly due to the fact that there were no measurable (that is, $> 10^{-6} \text{W cm}^{-2}$, the absolute detection limit of the OL754) differences in spectral irradiance amongst these treat-

ments indicating that, at least at the microalgal and rotifer particle counts used, there was no major effect on the ambient light quality or intensity. It is also possible that the high level of variability exhibited within one group of larvae, and/or the small number of replicates in this experiment, reduced our ability to discern any differences in behaviour. Alternately (or in addition), the water in which the larvae were placed in the rotifers-only treatment had the algae vacuum-filtered out of it: this filtrate water may have contained substances (e.g. microalgae-specific amino acids) that alter the behaviour of cod larvae. Finally, the result-conclusion may be different for other microalgal species. It would be useful to test all of these possibilities.

The results of this experiment (albeit as qualified above) illustrate that the effect of green water on the behaviour of cod larvae is subtle. Thus, any improvement in the growth and/or survivorship of cod larvae associated with green water is most likely a result of the indirect effects that the microalgae might have on the nutritional quality of their food, and/or on their physiology (reviewed by Reitan et al. 1997).

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Swimming, feeding and predator avoidance in cod larvae (*Gadus morhua* L.): trade-offs between hunger and predation risk

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Key words: cod larvae, swimming activity, feeding activity, starvation, risk of predation, trade-off, *Gadus morhua*

Abstract

Swimming and feeding activity of larval fish may influence predation mortality early in the predation process by changing encounter rate between predator and prey, as well as increasing probability of attack. Starvation-induced changes in behaviour of Norwegian coastal cod (*Gadus morhua* L.) larvae was investigated for five age groups ranging from early exogenous feeding period (8 days post hatch, dph) to metamorphosis (48 dph). The effects of predator presence (juvenile herring, in a two-chambered aquarium) on the starvation-induced behavioural changes was also investigated as well as the larvae's ability to avoid the area of high predation risk. The nutritional status of fed, and 3- and 5-days starved larvae was assessed by RNA : DNA ratios, and the toleration of starvation increased with age. Swimming bout rate decreased with deteriorating nutritional status in the three youngest age groups. In the two older age groups, the swimming bout rate initially increased and subsequently decreased. Feeding activity, measured as number of prey focuses, was low in the three youngest age groups, but increased markedly for the starved larvae in the older age groups. The presence of predators suppressed the feeding activity in the two oldest age groups, and the swimming activity in the oldest age group. The youngest larvae did not avoid the predators, as shown by a lower survival and a lower number of larvae in the safe side compared to in the control aquariums without predators. The older age groups seemed to perceive the predator presence and avoided the risky area. Nutritional status affected the safe-side distribution; the fed and moderately starved larvae likely had increased ability to actively seek refuge in the safe side compared to the longest starved group. Trade-offs in swimming and feeding behavior, where conflicting demands between predator avoidance and food intake existed, was evident in the older age group.

Introduction

The two main mortality agents in the early life history of marine fish larvae are predation and starvation (Bailey and Houde 1989), and as larval nutritional status may influence the predation mortality the two factors presumably interact (McNamara and Houston 1987; Miller et al. 1988). Starvation leads to decreased growth (Bisbal and Bengtson 1995; Ehrlich et al. 1976; Yin and Blaxter 1986), slower development (Høie et al. 1998; Kamler et al. 1990), and changes in behaviour for energy-saving or energy-searching purposes; all of which can indirectly affect predation mortality (Blaxter and Ehrlich 1974; Chick and Van den Avyle 2000; Munk 1995; Puvanendran et al. 2002; Ross et al. 1996; Skiftesvik 1992; Sogard and Olla 1996).

Predation mortality rate is the product of (1) the probability of being encountered by the predator, (2) the probability of being attacked, and (3) the probability of being caught (Fuiman and Magurran 1994). Starvation may affect all three phases of the predation process. The effects of starvation on events occurring before an attack (1, 2) are often the opposite of the effects on events occurring after initiation of the attack (3). A decrease in activity due to energy depletion will usually reduce encounter rate and attack probability, but decrease escape success (Bailey and Yen 1983). Reduced growth and slower development of starved larvae can in the short term decrease the probability of attack due to reduced conspicuousness, but increase escape failure because of less developed sensory and locomotory capabilities (Fuiman and Magurran 1994). A starved larva with a higher activity during search for food can experience an increased predator encounter rate and attack probability (Cowan and Houde 1992), and the escape success can be reduced by a decrease in predator vigilance (Milinski 1993; Skelly 1994). In addition to the starvation-induced effects on the predation process, reduced growth will increase the time a larva spends within a specific stage or size class and thus affect the cumulative predation mortality rate (Shepherd and Cushing 1980).

Most studies on fish larvae predation have focused on the effect of larval size and development on escape success (3) and predation mortality (product of 1, 2 and 3), and mortality is most often found to decrease with increasing larval size or to be dome shaped (e.g. Cowan and Houde 1992; Cowan et al. 1996; Folkvord and Hunter 1986; Lundvall et al. 1999; but see Pepin 1993; Pepin and Shears 1995). Larval behaviours affecting predator encounter (1) and attack probability (2) have rarely been investigated directly, but suggested as explanations to observed differences in predation mortality rates for larval fish (Cowan and Houde 1992; Fuiman 1989; Litvak and Leggett 1992). An exception is the study by Bailey and Yen (1983) where an initial increase in mortality of Pacific hake (*Merluccius productus*) larvae by predation by a marine copepod was caused by increased larval swimming activity.

Starvation influences not only the growth and development of the larvae, and in studies investigating the interaction effect of larval starvation and predation mortality, the reaction rate usually decreases with decreasing nutritional status (Booman et al. 1991; Chick and Van den Avyle 2000; Jonas and Wahl 1998). Predation mortality has often been found to increase with starvation (Bailey and Yen 1983; Gamble and Hay 1989; Margulies 1990; Purcell et al. 1987), but usually without identifying factors acting on the different phases in the predation process. An exception is the study of Chick and Van den Avyle (2000) where they found swimming speed, reactive distances and responsiveness to a model predator to decrease with lower feeding rations for larval striped bass (*Morone saxatilis*), and used these results to model effects of starvation on predation mortality. To our

knowledge the only study on nutritional status and anti-predation behaviour in cod larvae is the study by Neilson et al. (1986), where escape intensity was found to be positively correlated with food ration. Size-, developmental- and energy depletion-related effects could, however, not be separated, and starvation-induced changes in the earlier phases in the predation process were not investigated.

Predator presence may also modify the behaviour of fish prey before initiation of an attack in order to increase survival (Lima and Dill 1990). The most common changes involved are change of habitat, schooling, freezing and decreased feeding activity (Smith 1997). Ydenberg and Dill (1986) state that overt behaviour reflects costs and benefits of performing that or an alternative behaviour. Nutritional status may modify the costs and benefits, and therefore influence the behaviour in the presence of predators. Adult fish can effectively trade-off conflicting demands such as foraging and predator avoidance (Milinski 1993), but few studies have investigated behavioural trade-offs of larval fish in the presence of predators. Bishop and Brown (1992) found that stickleback larvae (*Gasterosteus aculeatus*) foraged less when predators were present, and the same was found for larval lumpfish (*Cyclopterus lumpus*) (Williams and Brown 1991), indicating that decision-making balancing different needs exists in larval fish. It is not known when the ability to make behavioural trade-offs arise during development since this requires that the larva has developed the ability to perceive predator presence at some distance. Fuiman and Magurran (1994) point out that more studies are needed to understand the development of behavioural decision-making where trade-offs between foraging and predator avoidance exist.

The aim of the current study was to investigate starvation-dependent behaviour of larval fish that might affect predation mortality, and further to determine if the behaviour was influenced by the presence of predators. The larva's ability to trade-off the behaviours associated with predator presence and food search, taking into account nutritional status, was also studied. Swimming and feeding behaviour of cod larvae, as well as the ability of the larvae to avoid the near field of a predator, was investigated for larvae with different nutritional status with and without predators present. Five different age groups were tested, as the behaviour, effect of starvation, and ability to sense a predator are expected to change with larval age and development. This study is a first attempt to investigate the complex behavioural basis for variations in predation mortality for cod larvae with varying nutritional status under the threat of a predator.

Materials and Methods

The cod larvae used in the experiment were raised from two stocks of Norwegian coastal cod. The eggs were collected from tanks with 25 cod (15 females and 10 males) in Austevoll Aquaculture station (southern Norway) and 45 cod (approximately 50:50% males and females) from the cod farm Troms Marin Yngel (northern Norway). The eggs were transferred to the Department of Fisheries and Marine Biology at the University of Bergen before the experiment. The eggs were kept at 5-6° C until approximately 2 days before hatching when the temperature gradually was raised to reach 10° C two days after hatching. The larvae from the 2 stocks were each kept in 2 green rearing tanks (1×1m, 500 l) at 10° C (Fig. 1). The light intensity fluctuated according to the seasonal and daily cycles in Bergen (60° N) using a computer controlled light system, Lysstyr

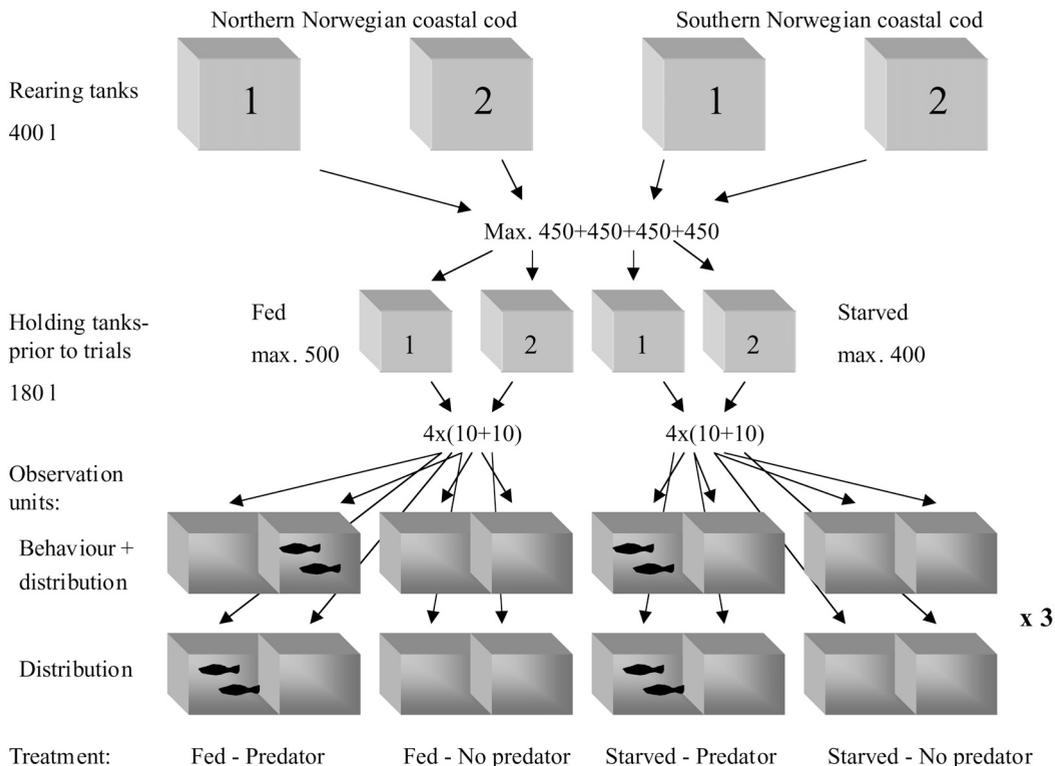


Figure 1. Set up of experiment. Behaviour and distribution were observed in 4 units (with 3 replicates over time) and in addition distribution was observed in 4 other units (with 3 replicates, i.e. 6 replicates for distribution data).

(Hansen 1990). The larvae were fed natural zooplankton collected at Espesrend field station outside Bergen. Every morning the density of zooplankton in each tank was recorded and adjusted to about 2000 prey l^{-1} . The zooplankton consisted mainly of nauplii and copepods filtered and retained initially by 80-250 μm mesh size filters, and as the larvae grew, by 80-1000 μm mesh size filters (see Otterlei et al. (1999) for details about the rearing procedure).

Before the trials equal numbers of larvae from the two stocks were mixed and carefully transferred to 4 smaller tanks (60x60x50 cm, 180 l, otherwise similar to the rearing tanks). This was done to ensure a broad genetic background of the larval cod used in this study. The larvae in 2 of the tanks were fed as previously while those in the other 2 tanks were starved. Larvae were taken out for experiments after 3 and 5 days. Experiments were run on 5 age groups ranging from age 8 to 48 dph (days post hatch, Table 1).

The observations were conducted in 8 glass aquaria (35x35x70 cm, 70 l) with one transparent side, 3 black sides and an off-white bottom. The aquaria were covered with a black light-proof tent with openings only for the transparent side. Above each aquarium inside the tent there was a light tube, illuminating the aquaria in the otherwise dark observation room. The behaviour of the

Table 1. Mean size (SL) of the different groups of fed (FL) and starved (SL) larvae used in the experiment ($n = 20$).

| Age group | 0 and 3 d starv. | | | | 0 and 5 d starv. | | | |
|-----------|------------------|----------|-------------------------|-------------------------|------------------|----------|-------------------------|-------------------------|
| | Age | Date | Mean size FL mm (sd) | Mean size SL mm (sd) | Age | Date | Mean size FL mm (sd) | Mean size SL mm (sd) |
| 8-d | | | | | 8 | 04. May | 5.26 (0.38) | 4.68 (0.23) |
| 13-d | 12 | 08. May | 5.43 (0.27) | 5.10 (0.16) | 14 | 10. May | 5.39 (0.35) | 5.32 (0.21) |
| 21-d | 20 | 16. May | 6.25 (0.53) | 5.78 (0.53) | 22 | 18. May | 6.78 (0.88) | 6.01 (0.40) |
| 33-d | 32 | 28. May | 8.16 (1.63) | 7.64 (1.17) | 34 | 30. May | 8.04 (1.58) | 7.53 (0.97) |
| 47-d | 46 | 11. June | 15.60 (2.61) | 14.22 (1.90) | 48 | 13. June | 13.85 (2.48) | 14.77 (1.85) |

larvae was then easy to record and any disturbance by the observer was minimised. Each aquarium was divided into 2 compartments by acid-proof metal netting with 7 mm wide openings. In four aquaria one of the compartments contained two predators (juvenile herring, 83 mm (± 0.8 SD)), and the larvae could thus choose between a risky and a safe side. The other aquaria served as controls with no predators. Juvenile herring was chosen because of its appropriate size and because it constantly moves around, thereby decreasing the variability between aquaria and increasing the chance of larval detection at some distance. Every day before the experiment started about 70 zooplankton l^{-1} were added to each aquarium (same size fraction as used in the rearing and holding tanks). The concentration of zooplankton was counted after the first trial and adjusted before the third trial (in the oldest age group it was adjusted also before the second trial). The estimated mean concentration in trial 2 and 3 was 60 ± 29 and 62 ± 28 zooplankton l^{-1} respectively.

The following four groups were set up using fed, and 3 and 5 d starved larvae on separate days (Fig. 1): starved larva with and without predator, and fed larva with and without predator. Eight aquaria were used. Behavioural observations were made in four aquaria, with three replicates over time (within 8 h), while larval distribution was recorded in all eight aquaria over three times giving six replicates per treatment (only 3 for the two youngest age groups). We aimed to run the experiment at the same time every day starting in the morning. The order of groups and aquaria was randomised.

Before the observations 10 larvae were carefully transferred in a beaker (0.8 l) to each of the two compartments (a total of 20 larvae per aquarium). To study behavioural changes acting early in the predation process the safe compartment was used for the observations. The behaviour of the larvae was recorded by observing 5 different individuals consecutively for 90 sec each (Focal animal sampling, (Altmann 1974)). A program was made (event recorder system) and a PC used to record the time when different behaviours occurred. A human-recorded voice spoke the behaviour for which each button was pre-programmed, to minimise error when recording. The following behaviours were recorded: 1) *Swimming*: recorded as swimming bout rate, as the larvae usually were swimming 1-2 body lengths followed by an inactive period. Swimming bouts in contact with the bottom were not distinct and thus not included. 2) *Feeding*: recorded as number of times a larva was focusing on a prey. Focusing was easily recognised as the larva used the tail- and pectoral fins to keep the position of the head and body fixed relative to the prey. When swimming, not only the fins, but also the whole body created forward movement. The focusing often led to an attack, but as the attacks were difficult to recognise for the younger age groups, number of focuses was

used throughout the experiment. Larval behaviour was recorded twice, starting approximately 90 s (obs 1) and 90 min (obs 2) after larval transfer respectively. The position of each larva relative to a grid system (5 cm in the vertical plane and in the depth dimension divided into front, mid or back) was noted before and after the 90 sec observation. *Swimming distance* was expressed as the distance moved in a straight line during the observation period and calculated using the larval position in the grid system before and after observation (from centre to centre of grid cell). We tried to pick larvae for observation with a vertical position that roughly reflected the vertical distribution of the larvae in the compartment.

The distribution of the larvae in the two compartments was found by counting the number of larvae in each compartment. The distribution was recorded after each behavioural observation, or at about the same time in the aquaria where behaviour was not recorded. For the youngest age groups the larvae were difficult to count and a daily counting accuracy was estimated by counting the predator-free aquaria ((20 larvae added - # of larvae counted)/100, the first compartment was not recounted when the number in both compartments did not sum up to 20). The data on distribution were used to estimate *survival* (proportion of larvae surviving predator attacks = (sum live larvae - carcasses) / (added larvae - carcasses)) and *avoidance tendency* (tendency to seek the safe compartment = proportion of live larvae found in the safe compartment). Based on the predator-free controls the carcasses (dead larvae) at the bottom were assumed to have suffered from handling mortality.

Every test day a sample of 10 larvae was randomly picked from each of the 4 holding tanks (20 from each starvation group). Standard length (SL, snout to end of notochord) was measured in a stereomicroscope on live larvae anaesthetised with Metacain, before they were stored individually in Eppendorf vials in liquid nitrogen and thereafter in -80°C . Individual larvae were later analysed for dry weight (DW) and total amount of RNA and DNA according to methods described in Imsland et al. (2002). A One-Way ANOVA was run to compare the $\log\text{DW} : \log\text{SL}$ and $\text{RNA} : \text{DNA}$ relationship between nutritional groups.

Mean swimming bout rate and mean number of focuses (log-transformed due to a non-normal distribution) was found for each replicate aquarium (5 larvae), and the data were analysed by Repeated Measures ANOVA using the data analysis software system STATISTICA (StatSoft, Inc. 2001). Because only fed and 5 d starved larvae were tested in the youngest age group the design was incomplete, and two analyses were performed for each of the behaviour categories. In the main analysis the youngest age group was excluded to evaluate the three starvation levels used. In addition, the behaviour of the youngest age group was analysed using only the fed and 5-d starved larvae in all age groups in the Repeated Measures ANOVA, and the results were compared to those from the main analysis. The 2 observation periods were used as the repeated variable and age group, nutritional status and predator presence were categorical predictors. Swimming distance was analysed using Kruskal-Wallis ANOVA due to a bimodal distribution.

The distribution data were adjusted by correcting for the daily counting accuracy. The survival and the avoidance data were analysed by Repeated Measures ANOVA, with observation being the repeating variable, and age, nutritional status and predator presence the categorical predictors (only age and nutritional status for the avoidance data). Natural log transformations were used for the survival data to obtain linearity and homogeneous variances (Zar 1999).

Results

The daily growth rate in weight ranged from 7 % in the beginning of the experiment to 12 % for the oldest age group. Nutritional status, indicated by RNA : DNA, was lower for starved than for fed larvae for all age groups (One-way ANOVA, $p < 0.001$, Fig. 2). For the fed larvae the RNA : DNA was relatively constant throughout the experiment, whereas for the starved larvae this ratio seemed to increase for the older age groups, indicating that they tolerated starvation better than the younger age groups. The condition of the starved larvae ($\log DW : \log SL$) compared to the fed larvae was significantly different for all age groups (One-way ANOVA, $p < 0.001$). Except for in one case (mean swimming bout rate for the 33 d age group, Students t-test, $t = 2.5$, $p < 0.05$) there was no difference in mean swimming bout rate or in mean number of attacks for fed larvae between the two test days within each age group, and the data for fed larvae was therefore merged in the subsequent analysis.

Swimming activity. Age and nutritional status and the interaction effect between them explained the main differences in mean swimming bout rate between groups (Repeated measurements ANOVA, $p < 0.0001$, Table 2). Mean swimming bout rate increased with age for both 3 and 5 day

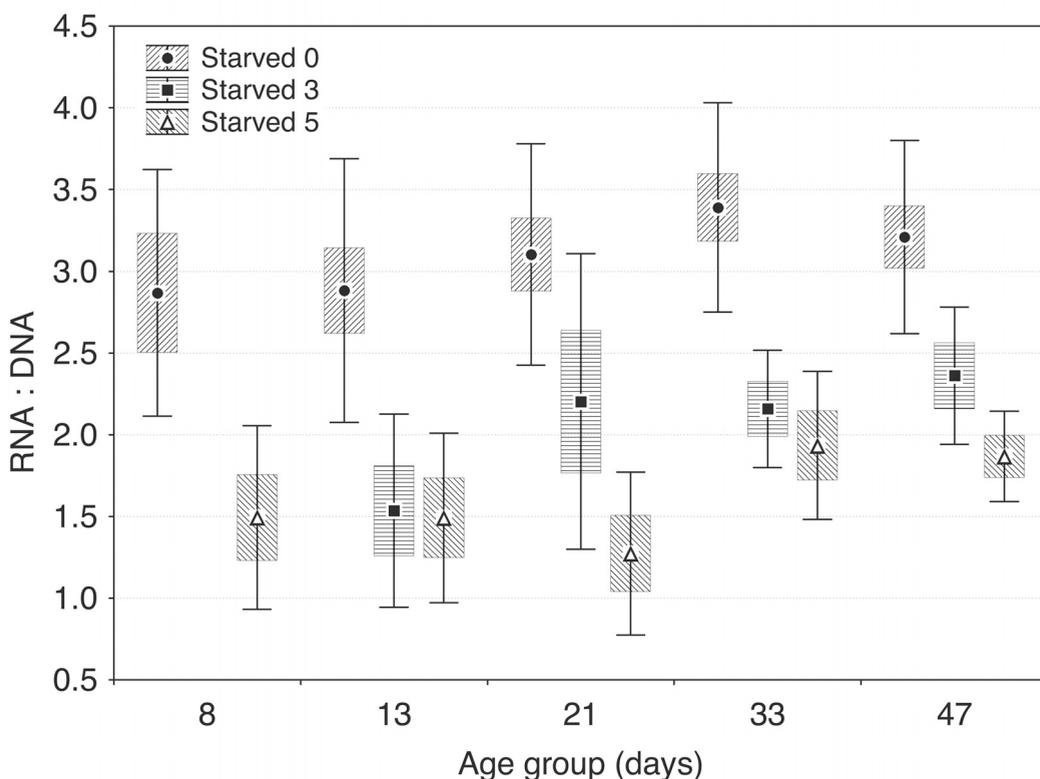


Figure 2. Mean (box: conf. Int, whiskers: SD) RNA : DNA ratio in fed, 3-d starved and 5-d starved larvae.

starved larvae, but not for the fed larvae. Swimming activity was influenced by nutritional status, but the effect depended on age. For the three youngest age groups the mean swimming bout rate decreased with increasing starvation, while for the two oldest age groups the swimming activity increased for the 3 d starved larvae and decreased for the 5 d starved larvae (Fig. 3). When the youngest age group was included, and the analysis performed on the fed and 5 d starved larvae alone, a similar interaction effect of nutritional status and age was evident (Repeated Measures ANOVA, $F_{4, 56} = 7.13$, $p < 0.0001$).

There were generally no differences in mean swimming bout rate between individual groups in the two observations. A significant interaction effect between observation, age and nutritional status was, however, found ($F_{6, 61} = 3.32$, $p < 0.01$), and the post hoc comparison revealed only minor differences in the effect of nutritional status between the two observations.

There was no clear general effect of predator presence on larval swimming activity. Since behaviour was recorded only in the safe compartment a change in behaviour could be expected only in those larvae that are able to sense the predator at some distance. Due to sensory development

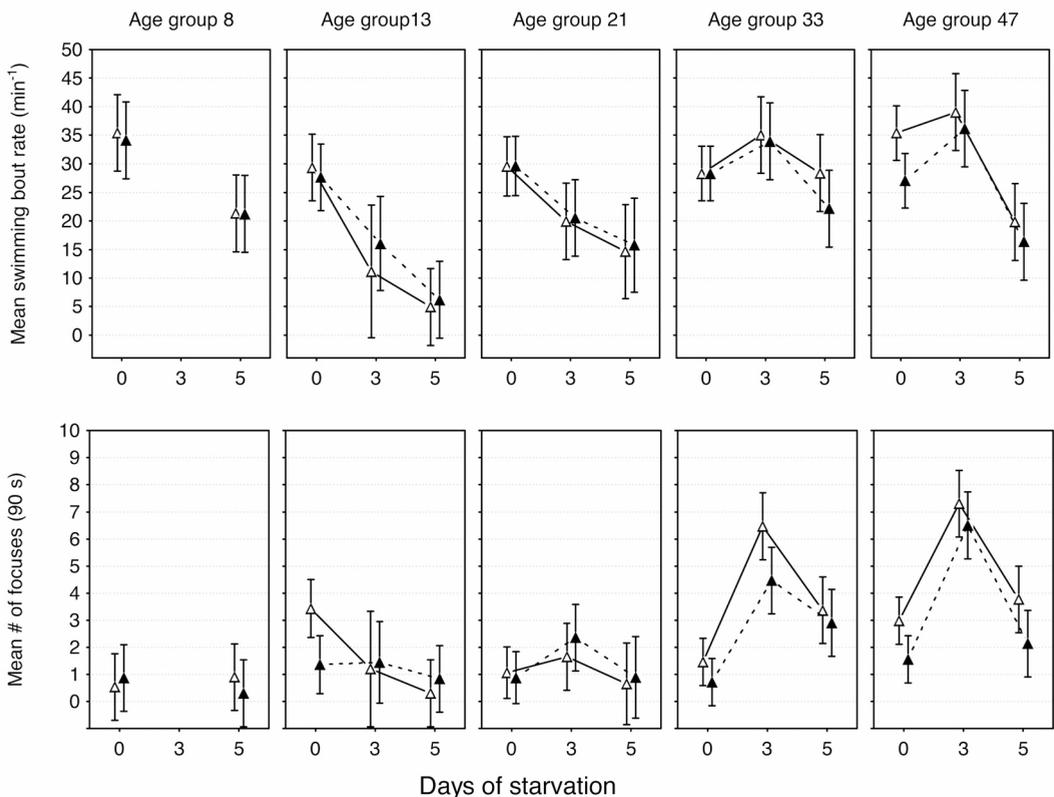


Figure 3. Mean (\pm conf. int) swimming bout rate (upper graph) and mean (\pm conf. int) # of focuses (lower graph) in each age group for larvae starved for 0, 3 and 5 days, with predators present (\blacktriangle , dashed line) and in controls (\triangle , solid line).

Table 2. Summary of 3-way and Repeated Measures ANOVA performed for the different behaviours and distribution data. Significant effects are marked with *. In testing survival only the data with predators present was used.

| | Mean swimming rate | | | | Mean feeding activity | | | | Prop. larvae in safe side | | | | Survival | | | |
|-------------------------|--------------------|---------|-------|---------|-----------------------|-------|-------|---------|---------------------------|-------|-------|---------|----------|-------|-------|---------|
| | df | MS | F | P | df | MS | F | P | df | MS | F | P | df | MS | F | P |
| Age | 3 | 1375.14 | 21.19 | <0.001* | 3 | 4.716 | 26.33 | <0.001* | 3 | 0.298 | 21.50 | <0.001* | 3 | 0.464 | 33.17 | <0.001* |
| Predator | 1 | 61.27 | 0.94 | 0.335 | 1 | 0.881 | 4.92 | 0.030* | 1 | 0.002 | 0.18 | 0.676 | | | | |
| Status | 2 | 2544.52 | 39.21 | <0.001* | 2 | 4.182 | 23.34 | <0.001* | 2 | 0.202 | 14.58 | <0.001* | 2 | 0.161 | 11.53 | <0.001* |
| Age*Predator | 3 | 76.88 | 1.18 | 0.323 | 3 | 0.355 | 1.98 | 0.126 | 3 | 0.344 | 24.82 | <0.001* | | | | |
| Age*Staus | 6 | 510.42 | 7.87 | <0.001* | 6 | 1.950 | 10.89 | <0.001* | 6 | 0.029 | 2.08 | 0.061 | 6 | 0.038 | 2.69 | 0.023* |
| Predator*Status | 2 | 26.06 | 0.40 | 0.671 | 2 | 0.520 | 2.90 | 0.062 | 2 | 0.047 | 3.40 | 0.037* | | | | |
| Age*Predator*Status | 6 | 28.05 | 0.43 | 0.855 | 6 | 0.224 | 1.25 | 0.294 | 6 | 0.044 | 3.15 | 0.007* | | | | |
| Error | 61 | 64.89 | 61 | 0.179 | 113 | 0.014 | 57 | 0.014 | | | | | | | | |
| OBS | 1 | 22.31 | 0.70 | 0.404 | 1 | 0.695 | 4.90 | 0.031* | 1 | 0.004 | 0.88 | 0.350 | 1 | 0.114 | 63.84 | <0.001* |
| OBS*Age | 3 | 3.03 | 0.10 | 0.962 | 3 | 0.080 | 0.56 | 0.641 | 3 | 0.020 | 4.48 | 0.005* | 3 | 0.019 | 10.83 | <0.001* |
| OBS*Predator | 1 | 0.24 | 0.01 | 0.931 | 1 | 0.201 | 1.42 | 0.239 | 1 | 0.011 | 2.58 | 0.111 | | | | |
| OBS*Status | 2 | 40.01 | 1.26 | 0.290 | 2 | 0.681 | 4.79 | 0.012* | 2 | 0.026 | 5.95 | 0.003* | 2 | 0.017 | 9.62 | <0.001* |
| OBS*Age*Predator | 3 | 0.96 | 0.03 | 0.993 | 3 | 0.034 | 0.24 | 0.869 | 3 | 0.049 | 10.96 | <0.001* | | | | |
| OBS*Age*Status | 6 | 105.15 | 3.32 | 0.007* | 6 | 0.138 | 0.97 | 0.450 | 6 | 0.019 | 4.35 | 0.001* | 6 | 0.009 | 5.17 | <0.001* |
| OBS*Predator*Status | 2 | 0.79 | 0.03 | 0.975 | 2 | 0.014 | 0.10 | 0.904 | 2 | 0.004 | 0.97 | 0.383 | | | | |
| OBS*Age*Predator*Status | 6 | 29.58 | 0.93 | 0.477 | 6 | 0.147 | 1.04 | 0.411 | 6 | 0.008 | 1.74 | 0.118 | | | | |
| Error | 61 | 31.65 | | | 61 | 0.142 | | | 113 | 0.004 | 57 | 0.002 | | | | |

this might be expected only in older larvae (see discussion) and then perhaps only in some nutritional groups. In Figure 3 an effect of predator presence on the two oldest age groups was indicated. A Repeated Measure ANOVA was thus performed on these age groups, it revealed a lower swimming activity in the oldest age group where predators were present ($F_{1, 18} = 4.95$, $p < 0.05$). The decrease in swimming activity was most pronounced for fed larvae.

Feeding related behaviour. Feeding activity was generally low and the mean number of focuses ranged from 0 to 11.6 during the 90 s. Age and nutritional status and the interaction effect (Repeated Measures ANOVA $p < 0.0001$) between these two explained the main differences in feeding behaviour between the groups (Table 2). In the 3 youngest age groups the feeding activity was low for all starvation groups, while for the two oldest age groups feeding activity increased for the 3 d starved larvae and then decreased for the 5 d starved larvae (Fig. 3), but was still higher than for the fed larvae. An interaction effect between observation period and nutritional status was found (Repeated Measures ANOVA, $p < 0.05$), and the post hoc comparison revealed a higher feeding activity in obs 2 than in obs 1 for the fed larvae, but not for the other nutritional groups (Unequal N HSD, $df = 120.39$, $p < 0.001$). The youngest age group did not modify the general results in the ANOVA when included in the analysis.

The presence of predators significantly suppressed the feeding activity (Repeated Measures ANOVA, $p < 0.05$, Fig. 3). Graphically there seemed to be an interaction effect between age and predator presence, with less effect in younger larvae. And when the youngest age group was included in the analysis the interaction effect between age, nutritional status and predator presence was significant (Repeated Measures ANOVA, $F_{4, 56} = 3.01$, $p < 0.05$).

Swimming distance. Swimming distance depended on age (Kruskal-Wallis ANOVA, $p < 0.001$). For the three youngest age groups swimming distance was short, but it increased markedly for the next two age groups (data not shown). Presence of predators did not influence swimming distance and there was no effect of nutritional status except for the 33 d age group (Kruskal-Wallis ANOVA, $p < 0.005$) with swimming distance first increasing and then decreasing with increasing starvation time.

Predator avoidance and survival. The tendency to seek refuge in the safe compartment was influenced by larval age, nutritional status and observation period, with a strong interaction effect between predator presence and age (Repeated Measure ANOVA, $p < 0.0001$, Table 2). In the youngest age group fewer larvae were in the safe compartment than in the control groups (Fig. 4), indicating that the larvae moved over to the risky compartment without returning. Older larvae gradually showed an increased tendency to seek refuge in the safe compartment. Nutritional status influenced the distribution, but depended on age, predator and observation. The 5 d starved larvae avoided the risky compartment less than the fed and 3 d starved larvae in some age groups. The interaction effect of the observation period with the other variables (status, age and predator) indicates that the larval distribution changed over time with predator exposure, with differences between the age groups and starvation groups in time of movement.

Mortality caused by larval transfer was generally low (age group 8-21: max 15%, mean = 1.8%, age group 33-47: max 5%, mean = 0.16%) and the counting accuracy high (never below 0.80).

Carcasses were not included when calculating proportion larvae surviving predation. The survival increased from about 50% in the youngest age groups, to up to 100% for the larvae in the oldest age group. Age and status both had a strong influence on survival, and there was a weak interacting effect between the two variables caused by the 3 d starved larvae showing a higher survival than fed larvae in the two oldest age groups (Repeated Measure ANOVA, $p < 0.05$, Table 2). Observation, as the repeating variable, interacted with age and status, indicating that the time when predation mortality occurred (before observation 1 or between observations 1 and 2) varied with age and nutritional status, and reflected the tendency of the larvae to seek refuge.

Discussion

The swimming and feeding activity of cod larvae in the current experiment was influenced by age, nutritional status and presence of predators. Swimming activity decreased with deteriorating nutritional status for the 3 youngest age groups, while for the two older age groups swimming activity initially increased and subsequently decreased with increasing starvation time. The RNA : DNA pattern confirmed differences in nutritional status between groups and that the fed groups were initially in good condition. Starvation eventually depletes the energy reserves, but before this, may initially lead to changes in behaviour for energy saving or energy searching purposes (Blaxter and Ehrlich 1974; Chick and Van den Avyle 2000; Munk 1995; Ross et al. 1996; Skiftesvik 1992; Sogard and Olla 1996). The decrease in swimming activity with starvation in younger larvae is likely due to energy saving, either because the costs of food search under the current prey density is too high, or because insufficient energy reserves to maintain activity are available. Food searching activities will in general be expected to increase during starvation (Werner and Anholt 1993) and an increase in swimming activity could have been expected for the three youngest age groups with a shorter starvation period than used here (Skiftesvik 1992) or with a higher prey density. In older larvae with more energy reserves, swimming activity did not decrease until after 5 days of starvation. The increase in swimming activity after 3 days of starvation likely reflects the search for food. Other studies have also shown an initial increase in activity for starved larvae or larvae reared at low prey density. Wyatt (1972) found an increase in the amount of time spent searching at low prey densities in plaice larvae (*Pleuronectes platessa* L.), and Munk (1995), argued that the increase in swimming activity in 2-3 weeks old cod larvae with low prey density was caused by a high hunger level. Puvanendran et al. (2002) also found a higher swimming activity at lower prey densities for cod larvae ranging from 0 to 6 weeks. The increase in swimming activity with age for starved larvae in the current experiment, and the relatively lower difference in RNA : DNA ratios between fed and starved larvae with age indicates that food depletion had less impact on the older larvae (see also Jonas and Wahl 1998; Yin and Blaxter 1987b).

The feeding activity was low for the 3 youngest age groups, while in the older age groups it increased markedly for the starved larvae. The prey density in the aquaria was very low (70 l^{-1}) compared to what is considered necessary to obtain adequate growth in newly hatched cod larvae ($>1000 \text{ l}^{-1}$, (Otterlei et al. 1999)), but was set at this level to increase swimming activity (Munk 1995) and to delay a change in nutritional condition over a short time. The feeding rate observed

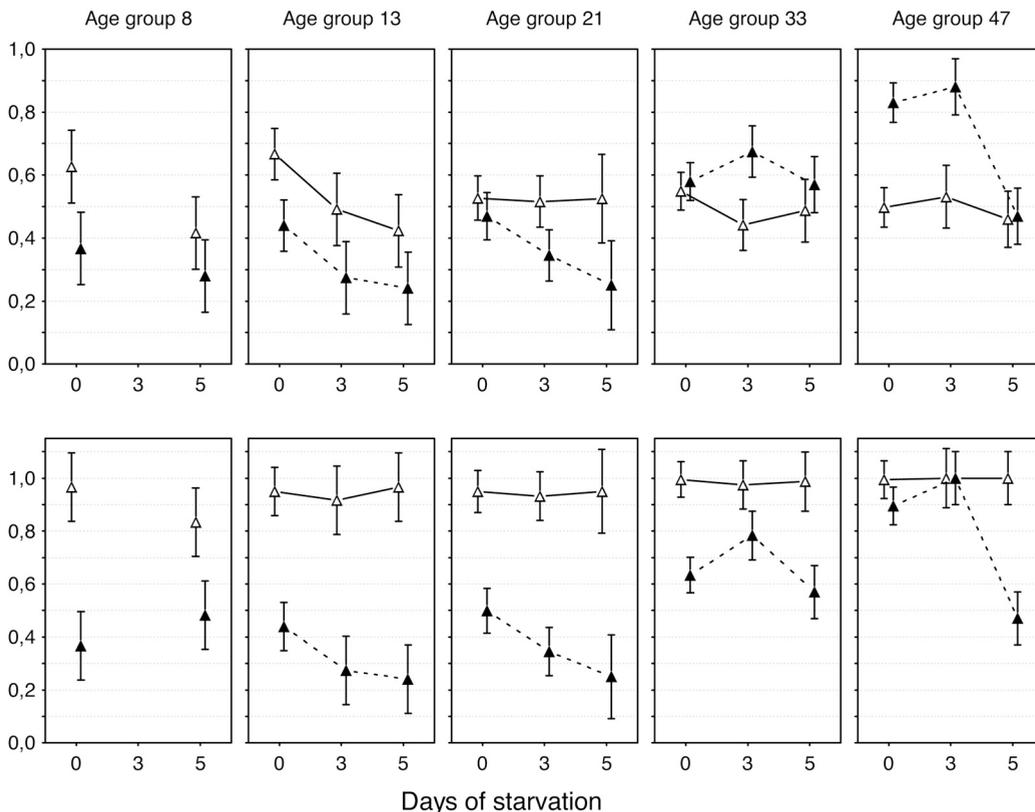


Figure 4. Mean (\pm conf. int) proportion of added larvae found in the safe side and mean survival after observation period 2 in each age group for larvae starved for 0, 3 and 5 days, with predators present (\blacktriangle , dashed line) and in controls (\triangle , solid line).

by Munk (1995) for 2-3 week old cod larvae at a similar prey density was within the range found in the current experiment. The low feeding activity in the youngest age groups was probably an effect of limited visual perception combined with the swimming activity being too low to increase food encounter. Prey detection ability and swimming capacity, as well as starvation tolerance, improve with age (Blaxter 1986; Fuiman and Delbos 1998), and this would explain the increased feeding motivation after a given starvation period for older age groups. With further deteriorating nutritional status, feeding activity was eventually limited by reduced swimming activity. Our results are in line with other studies that have shown an initial increase and a subsequent decrease in feeding activity with increasing starvation (Yin and Blaxter 1987b; Ross et al. 1996).

When starvation-induced changes in behaviour are used to explain variation in predation mortality one should know whether the behaviour is maintained also in the presence of predators. Predator presence affected the behaviour of the two oldest age groups with a decrease in feeding activity and a tendency towards a decrease in swimming activity. The larvae's ability to receive

visual, mechanoreceptive and auditory inputs improves with age (Blaxter 1986; Batty 1989; Fuiman and Magurran 1994) and sets the perception distance (Higgs and Fuiman 1998). Few studies have investigated the effect of predator presence on larval behaviour before an attack occurs (before the predator comes very close to the larvae). However, Lima (1998) and Bishop and Brown (1992) found a decrease in feeding activity in the presence of predators in 5-15 week old larval lumpfish (*Cyclopterus lumpus*) and stickleback larvae (*Gasterosteus aculeatus*) younger than 30 days. These studies indicate that larvae are able to alter the risk of predation by taking action early in the predation process. The current study supports this conclusion by showing a decrease in feeding activity in the presence of predators, and further suggests that the ability to do so arise in cod larvae after 21 dph. The effect of predator presence did not depend on larval nutritional status, as the decrease in feeding activity was similar for all groups. An effect of nutritional status was however indicated in the swimming activity in the oldest age group as the decrease here in the predator presence was most pronounced for the fed larvae. This is in agreement with the theory of balancing the benefits of feeding and risk of predation (Milinski 1993; Ydenberg and Dill 1986), with starved fish having a higher cost of reducing swimming and prey encounters than fed larvae.

The proportion of larvae found in the safe side provides information on the larvae's ability to sense a predator and to behave in an adaptive way to its presence. In the three youngest age groups almost all the surviving larvae were in the safe side. Except for in the youngest age group, starved larvae seemed more vulnerable to predation than fed larvae. The number of starved larvae found in the safe side was lower than in the control groups, indicating that more larvae moved over to the risky side than in the opposite direction. The starved larvae thus seemed either unable to sense the predator at the required distance or unable to keep distance. For the fed larvae the safe-side distribution was not different from the control groups. Two causal mechanisms might explain this. The fed larvae could either sense the predator presence and/or avoid the area of danger, or the escape success could be relatively higher, giving them time to move from the risky to the safe side by random movement or active avoidance at the same rate as they moved in the opposite direction. We favour the latter explanation because otherwise one could argue that fed larvae in the 13 d age group are more developed in regard to perceiving predator presence than the starved larvae in the 21 d age group. This is unlikely, since when the larvae in the 21 d age group were transferred to the starvation tanks they were older than the fed larvae in the 13 d age group.

For the two oldest age groups a gradually higher proportion of larvae were found in the safe side. Here we would argue that the safe side distribution is relatively more affected by the larvae being able to perceive the predator's presence. This is supported by the observation that feeding activity was influenced by predator presence in these older age groups. The safe side distribution would be the result of one or several of the following behavioural mechanisms: 1) The larvae showing a higher reaction rate with age (Folkvord and Hunter 1986; Margulies 1989) giving them more time to seek refuge by random movement, 2) the larvae sensing the predator and avoiding entering the risky area, and 3) the larvae actively seeking refuge in the safe compartment. With increasing age and nutritional status the relative importance of these mechanisms differ as a result of the ongoing development of the sense organs with larval size or age (Batty 1989; Fuiman and Magurran 1994) and of differences in motivation for predator avoidance. For instance in the oldest age group significantly more fed and moderately starved larvae were found in the safe side com-

pared to in the controls, while for the most starved larvae there was no difference. The fed and moderately starved showed (1) a high reaction rate, (2) sensed the predator and avoided entering the risky area and, (3) could likely actively seek refuge. The most starved larvae sensed the predator and avoided entering the risky area (2), but the escape success was too low to give the larvae time to seek refuge, or if they were able to actively seek shelter, the motivation to do so was too low.

The changes in behaviour observed in starved larvae could change predation mortality by influencing encounter rate between predator and prey, attack probability and escape rate (Fuiman and Magurran 1994). Changes in swimming activity would alter the encounter rate as well as the conspicuousness of the prey and thereby the probability of detection and attack (Bailey and Houde 1989). Feeding likely reduces the escape success as the larvae then focus on the food and reduce predator vigilance (Milinski 1993; Skelly 1994). An increase in both swimming and feeding activity would therefore be expected to increase risk of predation mortality by affecting the encounter rate, conspicuousness and escape success. The trade-off in favouring searching behaviour relative to anti-predation behaviour for moderately starved larvae in the presence of predators would further increase the risk of predation relative to the fed larvae. In the current study the moderately starved larvae in the older age groups showed a 20% increase in swimming activity and a 240% increase in feeding activity compared to fed larvae. For the 5 d starved larvae, swimming rate was reduced by 27% while feeding activity was increased by 67% relative to the fed larvae. Based on these results the change in vulnerability to predation with increasing starvation should thus be expected to be dome shaped, with moderately starved larvae showing increased risk of predation mortality compared to fed larvae and larvae starved for a longer period. An increased swimming and feeding activity could have been expected for the younger age groups if a shorter starvation period or higher prey density had been used (the 3 days starvation is presumably rather severe for these age groups) and the general dome shaped vulnerability should be expected here as well.

The dome shaped vulnerability curve is based on the changes in swimming and feeding activity in larvae with different nutritional status. However, several other factors influence the vulnerability curve. Firstly, a reduction in the escape-rate observed for starved larvae (Booman et al. 1991; Yin and Blaxter 1987a) is likely due to energy saving and not increased feeding activity. Strongly starved larvae would thus show a low reaction rate even though feeding activity decreases. Secondly, the vulnerability of a larva depends on the predator regime as the relative importance of the different factors affecting predation mortality would depend on the predator (Bailey and Houde 1989). Changes in larval behaviour affecting the encounter rate would for instance be much more important in the presence of a slow moving predator compared to a fast moving one. Chick and Van den Avyle (2000) modelled the increase in predation mortality for two different fish predators and found that larval swimming activity had little or no impact on the predation mortality, while Bailey and Yen (1983) showed that swimming activity in Pacific hake (*Merluccius productus*) larvae affected the predation mortality by a marine copepod. Vulnerability to predation also depends on the larva's absolute size, which again differs according to the predator regime (Bailey and Houde 1989).

The general dome shaped relation in vulnerability to predation with increasing starvation was not reflected in the current study. However, only the larvae in the risky compartment suffered from predation mortality and the distribution of larvae relative to the predators was thus not an ideal free distribution. This would affect the differences in survival between nutritional groups because

of variations in the larvae's ability to seek refuge. For instance, the increase in activity for the 3 d starved larvae in nature would increase predator encounter rate, but could in the current experiment increase the chance of the larvae moving to the safe side and thereby reduce predation mortality. Further, the visual perception of the predators far exceeds the range used in the aquaria, and as the dome shaped relation in vulnerability is largely explained by changes in encounter rate, the survival results can not be compared to the theoretic vulnerability curve. The survival and distribution data can, however, be used to identify larvae's ability to behave according to a predator, and to elucidate differences between groups at different developmental stages.

The results in this study contribute to our knowledge of predation mortality relative to nutritional status in fish larvae, to ontogenetic changes relating to predator evasion and avoidance, and to the ontogeny of decision-making during trade-offs between conflicting demands. The results agree largely with earlier studies investigating related aspects on larval behaviour, but provide additional knowledge especially regarding the effect of the presence of a predator on larval behaviour. The presence of a predator influenced the behaviour of older larvae in the current study. The ontogeny of the sense organs in young larvae is likely tuned on finding food at close range to ensure rapid growth, which in itself reduces the overall predation mortality (Blaxter 1986; Fuiman 1993). Their anti-predation behavior includes escape responses when the predator is close to the larva (Batty and Blaxter 1992), and presumably to a smaller degree changes in behaviour acting before an attack is commenced. The current experiment suggests that mainly larvae older than 3 weeks are able to perceive predators at some distance and make trade-offs between feeding and anti predator behavior.

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Morphogenesis of sense organs in the bluefin tuna *Thunnus orientalis*

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Abstract

Larvae of the bluefin tuna *Thunnus orientalis* were reared from hatching to day 30, sampled every day, and examined under light and electron microscopy for morphological development of the sense organs. The larvae came from fertilized eggs collected from a coastal broodstock pen and transferred to the hatchery of the Amami Station of the Japan Sea Farming Association. Growing larvae were sequentially fed rotifers, brine shrimp, and fish larvae. In this paper, morphological development is given by larval size (total length TL) and age (hours or days from hatching). Newly hatched larvae were 3.0 mm long, hung suspended head down in the water column, and avoided glass pipets put in their way. Yolk-sac larvae had well developed mechanoreceptors — a pair of large free neuromasts (45 µm diameter, with cilia and cupulae 40-50 µm thick and 250-310 µm long) behind the eyes, seven pairs of small neuromasts on the head, and seven pairs on the trunk. Yolk-sac larvae also had an inner ear — an ovate otic vesicle with two otoliths and an innervated ciliated epithelium; the inner ear formed three maculae after 18 h and three pockets after 2 d. The retina was innervated after 13 h, the retinal layers and the lens formed after 19 h, and the single cones and horizontal cells in the retina differentiated after 2 d. The olfactory pits also opened after 2 d and the olfactory epithelium had ciliated receptor cells, microvillous receptor cells, and ciliated nonsensory cells. Larvae 3.9 mm long (2 d) were rheotactic and could swim 10x their total length in a single burst. Larvae 4.0 mm long had well pigmented eyes when they started to feed at 3 d. Larvae of 4.5 mm (5 d) were slightly photopositive. Larvae 5.6 mm long (8 d) had a flexed notochord, small pelvic fins, and three partly ossified semicircular canals. The taste buds differentiated first in the epithelium of the upper pharynx of larvae 5.6 mm long, when canine-like teeth and pharyngeal teeth also appeared. Larvae of 16 mm (16 d) had complete fin rays and spines except in the pectorals, had nine pairs of neuromasts on the trunk and 11 pairs on the head, and showed optomotor reaction. The development of the sensory organs are accompanied by behavioral changes that have important implications to larval ecology at sea and to tuna hatchery operations.

Introduction

Ontogeny of the sense organs and behavior is important to feeding and predator avoidance of fish larvae at sea (Blaxter 1986). Such is true as well in fish farms. Successful farming depends on understanding the behavior of fish, especially during the early larval stages when technical difficulties result in high mortality in the hatchery. Changes in larval behavior are closely related to the development of the sense organs in marine fishes (Ishida 1987), in large-mouth bass *Micropterus salmoides* and Nile tilapia *Tilapia nilotica* (Kawamura and Washiyama 1989), and in reared marble goby *Oxyeleotris marmoratus* (Senoo et al. 1994).

Tunas are among the most important species in world fisheries but also among the least known in terms of early life history. Most studies of the early life history of tunas have depended on ichthyoplankton net samples. Yabe et al. (1966) collected larvae of the bluefin tuna *Thunnus orientalis* at high density in western Pacific waters, indicating spawning in the area. Davis et al. (1990) found diel patterns in the vertical distribution of larvae of southern bluefin *Thunnus maccoyii* and other tunas in the east Indian Ocean. Margulies (1997) examined the visual system of larvae and juveniles of *Euthynnus lineatus*, *Scomberomorus sierra*, and *Auxis* spp. caught at sea and inferred their performance capabilities. The recent success in breeding and rearing bluefin tuna in Japan (Miyashita et al., 2001) has made available valuable experimental material for many studies on larval biology: the digestive system (Takii et al. 1996; Miyashita et al. 1998); oxygen consumption (Miyashita et al. 1999); rotifer-size selectivity and optimum rotifer density (Sawada et al., 2000); trauma caused by collision with walls of tanks or cages (Miyashita et al., 2000); morphological development (Miyashita et al., 2001); muscle development (Hattori et al., 2001); and retinomotor responses (Masuma et al., 2001). We studied the morphogenesis of the sense organs in bluefin tuna to improve the knowledge about the early life history of this fish.

Materials and Methods

Fertilized bluefin eggs were collected from the coastal broodstock pen and transferred to a 500 l rearing tank in the laboratory of the Amami Station. Larvae and juveniles were fed S-type rotifer *Brachionus plicatilis* and L-type rotifer *B. rotundiformis* from first feeding to 25 d, *Artemia* nauplii from 9 d to 30 d, larvae of striped knifejaw *Oplegnathus fasciatus* from 10 d to 30 d, and minced and chopped fish meat from 15 d to 30 d. Water temperature in the tank ranged from 27.3 to 29.0°C. The juveniles were transferred to an outdoor fish cage after 30 d.

Bluefin larvae were sampled every day for 33 d (the oldest specimens were from the outdoor cage) and examined for morphological development of the sense organs. One or two specimens were anesthetized with MS 222 and illustrated with the aid of a camera lucida. Live yolk-sac larvae were anesthetized with MS 222 for observation of the cupulae of free neuromasts under a profile projector. Some specimens were preserved in Bouin's solution, embedded in paraffin, cut into 6-8 µm thick longitudinal and cross sections and stained with hematoxylin-eosin for histological examination under a photomicroscope. Other specimens were preserved in 4% glutaraldehyde or 4% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4), dehydrated in an ethanol series, freeze-

dried, and coated with platinum for examination of the olfactory epithelium, free neuromasts, and taste buds under a scanning electron microscope. As it was difficult to resolve the rod cells in the retina by photomicroscope, the nature of the visual cells was ascertained by making counts of the distinct ellipsoidal structures (the cone ellipsoids) in the visual cell layer, and of the cell nuclei in the outer nuclear layer in 3-5 adjacent cross-sections of the retina.

To study the functional development of the eyes, free neuromasts, and olfactory epithelium, the behavior patterns of larvae of 3.7 mm to 24 mm in total length (1 d to 21 d old) were observed both in the rearing tank and in a 2 l glass beaker in the laboratory:

- phototactic response to a 5-watt flashlight (method earlier described by Kawamura et al. 1983),
- optomotor reaction to a moving pattern of alternate black and white stripes 1 cm wide (Kawamura and Hara 1980),
- rheotaxis (Kawamura et al. 1984).

Results

Larval development and behavior. The typical pattern of early larval development of bluefin tuna is shown in Figure 1. The newly hatched yolk-sac larvae averaged 3.0 mm in total length TL. The pectoral fin buds appeared after one day. When the larvae were 3.9 mm long (2 d), the pectoral fins were formed, the yolk and oil globule were nearly resorbed, and the mouth and anus opened. Larvae avoided a moving glass pipet well; the single-burst avoidance movement increased to as much as 4 cm (ca. 10x TL) after 2 d. Larvae were also capable of horizontal movement, staying upright, and swimming against the current (positive rheotaxis). Larvae at 4.0 mm (3 d) had well pigmented eyes, a bent and swollen rather than straight gut, and small amounts of yolk and oil globule still remaining; these larvae started to feed on rotifers. Larvae 4.5 mm long (5 d) were positively phototactic and some had swimbladders inflated for the first time. The rudiments of the hypural plate and the anal fin appeared in larvae 5.8 mm (9 d), at the same time as four teeth on the upper jaw and three on the lower jaw. The notochord was slightly flexed in 5.8 mm larvae and fully flexed in 8.2 mm (12 d) larvae. All larvae 8.2 mm long had inflated swimbladders and forked caudal fins. Cannibalism was first observed among the flexion larvae. Larvae 16 mm (16 d) had the full complement of rays and spines in all fins except the pectorals, and showed optomotor reaction for the first time. The pectoral fin in most larvae larger than 24 mm (21 d) attained the full complement of 33 soft rays, and this marked the start of the juvenile stage. Scale formation began with the lateral line scales behind the operculum in 18 mm larvae (18-20 d), proceeded over the trunk, but was still not completed by 33 d in juveniles 29.8-45.5 mm.

Table 1 shows the chronology of morphological, sensory, and behavioral development of bluefin larvae. Details are given in the following sections.

Lateral line system. Bluefin larvae hatched with unpigmented eyes, closed olfactory pits, no taste buds, but well developed free neuromasts. Yolk-sac larvae hung suspended head down in the water column but were capable of avoiding a glass pipet gently placed in their way in the rearing tank. A pair of extremely large free neuromasts (45 μ m diameter, with cilia and cupulae 40-50 μ m thick

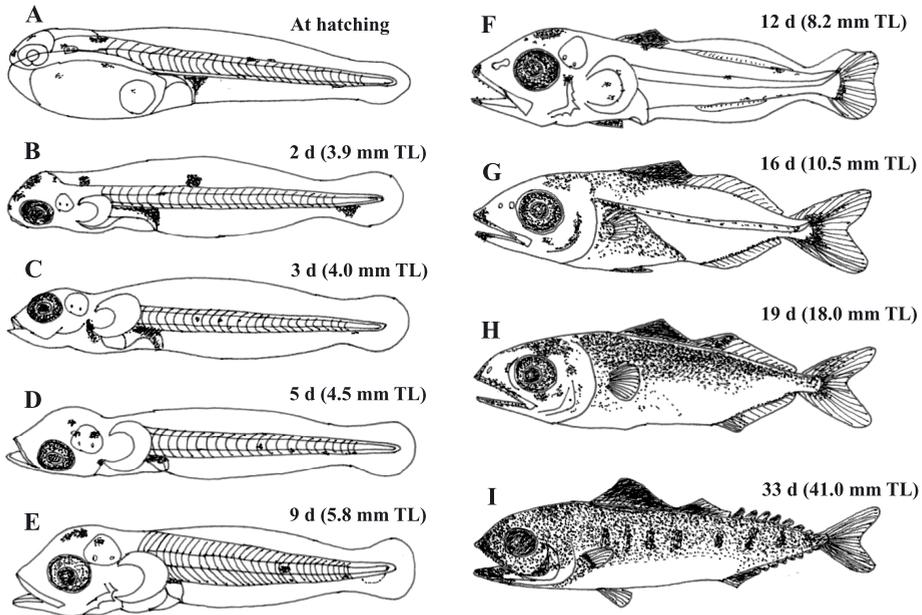


Figure 1. Bluefin tuna larvae and typical features of morphological development: A, newly hatched yolk-sac larvae 3.0 mm TL; B, larva 3.9 mm (2 d old) with pectoral fins formed, yolk and oil globule nearly resorbed, and mouth and anus open; C, larva 4.0 mm (3 d), eyes well pigmented, small amounts of yolk and oil globule still present, and bent and swollen gut with food; D, larva 4.5 mm (5 d) with first inflated swimbladder; E, larva 5.8 mm (9 d) with rudiments of the hypural plate and anal fin base, four teeth on the upper jaw, and three teeth on the lower jaw; F, larva 8.2 mm (12 d) with fully flexed notochord and forked caudal fin; G, larva 16 mm (16 d) with complete rays and spines in all fins except the pectorals; H, larva 18.0 mm (19 d) with almost fusiform body; I, Juvenile 41.0 mm (33 d) with scale formation in progress.

and 250-310 μm long) was present behind the eyes, seven pairs of smaller neuromasts (cupulae 20 μm thick and 30-40 μm long) on the trunk, one pair on the snout, and five pairs around the eyes (Figure 2). Bluefin larvae did not seem hindered by their large cupulae during burst swimming. Electron microscopy of the free neuromasts showed that the neighboring hair cells were arranged in opposite directions and a single neuromast had four polarities, cranial-caudal and dorsal-ventral (Figure 3). The neuromasts with four polarities in bluefin can detect four-directional water displacements. The free neuromasts with four polarities are also found around the eyes in Japanese parrotfish *Oplegnathus fasciatus* (Ishida and Kawamura 1985). The four polarities might be more advanced in detecting moving objects than two polarities, cranial-caudal or dorsal-ventral, which can detect only bidirectional water displacements.

The free neuromasts increased to 9 pairs on the trunk and 11 pairs on the head in larvae 16.0 mm long (16 d). In juveniles 30.4 mm (23 d), the epidermis folded to form the lateral recess in which the free neuromasts were lodged, but some free neuromasts remained exposed in the open grooves on the head. Juveniles 29.8-45.5 mm long and 33 d old no longer had free neuromasts in the epidermis, but almost complete lateral line canals.

Table 1. Correlation between larval morphology and behavior and the morphogenesis of the sense organs in bluefin tuna *Thunnus orientalis*.

| Morphology and behavior | Size (age) | Sense organs |
|---|------------------------------------|--|
| Hung suspended head down in the water column Capable of avoiding moving pipette | Hatching 3.0 mm | Eyes unpigmented, taste buds absent, olfactory pits closed Otic vesicle with two otoliths and ciliated epithelium Free neuromasts on the head and the trunk |
| | York-sack stage 3.4 mm (13h) | Eye lens formed and retina layered |
| Mouth and anus open Pectoral fins appear Capable of horizontal movement, upright position Positive rheotaxis | 3.9 mm (2d) | Olfactory pits opened and epithelium ciliated |
| | 4.0 mm (3d) | Eyes well pigmented, visual system morphologically complete |
| Feeding starts | Preflexion stage 4.5 mm (5d) | Area lateralis in the retina |
| First inflation of the swimbladder Positive phototaxis | 5.6 mm (8d) | First taste buds in the upper pharynx |
| Teeth on the upper and lower jaws Notochode slightly flexed | 5.8 mm (9d) | |
| | Flexion stage 8.2 mm (12d) | Inner ear fully ossified |
| Notochode fully flexed Caudal fin forked Cannibalism starts | 16.0 mm (16d) | Free neuromasts 9 pairs on the head, 11 pairs on the trunk Anterior and posterior nares were formed Olfactory epithelium folds Horizontal cells in three layers |
| | Postflexion stage 18.0 mm (18d) | |
| Optomotor reaction starts | 18.0 mm (19d) | First rod cells in the retina Olfactory epithelium with 5 lamellae |
| Scale formation starts | 24.0 mm (21d) | |
| Pectoral fins with adult complement | Juvenile stage 45.5 mm (33d) | Lateral line canal complete First twin cones in the retina Taste buds absent on lips |

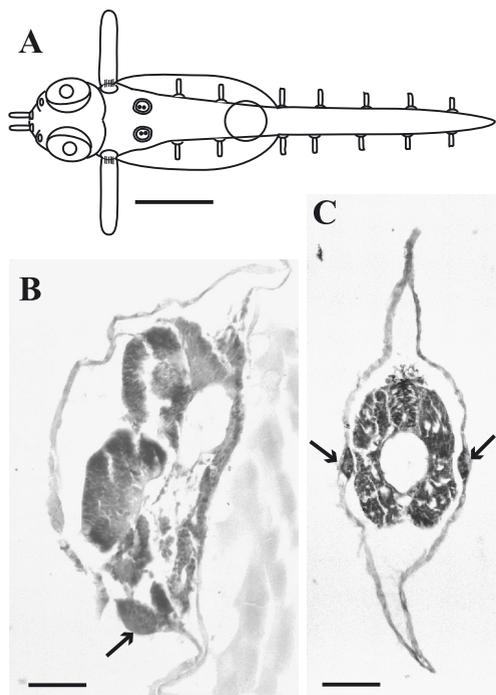


Figure 2. Free neuromasts in bluefin larvae 3 mm TL: A, arrangement of the free neuromasts around the body, except the five pairs of small neuromasts around the eyes not shown, scale bar 250 μm ; B, photomicrograph showing a large free neuromast behind the eye (arrow), scale bar 50 μm ; C, photomicrograph showing a pair of free neuromasts on the trunk (arrows), scale bar 50 μm .

Inner ear. At hatching, the inner ear of bluefin larvae was an ovate vesicle with two otoliths and an innervated ciliated epithelium (Figure 4). Three maculae were formed 18 h from hatching, and the semicircular canals and crus commune after 2 d. The canals were partly ossified in 5.6 mm larvae (8 d) and completely ossified in 8.2 mm larvae (12 d) when the notochord was fully flexed. The third otolith was not seen under a dissection microscope in larvae during the preflexion and flexion stages.

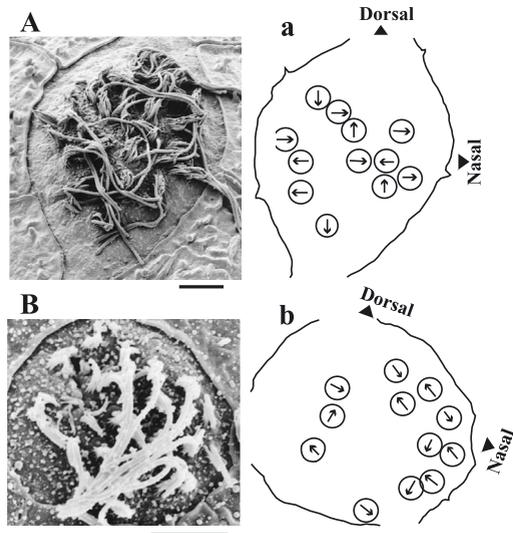


Figure 3. Scanning electron micrographs of the free neuromasts on the head (A) and trunk (B) of newly hatched bluefin larvae show the polarity of sensory hair cells, further illustrated in a and b, where each arrow points from the stereocilium to the kinocilia. Scale bar, 3 μm .

Eye. The visual system of bluefin larvae also developed rapidly (Figure 5). In larvae 3.4 mm long (13 h from hatching), the eye lens was formed, the outer nuclear layer of the retina had a single layer of cells and a thin inner plexiform layer was already recognizable. After 25 h, the 3.7 mm larvae had short but identifiable cone ellipsoids in the retina and a single layer of horizontal cells. The eyes were well pigmented in 4 mm larvae (3 d) and the visual system was morphologically complete, although the retina had only single cones. In larvae 4.5 mm (5 d), the retina thickened in the temporal area into an *area lateralis*, indicative of acute vision in the nasal direction. The horizontal cells formed three layers in the temporal and ventro-temporal regions of the retina in 10.5 mm larvae (16 d) and along the entire retina with an *area lateralis* in the ventro-temporal area in 30.4 mm larvae (23 d).

In the retina of 19 d old bluefin 18 mm long, the outer nuclear layer had two layers of

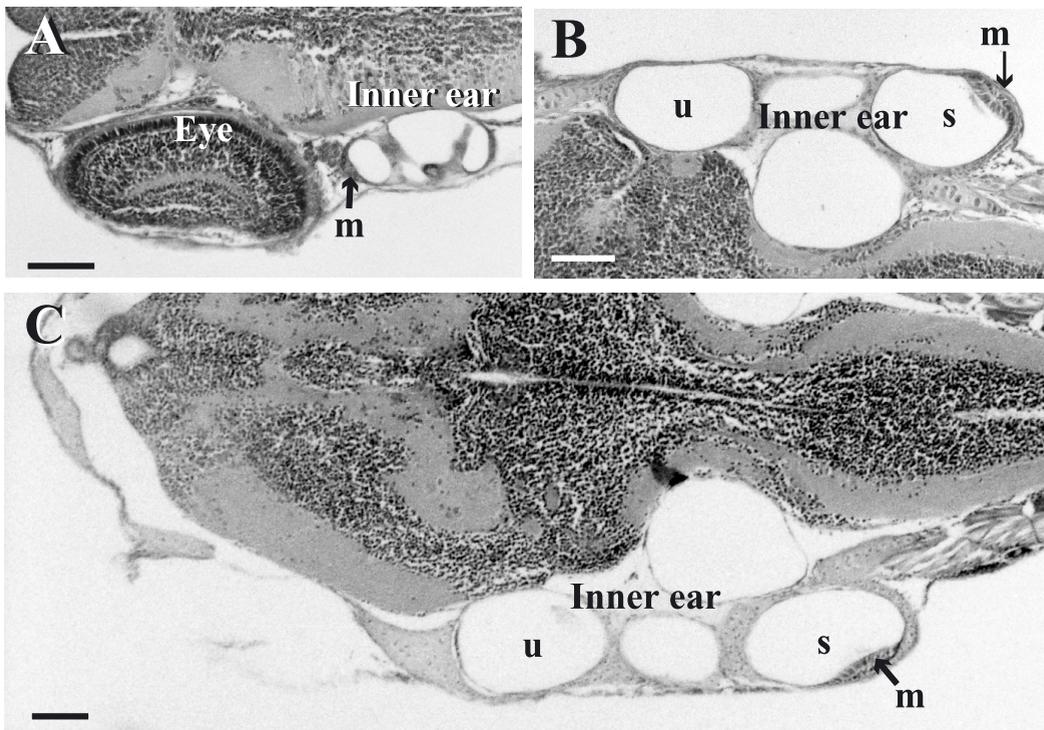


Figure 4. Development of the inner ear in bluefin larvae: A, larva 3.9 mm TL (2 d) with three pockets in the inner ear; B, larva 5.6 mm (8 d) with partly ossified semicircular canals; C, larva 8.2 mm (12 d) with completely ossified semicircular canals. m, macula; s, sacculus; u, utricle. Scale bar, 50 μ m.

cells and the ratio of the number of photoreceptor nuclei to the number of cone ellipsoids exceeded 1.0, indicative of the formation of rod cells. Rapid recruitment of rods was evident from the increase in this ratio from 4.4 in 30.4 mm juveniles (23 d) to 8.0 in 45.5 mm juveniles 33 d old. Twin cones were first seen in the retina of 33 d old juveniles (29.8 mm and 45.5 mm). The cones were arranged in a square mosaic (central single cone surrounded by four twin cones) in the *area lateralis* and in parallel mosaic in other areas of the retina.

Olfactory organ. Although the olfactory pits were still closed 13 h after hatching, the olfactory epithelium was already fused with the olfactory bulb of the brain, indicating early neural connection (Figure 6A). Larvae 3.9 mm (2 d) had open olfactory pits and the olfactory epithelium consisted of the ciliated receptor cells, microvillous receptor cells, and ciliated nonsensory cells (Figure 7). In first feeding larvae, the olfactory tracts were elongated and the olfactory bulb and lobes were well developed (Figure 6B). Anterior and posterior nares were fully formed in 11.1 mm larvae (16 d). The first folding of the central part of the olfactory epithelium was observed in 16 mm larvae (16 d) and five olfactory lamellae were formed in larvae 18 mm long (19 d).

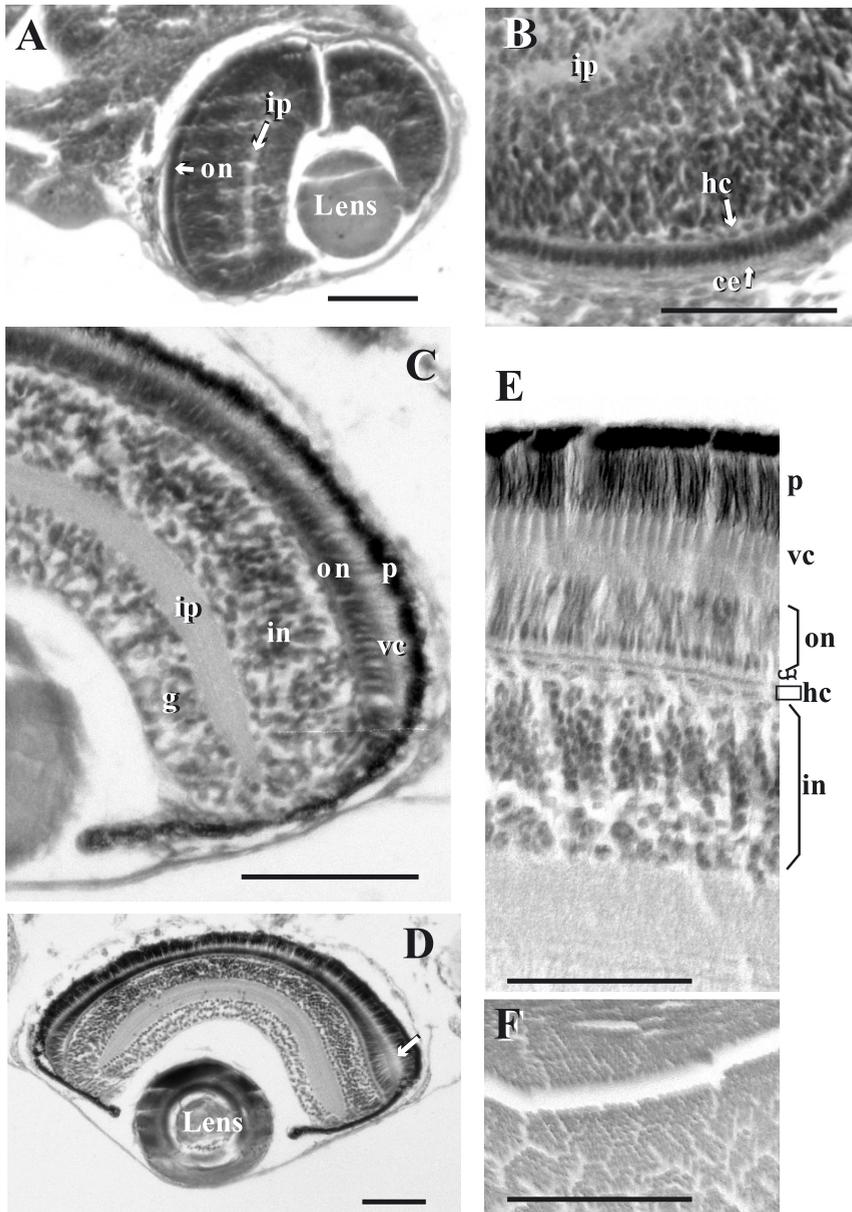


Figure 5. Development of the retina in bluefin larvae: A, larva 3.4 mm TL (13 h), lens is formed, retina with outer nuclear cells in a single layer and thin inner plexiform; B, larva 3.7 mm (25 h), retina with short but identifiable cone ellipsoids and single layer of horizontal cells; C, larva 4.0 mm (3 d), retina well pigmented; D, larva 4.5 mm (5 d), retina thickened in the temporal area (arrow) into an area lateralis; E, larva 18 mm (19 d), outer nuclear layer of retina with two layers – cone nuclei and rod nuclei; F, larva 18 mm (after 19 d), visual cell layer with single cones only. ce, cone ellipsoid; g, ganglion layer; hc, horizontal cell; in, inner nuclear layer; ip, inner plexiform layer; on, outer nuclear layer; p, pigment epithelium; vc, visual cell. Scale bar, 50 μm .

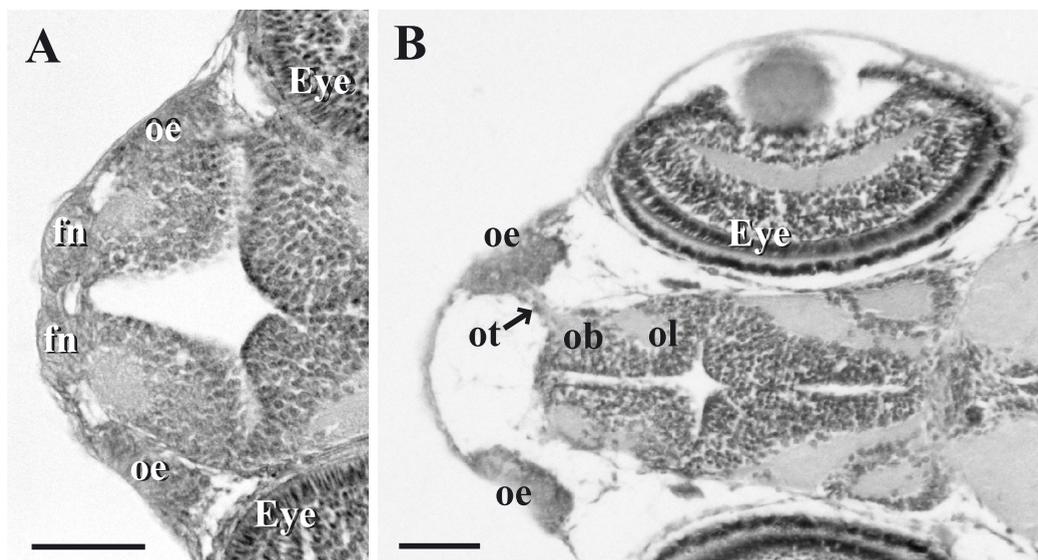


Figure 6. The olfactory system of bluefin larvae: A, larva 3.9 mm TL (2 d), olfactory epithelium fused with olfactory bulb; B, larva 4.0 mm (3 d), olfactory tracts elongated, olfactory bulb and lobes well developed; fn, free neuromast; ob, olfactory bulb; oe, olfactory epithelium; ol, olfactory lobe; ot, olfactory tract. Scale bar, 50 μ m.

Taste buds. The taste buds differentiated first in the upper pharynx of 5.8 mm larvae (8 d) and later in the epithelium of the oral cavity and gill arches in 6.7 mm larvae (10 d), when canine-like teeth and pharyngeal teeth appeared (Figure 8). The taste buds progressively became denser in the upper pharynx and the epithelium of the oral cavity and gill arches but were not present on the lips until 33 d.

Discussion

The results of this study contribute to a better understanding of the biology of tunas, particularly the sensory capabilities of the larvae of the bluefin *Thunnus orientalis*. The sensory systems developed at different times and rates, but they all ‘conspired’ (as it were) to maximize the chances of bluefin larvae to feed but avoid being eaten. The new information can be used to explain aspects of the early life history at sea as well as help develop better larval rearing techniques in the hatchery.

The early appearance of 14 pairs of free neuromasts in bluefin larvae suggests that mechanoreception is an important sense very early in life, presumably for predator avoidance and prey detection. The neuromasts in bluefin larvae had four polarities, cranial-caudal and dorsal-ventral, and can detect four-directional water displacements (similar to the neuromasts around the eyes in Japanese parrotfish; Ishida and Kawamura 1985). Neuromasts with four polarities are presumably more effective in detecting moving objects than those with two polarities, cranial-caudal or dorsal-ventral. Bluefin larvae in the rearing tank avoided a glass pipet by swimming away at burst speeds up to 10x the total length. Such burst speed can help larvae evade predators (or plankton nets) at

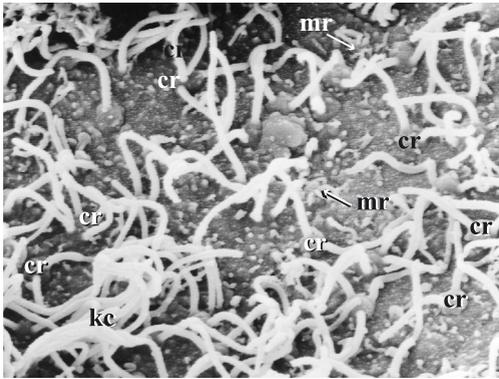


Figure 7. Scanning electron micrograph of the olfactory epithelium of bluefin larva 3.9 mm TL (2 d). cr, ciliated receptor cell; kc, kinocilia of nonsensory cell; mr, microvillous receptor cell. Scale bar, 3 μ m.

phus sajori, and Spanish mackerel *Scomberomorus niphonius* hatch with 9 to 12 pairs of free neuromasts, whereas those of the red sea bream *Pagrus major*, Japanese parrotfish, and Japanese flounder *Paralichthys olivaceus* have only one pair on the head, and those of the tiger puffer *Takifugu rubripes* have none at all (Ishida 1987). All these larvae are pelagic and it is not clear why there is such a difference in the number and size of free neuromasts. However, such differences must be taken into account during larval rearing. Larvae of species such as the bluefin with so many free neuromasts can not be handled without causing undue stress and it is advisable not to agitate or transfer larvae when the free neuromasts are still too numerous or still fully exposed. For several fish species reared in the hatchery, the high mortality experienced with early larvae, even before yolk resorption, may be due at least partly to stress from strong aeration, vibrations, or other disturbance overloading the sensitive mechanoreceptors.

In addition to the free neuromasts, a ciliated epithelium in the inner ear allowed bluefin larvae at hatching to detect vibrations in the water and avoid a moving pipet. The inner ear is able to detect vibrations in the absence of, or independent of the free neuromasts (Kawamura and Washiyama 1989). When it develops, the swimbladder becomes an important component of the auditory system of fish larvae because it converts variation in sound pressure into fluid displacements inside the body (Fuiman and Magurran 1994). Inflation of the swimbladder took place in bluefin larvae 8.2 mm long and 12 d old, coinciding with the full ossification of the semicircular canals of the inner ear. Full auditory function requires fully ossified semicircular canals (Ishida and Kawamura 1985).

Vision is the major sense used by fish larvae in feeding and the eye becomes well pigmented and functional before the onset of feeding (Kawamura and Washiyama 1989). Most fish larvae show positive phototaxis towards an artificial light source at the time of first feeding (Ishida 1987), but bluefin larvae showed phototaxis two days after first feeding. Several changes in the bluefin retina improved vision with growth. The area lateralis allowed acute vision and prey attack in the dorso-nasal direction (similar to the larvae of the black bream *Acanthopagrus butcheri*; Shand et al., 2000). In

sea (Fuiman and Magurran 1994; Davis et al. 1990). The ability of fish larvae to avoid plankton nets is probably due to both the free neuromasts (initiating the startle response) and the auditory system (detection of items at a distance) (Fuiman and Magurran 1994).

The number of free neuromasts and the size of the cupulae determine the sensitivity of mechanoreception (Mukai et al. 1994). Larvae of pelagic fishes tend to have more free neuromasts at hatching than larvae of demersal fishes; the former are capable of avoiding a moving glass pipet but the latter are not (Ishida 1987). The newly hatched larvae of the Japanese sardine *Sardinops melanostictus*, Japanese anchovy *Engraulis japonica*, halfbeak *Hemiram-*

the hatchery, the rotifers taken by 5-7d old bluefin larvae were larger than the average size available and the size-selective feeding was not due to change in mouth size (Sawada et al., 2000). The formation of the area lateralis probably enabled the size-selective feeding. The horizontal cells formed three layers in the area lateralis in 16 mm larvae (16 d), and throughout the entire retina in 30.4 mm larvae (23 d). Since the horizontal cells allow movement perception (Kawamura and Tamura 1973), bluefin larvae became better able to detect fast-moving prey, as evidenced by a well developed optomotor reaction at age 16 d.

Development of the retina differs among fish species and rods may appear before twin cones or vice versa (Omura et al. 1997). Bluefin larvae developed rods at 18.0 mm (19 d) and the number of rods quickly increased. The twin cones formed in early juvenile bluefin about 14 d after the rods, much later than in other tunas (postflexion stage in the scombrids studied by Margulies 1997). High visual sensitivity due to rods may be more important to bluefin larvae than acute form vision made possible by twin cones. Since rods are much more photosensitive than single and twin cones (Kawamura et al. 1984), bluefin larvae 18 mm and larger may inhabit deeper waters and be able to feed in dim light at dawn and dusk. Indeed, 18.1 mm was the maximum size of bluefin larvae collected by Yabe et al. (1966) from surface and subsurface (20-30 m) plankton tows in the western Pacific.

Chemoreception is also important to feeding larvae (Blaxter 1986; Fuiman and Magurran 1994). In bluefin larvae, the olfactory pits were open and the ciliated olfactory epithelium was externally exposed many days before the taste buds appeared. The yolk-sac larvae had two types of olfactory neurons, the ciliated receptor cells and the microvillous receptor cells, similar to other marine teleosts (Ishida 1987). Folding of the olfactory epithelium occurred earlier in bluefin tuna (at 16 mm, 16 d after hatching) than in several other marine teleosts from Japanese waters such as Japanese flounder (71 d), tiger puffer (57 d), Japanese parrotfish (36 d) and yellowtail *Seriola quinqueradiata* (31d) (Ishida 1987). Bluefin larvae developed taste buds at about the same age as other marine teleosts (6-13 d after first feeding; Ishida 1987). Taste buds allow fish to choose among food items and food preference has been observed in the larvae of Japanese flounder

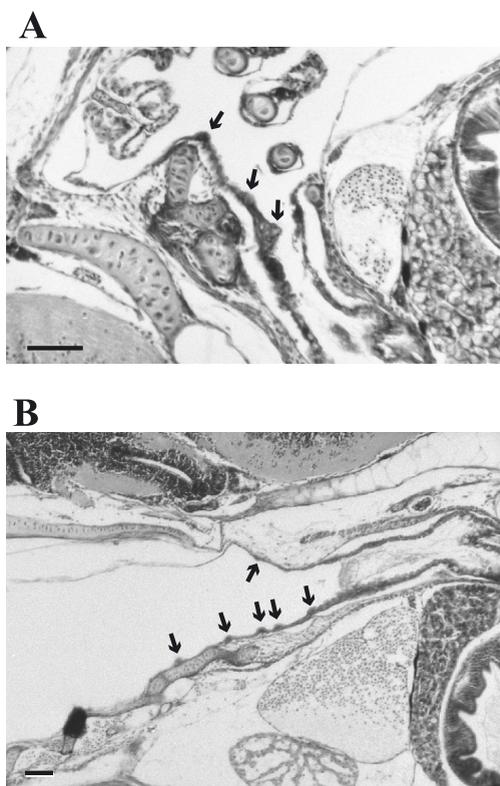


Figure 8. Taste buds in bluefin larvae: A, larva 5.6 mm TL (8 d), first taste bud (arrows) in the upper pharynx; B, larva 6.7 mm (10 d), first taste buds (arrows) in the epithelium of the oral cavity. Scale bar, 50 μ m.

(Kawamura et al. 1989) and largemouth bass (Kawamura and Wshiyama 1989) after the taste buds formed. Hatchery-reared bluefin larvae do not accept artificial feeds, even those with the stimulant L-glutamine (our unpublished observations), and it would be useful to study further olfaction and gustation in bluefin larvae to refine the hatchery technology.

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Development of the lateral line system in the channel catfish

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Abstract

Primitively, the lateral line system of jawed fishes consisted of mechanoreceptive neuromasts and electroreceptive ampullary organs. Both receptor types were retained in the earliest ray-finned fishes, but ampullary organs were lost with the origin of neopterygians and subsequently re-evolved at least twice among teleost fishes. The development of the lateral line system of the channel catfish, a member of one of the teleost groups having re-evolved electroreceptors, is described in order to complete an out-group analysis of the ontogenies of taxa from three groups: taxa that possess primitive electroreceptors, teleosts that have re-evolved electroreceptors, and teleosts that have not re-evolved electroreceptors. This type of analysis should identify where in development relevant changes have occurred and whether these changes involved the source tissue or the process. The lateral line system in channel catfish, as in the other taxa being compared, develops from a series of lateral line placodes that initially give rise to lateral line nerves, which subsequently innervate neuromasts that differentiate from elongations of the placodes termed sensory ridges. In the channel catfish, ampullary organ primordia appear to differentiate from the lateral zones of these sensory ridges, following the differentiation of the neuromasts, as do primitive electroreceptors in those taxa that retain them. This observation should be experimentally confirmed, as regards catfish, however. If catfish ampullary organs do arise from placodal cells, the re-evolution of electroreceptors in catfish would not involve a change in the embryonic source of these receptors but a change in the process of patterning, as compared to the placodes of teleosts that have not re-evolved electroreceptors.

Introduction

There are a number of reasons for studying the development of the lateral line system in catfishes. First of all, this system is an important sensory modality in most stages of the life history of fishes, but its development, particularly in teleosts, is poorly understood. Secondly, catfishes are an excellent group of teleosts in which to study the development of this system, because these fishes produce large numbers of very large embryos which facilitates both descriptive and experimental studies. Finally, an examination of one class of lateral line receptors, the electroreceptive ampullary organs, in catfishes reveals a fascinating evolutionary story.

An out-group analysis (i.e., a step-wise examination of a given trait, beginning with the most closely related taxon and progressing to more distantly related taxa) of electroreceptors among jawed fishes (Northcutt 1986) indicates that the earliest jawed fishes must have been electroreceptive, since all living cartilaginous fishes possess electroreceptive ampullary organs (Bodznick and Boord 1986), as do most groups of bony fishes and their descendents, the amphibians (Northcutt 1986; Fritzsche and Münz 1986). Primitive ampullary organs occur in two of the three living orders of amphibians, in lungfishes and the living coelacanth, *Latimeria*, as well as in cladistian and chondrosteian ray-finned fishes (Fig. 1). Ampullary organs appear to have been lost with the origin of neopterygian bony fishes, however, as bowfins (halecomorphs) and gars (ginglymodes) do not possess these organs (Bullock 1982; McCormick 1982; Bullock et al. 1983). Although most groups of living teleosts do not possess electroreceptors, two groups, i.e., some osteoglossomorph and euteleost fishes (Fig. 1), do have electroreceptors, which appear to have independently re-evolved, based on marked differences in their structure, innervation, and physiology (reviewed in Bullock and Heiligenberg 1986).

How is it possible to understand the multiple evolution of electroreceptors? The answer is relatively simple, but the process is difficult to implement. Because ontogenetic changes produce phylogenies (Garstang 1922; Northcutt 1990), it is necessary to perform an out-group analysis (Fig. 2) of multiple ontogenies, and at least three ontogenies must be compared: 1) the ontogeny of a taxon whose lateral line system retains primitive electroreceptors (cartilaginous fishes, lungfishes, cladistian and chondrosteian ray-finned fishes, and apodan and urodel amphibians); 2) a taxon whose lateral line system does not possess electroreceptors (bowfins, gars, and most teleosts); and 3) a teleost that has re-evolved electroreceptors (African notoapterids, gymnarchids, gymnotiforms, mormyrids, and siluriforms). By comparing ontogenies from each of these three groups, it should be possible to identify where in development changes have occurred and begin to focus on the genetic bases of these changes.

As noted by Northcutt (1990), ontogenetic changes can occur in the source or precursor of a trait, or changes can occur in ontogenetic processes (patterning, sequence, and/or timing). For example, electroreceptors in teleosts might develop from an embryonic tissue that differs from the embryonic tissue that gives rise to primitive electroreceptors in other taxa. It is also possible that both primitive electroreceptors and the derived electroreceptors of teleosts arise from the same embryonic tissue but that this tissue is patterned differently or that electroreceptors develop at a different time in the differentiation of that tissue.

An out-group analysis (Northcutt 1997) of data from a number of taxa (Stone 1922; Holmgren 1940; Pehrson 1949; Metcalfe et al. 1985; Northcutt et al. 1994 1995; Schlosser and Northcutt 2000)

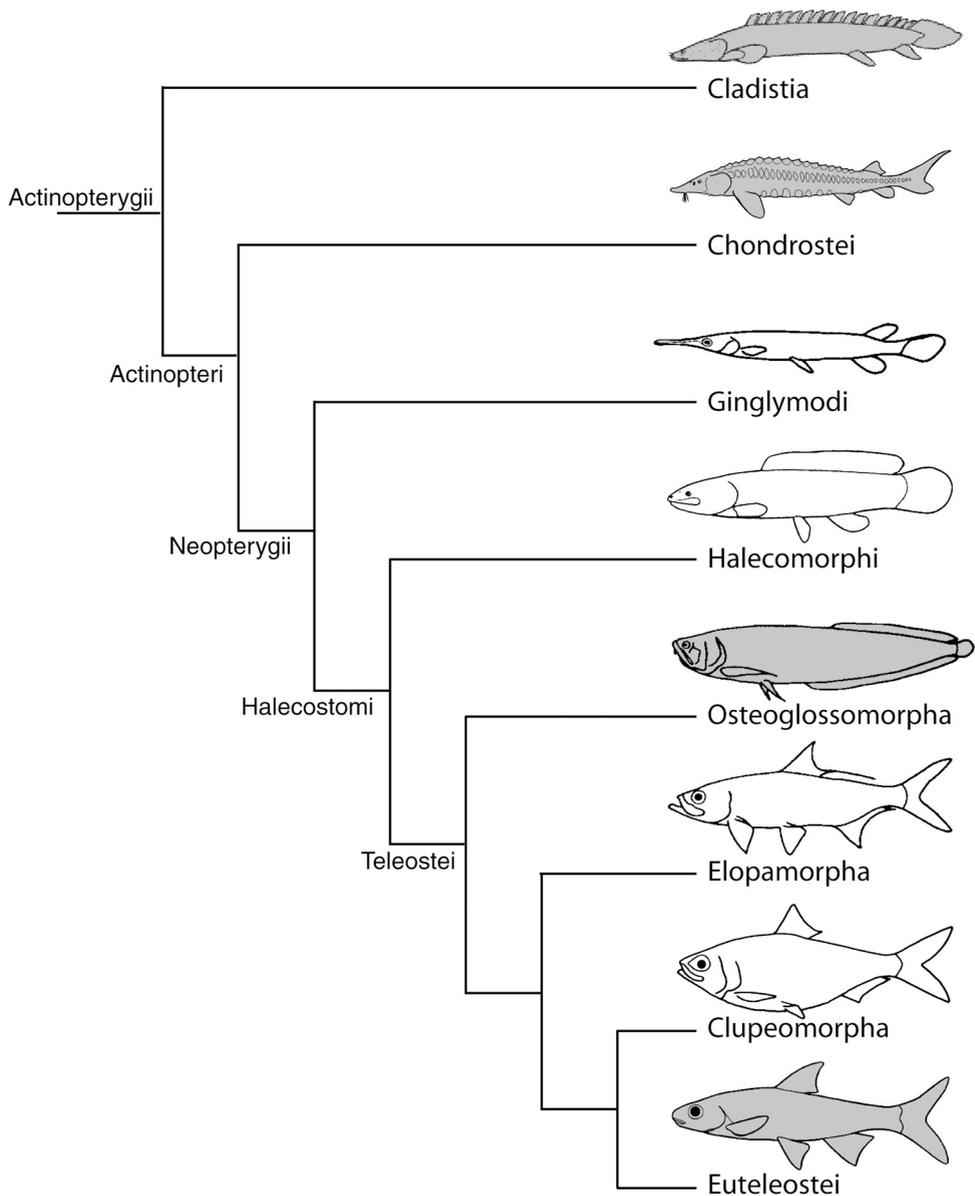


Figure 1. A current hypothesis of the phylogenetic relationships of living ray-finned fishes (electroreceptive groups are shaded). The cladistians and chondrosteans possess electroreceptive ampullary organs, whereas the ginglymodes (gars) and halecomorphs (bowfins) do not. Electroreceptors appear to have re-evolved at least twice among teleosts: once among some osteoglossomorphs (gymnarchids, mormyrids and African notopterids), and again in some euteleosts (gymnotiforms and siluriforms).

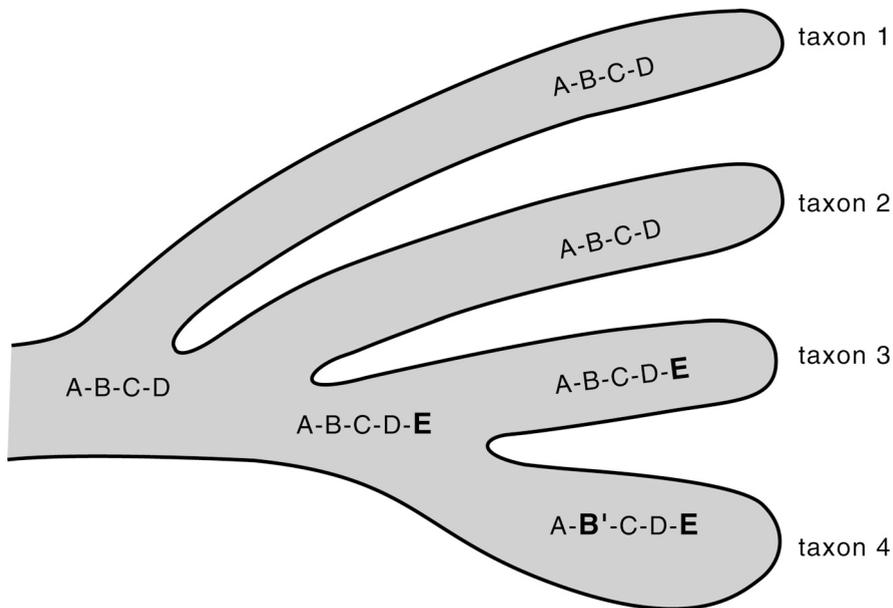


Figure 2. An out-group analysis of the stages (A-E) in the development of a theoretical trait in four taxa. If a highly corroborated hypothesis of the phylogeny of these taxa (indicated in gray) exists, it is possible to determine which stages are primitive (normal print) and which are derived (bold print). In this example, the primitive sequence of development of the theoretical trait in the last common ancestor of the four taxa must have been A-B-C-D. Stage E in taxa 3 and 4, however, must have arisen as a new terminal stage in the common ancestor shared by taxa 3 and 4. Finally, stage B' in taxon 4 is uniquely derived and represents the addition of a nonterminal change. Figure from Northcutt (2002).

indicates that a lateral line placode (a localized thickening in the inner layer of cephalic ectoderm) is the ontogenetic unit, and thus the phylogenetic unit, for the formation of the lateral line system. Primitively, the lateral line system of the earliest jawed fishes appears to have developed from six pairs of cephalic lateral line placodes (Fig. 3). These placodes can be divided into a preotic and a postotic series, relative to the octaval (otic) placode, which gives rise to the inner ear and the eighth cranial nerve. Each of the lateral line placodes exhibits a similar pattern of development (Fig. 4). Following the initial formation of the placode (Figs. 4A, 5A), ganglionic cells are generated (Fig. 5B), and the neurites of these cells enter the medulla and also maintain contact with the placode (Fig. 4B). At this point, mitotic activity of placodal cells increases, and placodes that form cephalic lines elongate (Figs. 4C, 5C), whereas placodes that form trunk lines actively migrate onto the trunk. Cell division throughout the rostrocaudal extent of a placode can result in the placode growing longer (elongation), or the cells that form a placode may actively move from one locus to another (migration). Following this period of elongation or migration, each elongated placode – now termed a sensory ridge – begins to form receptor primordia (Fig. 4D). Neuromast primordia

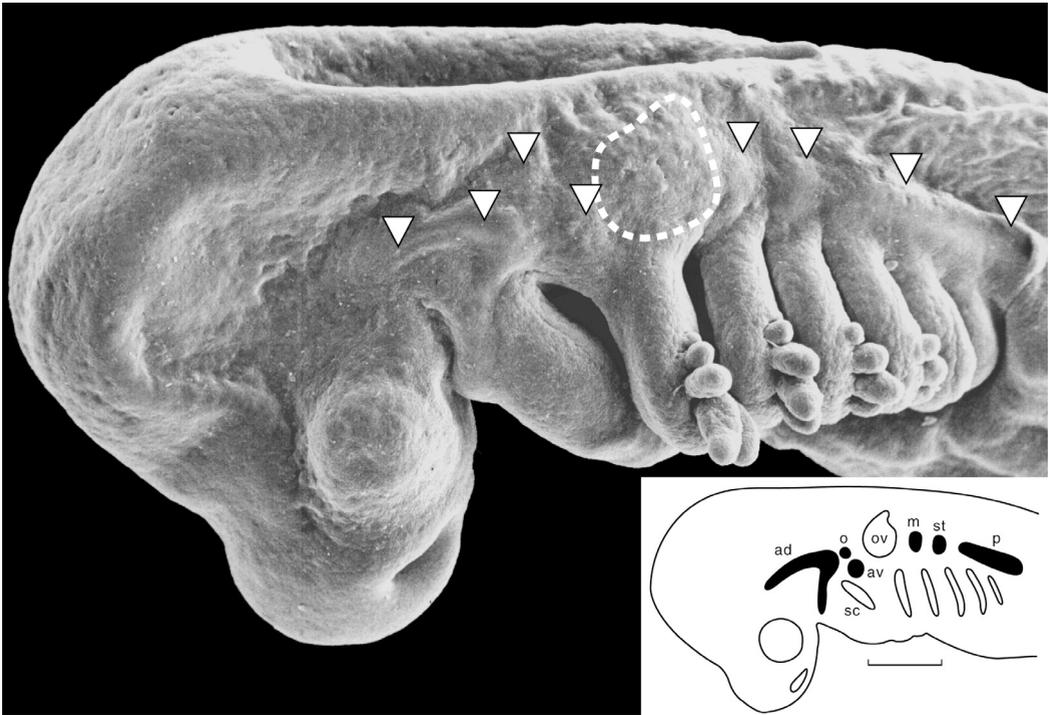


Figure 3. Scanning electron micrograph of the lateral surface of the head of a 17-day-old embryo of the clearnose skate, *Raja eglanteria*, illustrating the position and primitive number of placodes. The lateral line placodes can be recognized as distinct hillocks whose dorsal edges are indicated by white arrow heads. The borders of the otic placode are outlined by the white dashed line. *ad*, anterodorsal; *av*, anteroventral; *m*, middle; *o*, otic; *ov*, octaval; *p*, posterior; and *st*, supratemporal lateral line placodes; *sc*, spiracular cleft. Bar scale equals 500 μ m. From Northcutt (1997).

form first within the central zone of a sensory ridge, and electroreceptor primordia form subsequently within the lateral zones of a sensory ridge (Figs. 4E, 5D). Electroreceptor primordia are distinctly different from neuromast primordia in both their morphology and their origin within a sensory ridge, and thus there is no evidence that electroreceptor primordia arise from neuromast primordia.

Both electroreceptor and neuromast primordia subsequently erupt to the surface and form lines of superficial receptors (Fig. 4F). In amphibians these neuromasts remain superficial; in most other jawed vertebrates, however, ectodermal ridges form adjacent to the neuromasts (Fig. 4G) and subsequently fuse above these receptors, enclosing them within an epithelial canal which opens to the surface between adjacent neuromasts (Vischer 1989a, Webb 1989). Not all neuromasts are housed in canals, however, as some neuromasts remain superficial and form the so-called pit lines. Finally, the electroreceptors invaginate, forming the distinctive ampullary pattern of primitive electroreceptors, and the epithelial canals that contain neuromasts are subsequently surrounded by cartilage or bone (Fig. 4H).

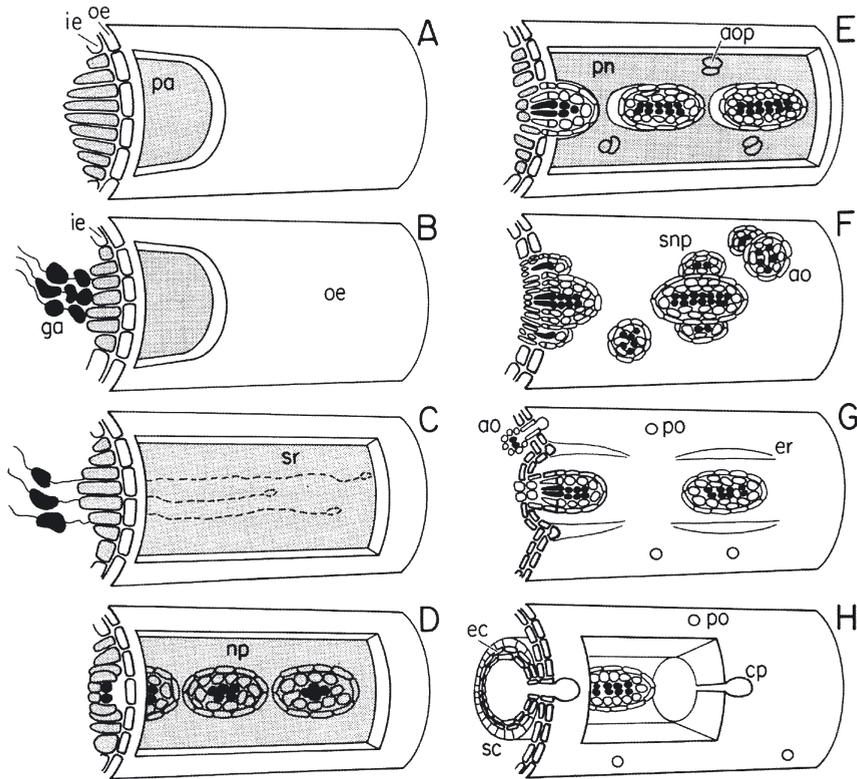
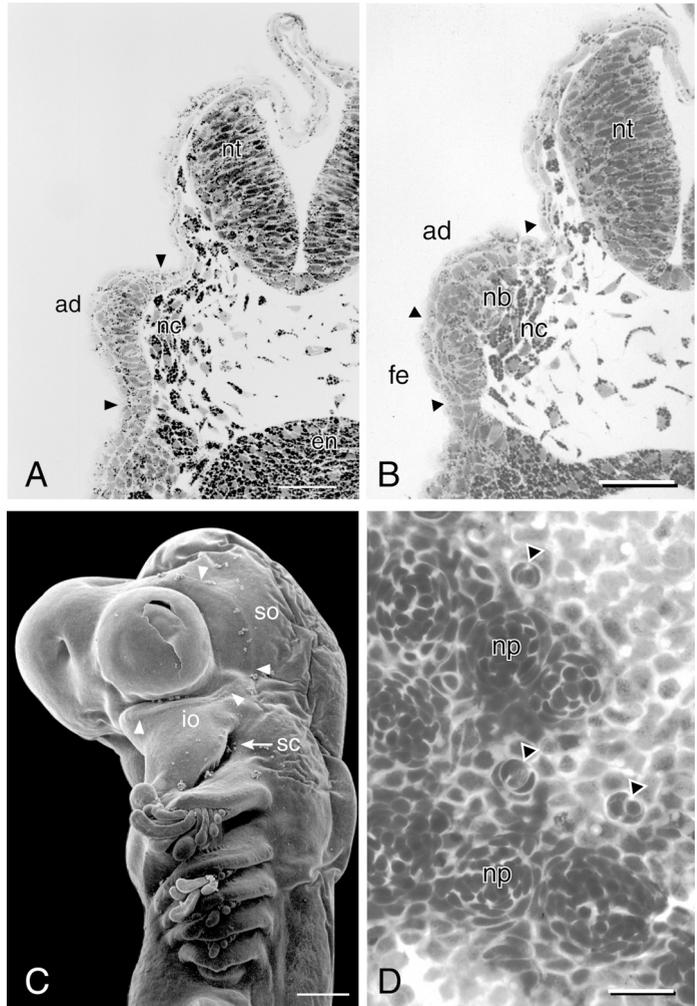


Figure 4. Stages in the development of a primitive lateral line placode. *ao*, ampullary organ; *aop*, ampullary organ primordium; *cp*, canal pore; *ec*, epithelial canal; *er*, ectodermal ridge; *ga*, ganglionic cells of lateral line nerve; *ie*, inner layer of ectoderm; *np*, primary neuromast primordium; *oe*, outer layer of ectoderm; *pa*, placode; *pn*, primary neuromast; *po*, ampullary pore; *sc*, secondary connective tissue, canal; *snp*, secondary neuromast primordium; *sr*, sensory ridge. From Northcutt et al. (1994).

All pre- and postotic lateral line placodes give rise initially to neuroblasts, which form the sensory ganglion of a lateral line nerve, and subsequently to receptors. Although it is not known whether a given placodal cell can give rise to both ganglionic cells and receptor cells, it is unlikely, as all ganglionic cells arise from a portion of the placode that is closest to the otic vesicle (Northcutt and Brändle 1995). Not all placodes give rise to electroreceptors, however, and not all electroreceptors are innervated by the lateral line nerve that arises from the parent placode. Primitively, three preotic placodes occur rostral to the octaval placode: 1) an anterodorsal placode (Fig. 3) gives rise to an anterodorsal lateral line nerve, which innervates the electroreceptors and neuromasts of the supraorbital and infraorbital lines, which arise from dorsal and ventral sensory ridges of the anterodorsal placode; 2) An anteroventral placode (Fig. 3) gives rise to the anteroventral lateral line nerve, which innervates the electroreceptors and neuromasts of the cheek and lower jaw lines, which arise from several sensory ridges formed by the anteroventral placode; and 3) An otic lateral line placode (Fig. 3; not to be confused with the octaval-otic placode) gives rise to an otic

Figure 5. Photomicrographs of transverse sections through rostral (A) and caudal (B) segments of the anterodorsal lateral line placode of a stage 35 embryonic axolotl, *Ambystoma mexicanum*; scanning electron micrograph of the lateral surface of the head (C) of a Scammon stage 26 embryonic dogfish, *Squalus acanthias*; and an ectodermal flat mount (D) from a stage 36 axolotl embryo showing a segment of a sensory ridge. In both A and B, neural crest cells (nc) are streaming ventrally along the lateral edge of the neural tube (nt) and along the ventral edge of the placode (upper and lower boundaries denoted by arrowheads) toward the endodermal (en) pharynx. In A, the anterodorsal placode (ad) has a distinct basement membrane, but in B, neuroblasts (nb) that will form the sensory ganglion of the lateral line nerve that innervates the receptors derived from the anterodorsal placode are in the process of delaminating from the placode. A second placode, the facial epibranchial placode (fe), is located immediately ventral to the anterodorsal placode. In C, the dogfish embryo illustrates a later stage in the development of lateral line placodes, in which the supra- (so) and infraorbital (io) sensory ridges (extent of ridges denoted by arrowheads) of the anterodorsal placode have elongated above and below the eye, respectively. The infraorbital sensory ridge is elongating along the length of the future upper jaw, and the spiracular cleft (sc) occurs along the caudal border of the lower jaw. In D, both neuromast (np) and ampullary organ primordia (denoted by arrowheads) have formed within the sensory ridge. Note that all ampullary organ primordia are forming on the lateral edge of the sensory ridge. Bar scales equal 100 μ m (A,B), 500 μ m (C) and 50 μ m (D). Panels A and B from Northcutt et al. (1994) and panel C from Northcutt (1997).



lateral line nerve, which innervates the neuromasts that arise from the otic lateral line placode. The otic lateral line placode forms electroreceptors in many taxa, but it is presently unclear whether these electroreceptors are innervated by the anteroventral or otic lateral line nerve.

Primitively, only the two most rostral postotic placodes, the middle and supratemporal placodes (Fig. 3), form both electroreceptors and neuromasts. In this case, a middle lateral line nerve

forms both electroreceptors and neuromasts. In this case, a middle lateral line nerve

and a supratemporal lateral line nerve are generated by the middle and supratemporal placodes, respectively. These lateral line nerves innervate the neuromasts that arise from their respective placodes, but the anteroventral lateral line nerve innervates all electroreceptors that arise from the middle and supratemporal placodes. The third member of the postotic series, the posterior lateral line placode (Fig. 3), gives rise to the posterior lateral line nerve, which innervates all trunk neuromasts that arise from this placode. Primitively, however, this placode does not give rise to electroreceptors.

There are fewer details regarding the development of lateral line receptors and nerves in neopterygian ray-finned fishes, which lack electroreceptors. It is clear, however, that in gars (Landacre and Conger 1913) and bowfins (Allis, 1889; and Beckwith 1907) the various lines of neuromasts and their nerves also arise from lateral line placodes. Therefore, loss of primitive electroreceptors in early neopterygian fishes must have occurred as a result of one of two phenomena: an alteration in a nonterminal stage (change in sequence) in which the developing sensory ridges of the lateral line placodes fail to differentiate lateral zones, which normally give rise to electroreceptor primordia (Fig. 4E); or an even earlier ontogenetic change in which sensory neuroblasts that might induce electroreceptor primordia, or the lateral zone from which they arise, fail to form.

Unfortunately, there is little information regarding the embryonic origin and subsequent development of electroreceptors in teleosts. Although electroreceptor primordia have been described adjacent to the developing lines of neuromasts in teleost larvae (Sato 1956; Northcutt 1987; Vischer 1989a,b), suggesting that both types of receptors differentiate from single lateral line placodes, there is no agreement that such placodes exist in teleosts (Landacre 1910; Lekander 1949; Sato 1955; Metcalfe et al. 1985; Vischer 1989a; Sahly et al. 1999; Gompel et al. 2001a,b.). Landacre (1910) observed lateral line placodes in *Ictalurus nebulosus* but reported that they disappeared after giving rise to the sensory ganglia of the lateral line nerves. Therefore, he thought that the placodes do not form sensory ridges from which lateral line receptors arise but that these receptors arise directly from general ectoderm. Similar claims have been made for electroreceptors in catfishes (Roth 1986), as well as both neuromasts and electroreceptors in *Eigenmannia* (Vischer et al. 1989). Lekander (1949) described the development of neuromasts in cyprinid teleosts and claimed that lateral line placodes exist in these taxa, but her illustrations clearly indicate that she was describing rows of neuromast primordia rather than lateral line placodes. Sato's (1955) work on cyprinid teleosts is more convincing. He illustrates ectodermal thickenings in early stages of development that are comparable in number and topography to the lateral line placodes of other fishes. Later studies (Metcalfe et al. 1985; Vischer 1989a; Gompel et al. 2001a,b.) have recognized a posterior placode that migrates onto the trunk and gives rise to neuromasts, but they have not addressed the question of whether additional lateral line placodes exist in teleosts. Although additional lateral line placodes have been identified in zebrafish, where they express *eya1* (Sahly et al. 1999), these placodes have been observed only in whole mount, and unfortunately no histological details are presently available.

The development of the lateral line system of the channel catfish is described in order to address many of the questions regarding the development of this system in teleosts in general. In addition, more details are needed regarding the development of teleost electroreceptors in order to complete an out-group analysis of lateral line ontogenies as outlined at the beginning of this section.

Materials and Methods

All observations are based on embryonic and larval channel catfish, *Ictalurus punctatus*, incubated at 24–26° C from fertilized eggs supplied by Osage Catfisheries Inc. located at Osage Beach, MO. All procedures were approved by the UCSD Animal Care and Use Committee and conform to NIH guidelines. These embryos and yolk-sac larvae were staged (Fig. 6) by modifying the normal tables of development for *Ictalurus nebulosus* (Armstrong and Child 1962). Most conclusions are based on observations of a number of individuals from at least four different egg clutches obtained over several years.

To visualize the early stages of placode and receptor organ development, channel catfish from stage 21 embryos through 3 week-old larvae were anesthetized in a phosphate-buffered solution (0.02%) of tricaine methanesulfonate (Sigma Chemical Co., St. Louis, MO) and fixed in 4% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). Following fixation for at least 12 hours, the specimens were rinsed in phosphate buffer and transferred through a graded ethanol series into absolute ethanol. After two changes in absolute ethanol, the specimens were critical-point dried in CO₂, mounted on aluminum stubs, sputter coated with gold-palladium alloy, and viewed with a Cambridge 360 Stereoscan scanning electron microscope.

More detailed histological observations of the lateral line placodes and their development were made by fixing additional channel catfish (stage 21 embryos through 8-week-old larvae) in 4% glutaraldehyde in 0.1M phosphate buffer. These specimens were rinsed in distilled water, dehydrated in an ethanol series, and embedded in glycol methacrylate (LKB Historesin). Serial sections (5 µm) were cut in the horizontal and transverse planes and stained with 0.1% cresyl violet.

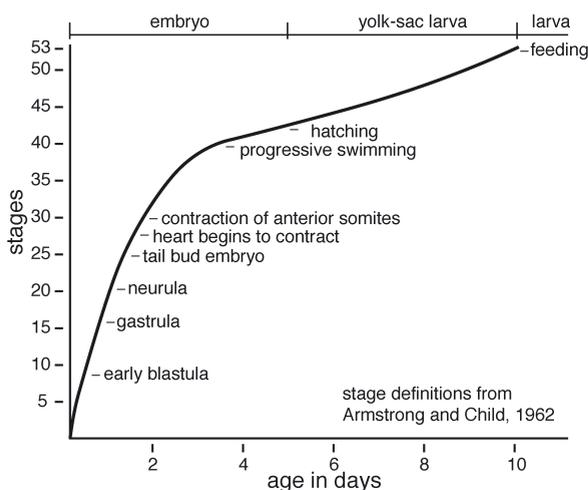


Figure 6. Timing of some major events in the development of the channel catfish. Times were established for embryos incubated at 26° C, but the duration of development can increase by two to three days if the embryos are incubated at 24° C.

Maps of the distribution of the lateral line receptors were constructed from ectodermal flat mounts (Stone 1922; Northcutt et al. 1994). Channel catfish from stage 43 embryos through 8-week-old larvae were anesthetized in tricaine methanesulfonate and fixed by immersion in 4% glutaraldehyde in 0.1M phosphate buffer. Following fixation for at least 24 hours, the animals were rinsed in distilled water and blotted dry. The dried specimens were then attached on one of their sides to a dry microscope slide using cyanoacrylate (Borden Inc., Columbus, OH), and the slides with the affixed specimens were immersed in phosphate buffer. This method of mounting the specimens held them firmly in place, without damaging the surface ectoderm, and fa-

cilitated subsequent manipulations. Mid-dorsal and mid-ventral incisions were then made along the entire length of the specimens, and the ectoderm was lifted free with the aid of tungsten microneedles. The freed ectodermal sheets were then placed in 30% hydrogen peroxide for 30-60 minutes to bleach pigment cells, rinsed in distilled water, and stained in 1% methylene green. After differentiation, the ectodermal sheets were cleared and coverslipped. Maps of the distribution of the lateral line receptors were drawn with the aid of a camera lucida attached to a BH-2 Olympus microscope.

Although taste buds also occur on the body of catfishes, there is no problem in distinguishing lateral line receptors from taste buds (Northcutt et al. 2000). Neuromasts are oval-shaped receptors formed by large columnar hair and support cells, whereas taste buds consist of much smaller cells that surround a distinct dermal papilla.

All illustrations were composed in either Adobe Photoshop or Adobe Illustrator (Adobe Systems, San Jose, CA). Manipulation of the digital images involved enhancement of brightness and/or contrast, isolation of specimen tissues from the background, and labeling of structures.

Results

The development of the lateral line receptors and the cranial nerves that innervate them is described in chronological order with reference to the stages listed in Figure 6. In fact, however, the development of the receptors and their nerves was traced in reverse chronological order, for ease of recognition, as mature electroreceptors and neuromasts are distinctly different in size and morphology (Northcutt et al. 2000). This approach hopefully minimizes errors of identification, but it should be noted that the beginning of major developmental events probably occurs slightly earlier than can be recognized solely on the basis of morphological criteria. For example, as mature neuromasts are traced back to the stage where they are beginning to form, i. e., the neuromast primordia, the stage at which such primordia can be recognized is almost certainly later than the stage at which cells are committed to forming such primordia.

A map of the distribution of the lateral line receptors on the head of a juvenile channel catfish (Fig. 7) illustrates the pattern of the mature system and is included to facilitate understanding of how the system develops. The present account is limited to describing major events in the development of the receptors and associated nerves of the head. Analysis of the development of this system on the trunk is still in progress and will be published at a later date. There are four major events described in the development of the cephalic lateral line system of channel catfish: 1) placode formation and neurogenesis; 2) sensory ridge formation; 3) receptor formation; and 4) canal formation.

Placode formation and neurogenesis. Gastrulation is essentially complete by stage 22, and the three basic germ layers can be recognized. At this time the neurectoderm consists of a T-shaped mass of cells comprising a ventrally directed core of cells (the neural keel) capped dorsally and laterally by the paired lateral masses. The neural keel will become the neural tube by a process of delamination, which begins by stage 24, but cells of the lateral masses are already streaming ventrally within

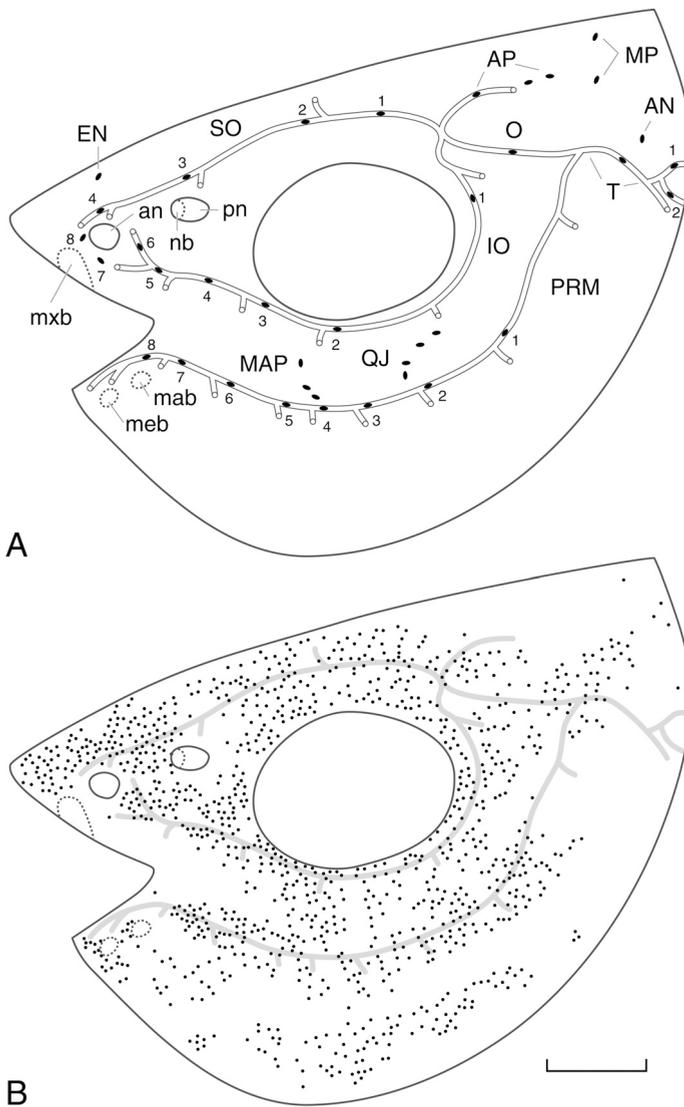


Figure 7. Maps of the distribution of neuromasts (A) and ampullary organs (B) based on an ectodermal flat mount of the head of a juvenile channel catfish (6.8 cm total length). In A, each neuromast, whether in a canal or on the surface of the skin, is indicated by a solid oval. The short tubes branching from the main canals are pores where the canals open to the surface. In B, each ampullary organ (indicated as a black dot) is mapped with reference to the more deeply located lateral line canals indicated in gray. The ampullary organs do not appear to be evenly distributed on the head but are clustered near the lateral line canals except for those located on the gular surface. Bar scale equals 2mm. AN, first accessory trunk neuromast; an, anterior naris; AP, anterior pit line; EN, ethmoid neuromasts; IO, infraorbital canal; mab, mandibular barbel; MAP, mandibular pit line; meb, mental barbel; MP, middle pit line; mxb, maxillary barbel; nb, nasal barbel; PRM, preoperculomandibular canal; pn, posterior naris; QJ, quadratojugal pit line; SO, supraorbital canal; and T, temporal canal. From Northcutt et al. (2000).

the space between the general ectoderm and neural keel. These migrating cells exiting the lateral masses appear to be neural crest cells, as they form the visceral arches and contribute to the formation of the sensory ganglia of the branchiomeric cranial nerves, as do crest cells in other vertebrates. The migration of crest cells from the lateral masses rapidly reduces the latter's size and rostrocaudal extent. By stage 24, some four hours later, the unmigrated segment of the lateral mass has differentiated into the otic vesicle and two closely associated placodes, the preauditory and postauditory placodes. The preauditory placode is an ectodermal thickening extending rostrally from the otic vesicle, and it appears to be a transient placode that does not give rise to any larval structures, as it can no longer be recognized by stage 28. A similar ectodermal thickening that extends a short distance caudally from the otic vesicle appears to be the primordium of the posterior lateral line placode, which occupies a comparable position in stage 28 embryos.

Three preotic lateral line placodes (anterodorsal, anteroventral, and otic) can be recognized for the first time at stage 24. At this time, neurogenesis has commenced in the anterodorsal placode (Fig. 8A) but not in the anteroventral and otic placodes. Neurogenesis is underway in the anteroventral and otic placodes by stage 28, and two postotic lateral line placodes, the middle and posterior placodes, can be recognized at this time. Sensory neuroblasts continue to be generated from the lateral line placodes through stage 30, and the placodes become more distinct in subsequent stages, so that by stage 35, each of the placodes can be recognized in SEM preparations as a distinct hillock in the ectoderm (Fig. 9).

Sensory ridge formation The sensory ridges begin to form around stage 34, as the anterodorsal placode at stage 35 has already begun to elongate to form short supraorbital and infraorbital sensory ridges (Figs. 8B, 9). The posterior lateral line placode has also begun to migrate at stage 34 and has moved caudally by some three to four somites by stage 35 (Fig. 9). The posterior lateral line placode is the most distinct of all the six lateral line placodes in embryonic channel catfish and is the only lateral line placode that migrates between the ectoderm and underlying mesenchyme (Fig. 9B). The other placodes that elongate remain within the inner layer of ectoderm and can be recognized from the other ectodermal cells because of their increased height. The cells of the remaining sensory ridges appear to divide more rapidly than the adjacent cells of the inner layer of ectoderm at stage 35, as the sensory ridges are bowed ventrally, so that a distinct cavity is formed between the sensory ridges and the overlying layer of ectoderm.

At stage 36, the supraorbital and infraorbital sensory ridges have elongated so that their rostral tips have reached the caudal pole of the olfactory organ and the rostral pole of the eye, respectively. The sensory ridge of the otic placode now almost touches the caudal end of the combined supra- and infraorbital sensory ridges, and its caudal tip is passing around the otic vesicle. The middle lateral line placode is also elongating, with a rostrocaudal orientation, and will continue to elongate until it fills in the gap from where the otic sensory ridge stops and the more caudal point where the posterior lateral line placode began its migration. Finally, the sensory ridge of the anteroventral lateral line placode has elongated until its rostral tip ends on the future lower jaw at the level of the overlying maxillary barbel; its caudal tip has continued to elongate dorsally, stopping just short of the elongating otic sensory ridge.

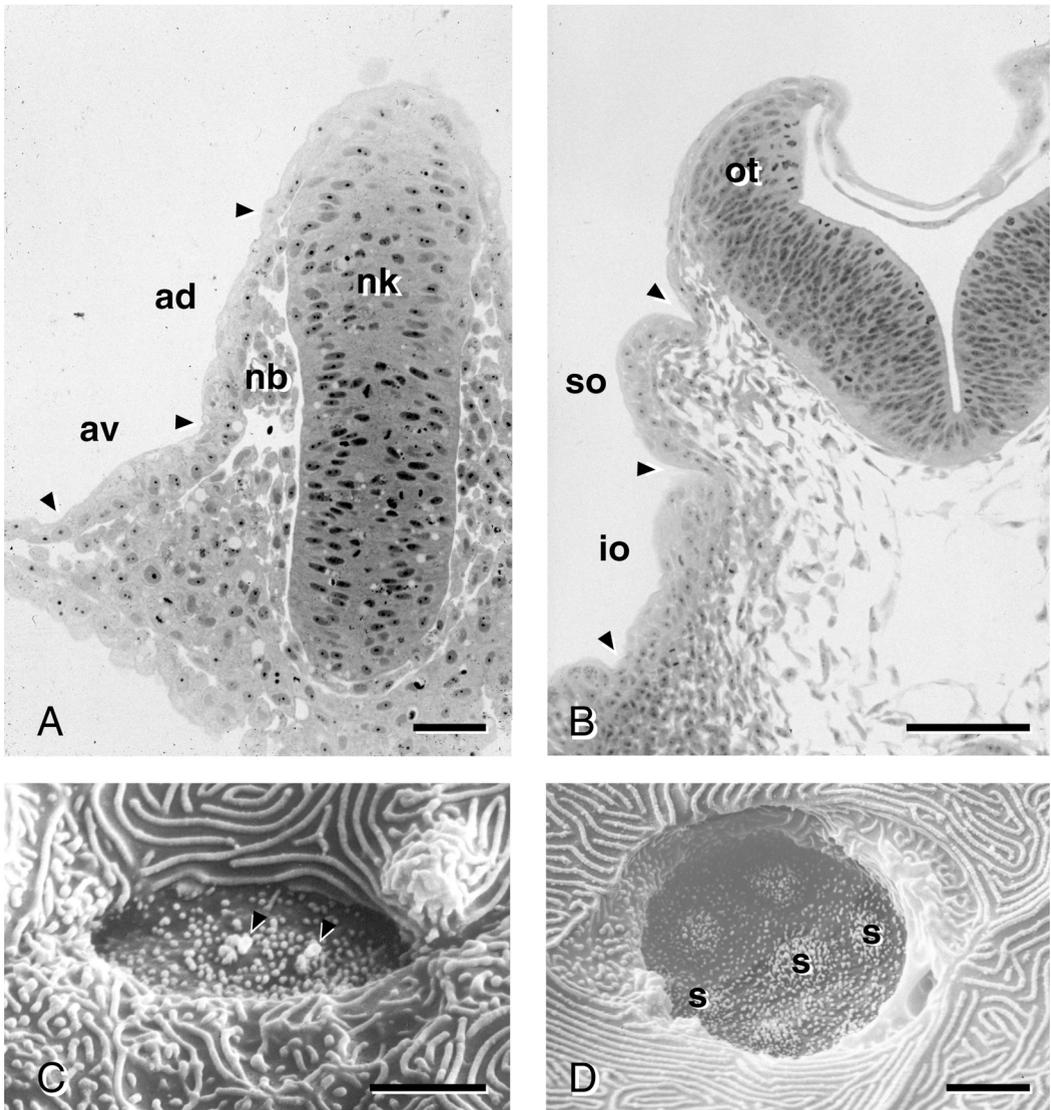


Figure 8. Photomicrographs of transverse sections through the neural keel (nk) of a channel catfish embryo at stage 24 (A) and the optic tectum at stage 35 (B); scanning electromicrographs of an erupting neuromast in a channel catfish embryo at stage 43 (C) and an ampullary organ opening to the surface at stage 49 (D). In A, the anterodorsal (ad) and anteroventral (av) lateral line placodes (upper and lower boundaries denoted by arrowheads) can be recognized. Ganglionic neuroblasts (nb) are delaminating from the anterodorsal but not the anteroventral placode at this time. In B, the supraorbital (so) and infraorbital (io) sensory ridges (upper and lower boundaries denoted by arrowheads) of the anterodorsal lateral line placode are illustrated just rostral to their origin from the placode. In C, the cells of the outer layer of ectoderm are retracting from over the apical surface of a neuromast primordium, with the tips (arrowheads) of two hair cells just developing. In D, an ampullary organ has just opened to the surface, and it is possible to look through the pore and see the apical surface of a number of sensory cells (s) in the floor of the ampulla. Bar scales equal 50 μ m (A); 100 μ m (B); and 3 μ m (C and D).

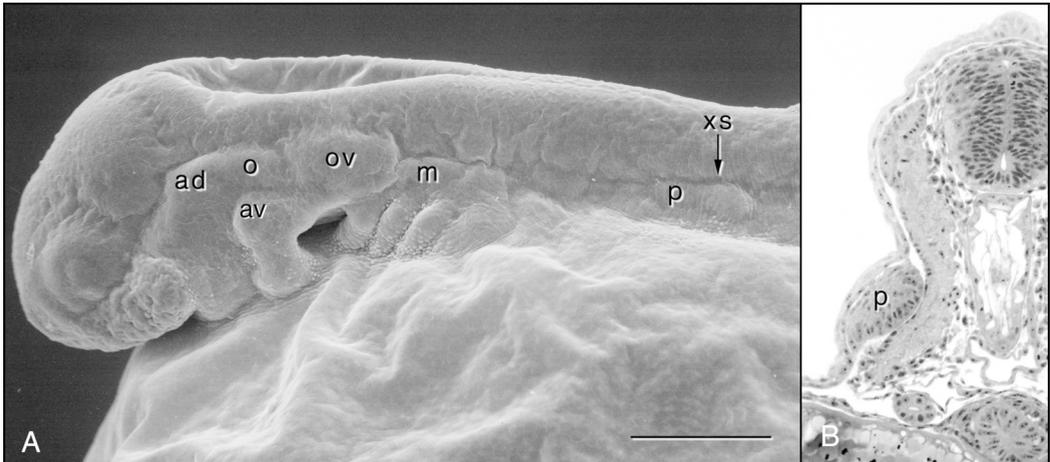


Figure 9. Scanning electron micrograph of the lateral surface of the head of a stage 35 channel catfish embryo (A) and a transverse section (B) from a similarly staged embryo that was plastic embedded. In A, all of the lateral line placodes (see Figure 3 for abbreviations) can be recognized as distinct bulges in the ectoderm. The section in B was taken through the posterior lateral line placode (p) at a level indicated by xs in A. Bar scale equals 250 μ m.

Receptor formation. Neuromast primordia form within the cephalic sensory ridges during stage 37. At this time, the sensory ridges are some 15 to 20 cells in width, and the neuromast primordia, when seen in transverse section, consist of approximately 9 to 10 taller columnar cells forming distinct dome-shaped primordia. By stage 39, cellular differentiation within the primordia has progressed to the point that spindle-shaped support cells and spherical-shaped hair cells can be recognized. Neuromasts begin to erupt to the surface at stage 41 (Fig. 8C), and by stage 45 all cephalic lines are characterized by erupted superficial neuromasts.

The formation and eruption of neuromast primordia is so rapid that gradients can not be recognized in this process within a given sensory ridge, nor can the sequence of this process among the various sensory ridges be determined. For example, at stage 37, the first stage in which neuromast primordia can be recognized, these primordia are seen in all the cephalic sensory ridges. Furthermore, the full complement of neuromast primordia for some sensory ridges can already be recognized at this stage.

Ampullary organ primordia have begun to form by stage 43, at which time they can be recognized within the lateral zones of the supraorbital and infraorbital ridges. In no case were these primordia ever seen to form outside of the sensory ridges. They can be recognized easily from neuromasts, as the ampullary organ primordia are spherical in shape and comprise far fewer cells than the larger neuromasts, which have erupted at this point with recognizable kinocilia. During stages 43 to 48, the number of ampullary organ primordia increases within the supraorbital and infraorbital sensory ridges, and they also begin to form within the sensory ridges of the remaining cephalic lateral lines.

The first ampullary organ primordia open to the surface at stage 49 (Fig 8D), and by stage 51 (Fig. 10), flat mounts of the head ectoderm (Fig. 10B) reveal mature ampullary organs with open pores, as

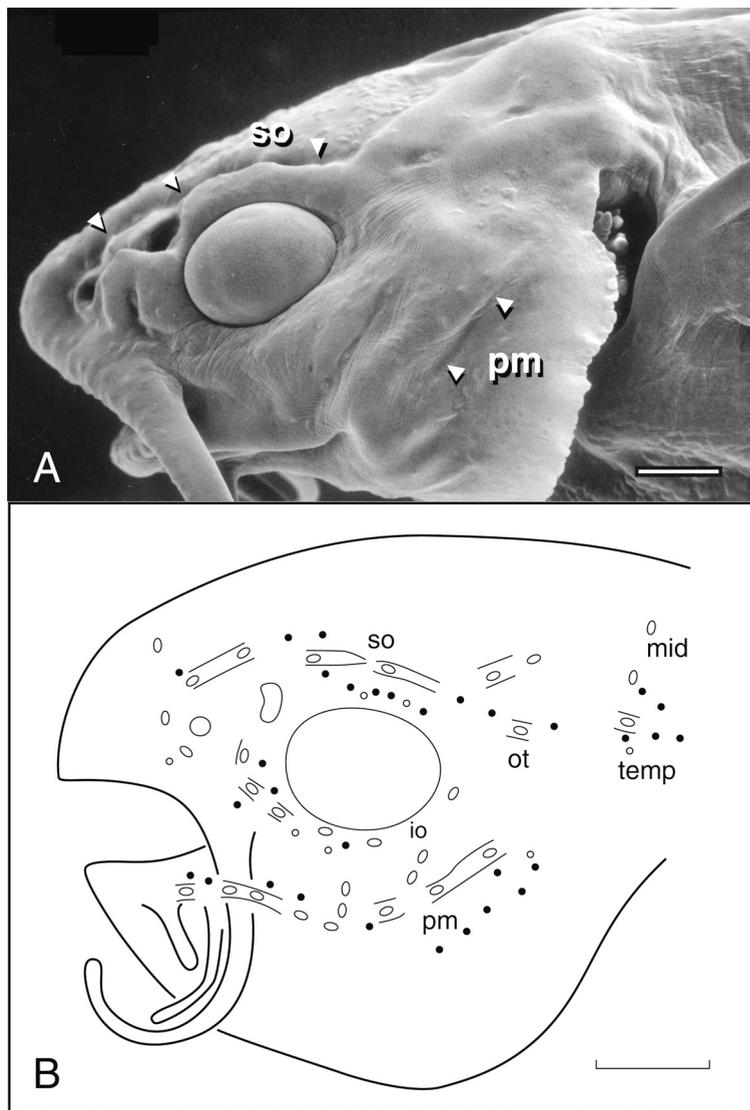


Figure 10. Scanning electron micrograph of the lateral surface of the head of a stage 51 channel catfish embryo (A) and a map (B) of the flattened head ectoderm from a second embryo of the same age. In A, the neuromasts of the supraorbital (so) and the preoperculo-mandibular (pm) lines are already enclosed in grooves (one lip of which is marked by arrowheads). In B, the positions of erupted neuromasts of the supraorbital (so), infraorbital (io), otic (ot), middle pit (mid), temporal (temp), and preoperculo-mandibular (pm) lines are indicated by open ovals. The parallel lines indicate the extent of the lips of ectodermal grooves that are beginning to enclose most neuromasts within canals, and the small open and solid circles indicate the position of immature and mature ampullary organs, respectively. Note that all ampullary organs at this stage are immediately adjacent to the neuromast lines. Bar scales equal $250\mu\text{m}$ (A) and $500\mu\text{m}$ (B).

well as a small number of primordia that are just beginning to develop adjacent to each of the cephalic lateral lines. The number of ampullary organs continues to increase, and the distribution of these organs in one-week-old feeding larvae (Fig. 11) is comparable to that in older juveniles (Fig. 7).

Canal formation. At the time when neuromasts of the cephalic lateral lines begin to erupt (stage 41), they form lines of superficial neuromasts. By stage 43, however, the ectoderm adjacent to these lines begins to elevate so that the neuromasts of many of the lines now occupy shallow grooves. At present, it is unclear whether the ectoderm that forms the walls of the grooves and eventually roofs

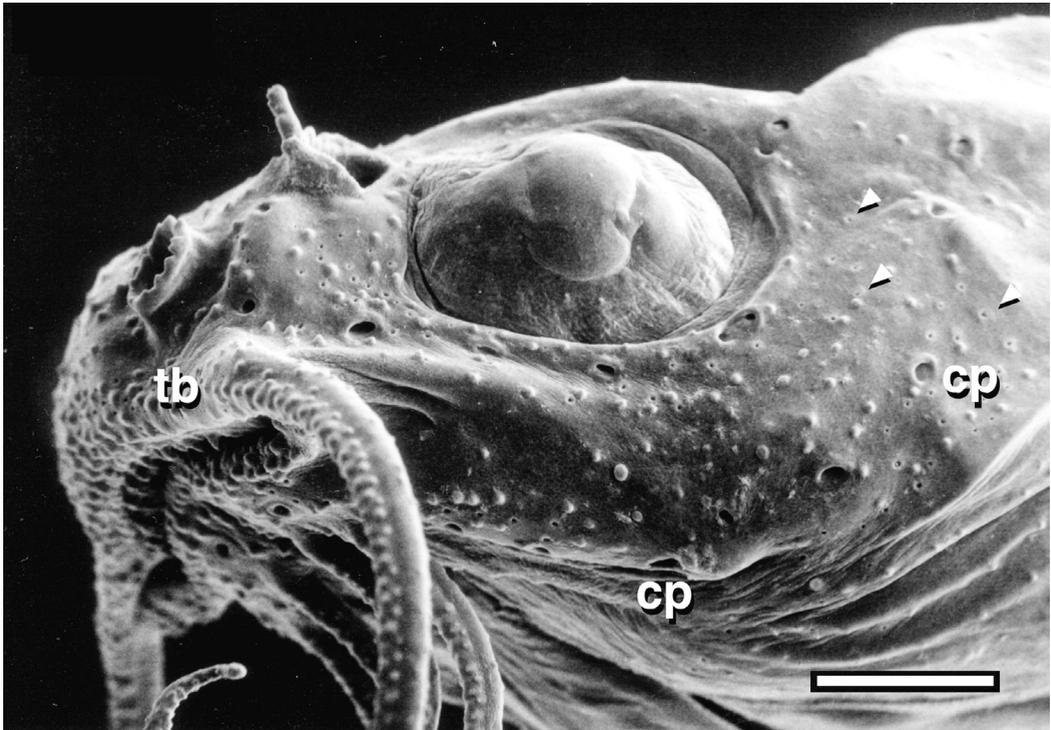


Figure 11. Scanning electron micrograph of the lateral surface of the head of a one-week-old, feeding, larval channel catfish. Note the large number of taste buds on the lips and barbels. Taste buds (tb) also occur in the skin of the head and trunk but can be easily distinguished from the lateral line system. The taste buds form small elevated tubercles, whereas the largest pores are canal pores (cp), and the smaller, more numerous pores are the pores of ampullary organs (denoted by arrowheads). Bar scale equals 500 μ m.

over the neuromasts is of placodal or general ectodermal origin. In either case, the grooves continue to deepen, and by stage 51 (Fig. 10A), the grooves are so deep and narrow that it is often difficult to visualize the neuromasts within the floor of these grooves by scanning electron microscopy. By stage 53, the lips of the supraorbital and preoperculo-mandibular grooves begin to fuse above each neuromast, but the infraorbital, otic and temporal lines are still characterized by open grooves. All cephalic grooves, however, have closed in one-week-old feeding larvae (Fig. 11), and the canals and their pores are essentially identical to those of juveniles (Fig. 7) and adults.

Discussion

The major events in the development of the lateral line system of the channel catfish are summarized in Figure 12. Placode formation and neurogenesis begin around stage 24, and all placodes and their associated cranial nerves can be recognized by stage 32, approximately 16 hours later.

The placodes begin to elongate at stage 33-34, and neuromast primordia begin to differentiate within the newly formed sensory ridges by stage 37 in another 16 hours. The primordia begin to erupt at stage 41, in approximately another 24 hours. Thus, most events in the development of the lateral line system in channel catfish span only about three days. This rate of development is almost two times that in axolotls (Northcutt et al. 1994) and in paddlefishes (unpublished observations). This rapid rate of development, plus the highly derived nature of neural crest development as part of the lateral masses, have almost certainly contributed to the confusion surrounding the development of the lateral line system in teleosts.

Except for its accelerated rate, development of the lateral line system in the channel catfish, as described herein, is remarkably similar to that reported in many other aquatic anamniotes (Beckwith 1907; Landacre and Conger 1913; Stone 1922; Holmgren 1940; Pehrson 1949; Northcutt et al. 1994; Schlosser and Northcutt 2000). It differs substantially, however, from lateral line development described by Wilson (1891) and Wilson and Mattocks (1897) and even Landacre (1910), who was describing catfish.

In 1891, Wilson described the development of the lateral line system in a sea bass, *Serranus atrarius*, as part of a description of the general development of this species. Although he noted the lateral masses associated with the neural keel, he believed that the cells of these masses were incorporated into the neural keel as part of the development of the brain. He thus traced the development of the lateral line system not to the lateral masses but to a single pair of rostrocaudal ectodermal thickenings, which he termed the sensory furrows and described as flanking the developing brain. He described the otic vesicle as forming by an invagination of the middle third of the sensory furrow, a process that left the anterior and posterior segments as isolated ectodermal thickenings, which he termed a branchial sense organ and a lateral line anlage, respectively. His lateral line anlage is almost certainly the posterior lateral line placode of other accounts, since he described this ectodermal thickening as moving caudally to “divide” into the trunk neuromasts.

Relating Wilson’s branchial sense organ to other accounts of lateral line development is more problematic, however. This organ is described as a sac early in development, but his drawings of this sac are, in fact, almost identical to the structures this author has described herein as sensory ridges. This interpretation is further reinforced by his describing the sac as changing shape and forming a sensory organ identical to a neuromast. It would appear that Wilson called this organ a branchial sense organ, rather than a neuromast, because of its early appearance, i.e. before that of other neuromasts. He notes that this “neuromast” is located rostral to the first gill slit, which forms immediately adjacent to the developing inner ear (see Fig. 9 of the present account), and that it is retained in larval sea bass. All in all, his description is consistent with his branchial sense organ being the single otic neuromast that forms from the otic placode immediately rostral to the otic vesicle. In this context, it is important to note that the otic neuromast is the first to erupt in at least one other teleost (Shardo 1995).

Wilson (1891) also described the development of two additional structures from the sac-like branchial organ. He noted that a cellular cord arose from the rostral surface of the sac as it was transforming into his branchial organ. He termed this cord the forward sensory tract and noted that it bifurcated into a dorsally directed dorsolateral tract and a rostrally directed anterior sensory tract. He further noted that neuromasts arose from both tracts after hatching. Although it is diffi-

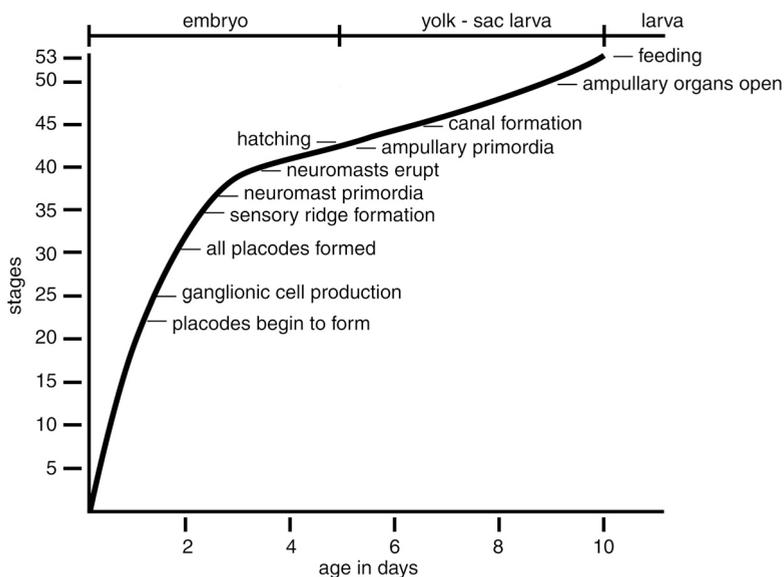


Figure 12. Timing of major events in the development of cephalic lateral line placodes (excluding the posterior placode) of the channel catfish.

cult to visualize these tracts from Wilson's description, it is possible that he was describing the development of neuromasts from the supra- and infraorbital sensory ridges of the anterodorsal lateral line placode. This interpretation is supported in a subsequent paper (Wilson and Mattocks, 1897), where the authors briefly describe the development of the lateral line system in a salmon (species not given) and state that the sensory furrow, which they now term the lateral sensory anlage, appears to be what is left of the lateral masses after most of the cells of the masses have "thinned" out. Here too the authors indicate that the lateral sensory anlage divides into three segments: 1) an anterior segment, which they now term a preauditory placode; 2) a middle segment, the otic vesicle; and 3) a posterior segment, which is termed the postauditory placode. Although they do not describe the development of a "branchial sense organ" in salmon, they do note that their preauditory placode bifurcates, as in Serranus, and their illustration (their Fig. 2) clearly indicates that they are describing the supra- and infraorbital sensory ridges.

Taking the two papers together (Wilson 1891; Wilson and Mattocks 1897), it is possible to interpret Wilson's and Mattocks' model of lateral line development: 1) A lateral sensory anlage is formed by at least a part of the cells of the lateral masses that do not migrate away; 2) The otic vesicle invaginates from the middle one-third of the anlage, with preauditory and postauditory placodes forming from the remaining segments of the anlage; 3) The preauditory placode will give rise to the otic neuromast, when present, and the neuromasts of the supra- and infraorbital lines; 4) The postauditory placode, as it begins to migrate onto the trunk, is now termed a posterior placode, and it forms the neuromasts of the trunk. This model, however, does not account for the

complexity of the lateral lines in catfishes or other teleosts. Nor does it account for the origin of the preoperculumandibular, middle, supratemporal, and temporal lateral lines or explain how five to six distinct lateral line nerves are generated. Clearly, the model is incomplete.

Landacre's (1910) description of the development of the cranial ganglia and lateral line system in *Ictalurus (Ameiurus) melas* and *I. nebulosus* comes closer to accounting for the known complexity of the lateral line system in catfishes and other teleosts. Landacre concluded that the lateral masses of teleost embryos consist of cells that are homologous to the cells of the neural crest and dorsolateral (octavolateral) placodes of other vertebrates. He believed that the lateral masses could be divided into dorsomedial and ventrolateral segments, which, unfortunately, he termed the dorsolateral mass and ventral mass (or dorsolateral placode), respectively. He described the dorsomedial segment (his dorsolateral mass) as being subdivided or sculpted into a number of rostrocaudal ganglia due to the differential migration of neural crest cells from this segment to form the branchial arches. He believed that the unmigrated cells of the dorsomedial segment then formed, in a rostrocaudal direction, the trigeminal, anterodorsal lateral line (his lateral line component of the facial), the anterior part of the otic (eighth) ganglion and the proximal glossopharyngeal and vagal ganglia. He concluded, however, that the ventral mass (dorsolateral placode) did not contain neural crest cells and that it simply differentiated into a preauditory placode, optic vesicle, and postauditory placode. Although he noted the sac-like appearance of the preauditory placode, he concluded that it did not form any sensory organs but either degenerated or consisted of crest cells that migrated away. Further, he believed that the otic vesicle gave rise to the posterior part of the otic (eighth) ganglion, as well as the sensory ganglion of the middle lateral line nerve (his lateral line component of the glossopharyngeal nerve). Finally, he concluded that the postauditory placode gave rise to the sensory ganglion of the posterior lateral line nerve (his lateral line component of the vagal nerve). Remarkably, he concluded that no sensory organs of the lateral line system arose from any part of the lateral masses but were induced from general ectoderm by the peripheral branches of the lateral line nerves. Although Landacre's observations support Wilson and Mattocks' (1897) claim that a common anlage forms from part of the lateral mass and that this anlage can be divided into preauditory, otic (octaval) and postauditory segments, his observations do not support their claim regarding the fate of the preauditory and postauditory placodes. Landacre did not describe the preauditory placode as giving rise to the otic and supra- and infraorbital neuromasts, nor did he describe the postauditory placode as migrating onto the trunk, in the form of a posterior lateral line placode, to give rise to neuromasts.

This author's observations on the development of the lateral line system in channel catfish differ substantially from those of Landacre. There is no question that a large part of the lateral masses consists of migrating neural crest cells, or that the otic (octaval) vesicle differentiates from a middle segment of the lateral mass. Landacre's divisions of the lateral mass were not seen by this author, however, nor was their evidence that any of the sensory ganglia of the lateral line nerves differentiate in situ from cells of the lateral mass. These observations are more consistent with the interpretation that the lateral mass rapidly disappears as most of its cells migrate away to form the branchial arches. It is also likely that the cells that do not migrate away do form the ganglionic components of the branchiomic cranial nerves that in other vertebrates are of neural crest origin. Furthermore, it is possible to trace the origin of the sensory ganglia of the lateral line nerves

to ectodermal placodes that form after the lateral masses cease to exist as cellular aggregates. In several cases, for example the anteroventral placode and sensory ganglion, the position of the lateral line placodes are so far ventral to the earlier position of the lateral masses that it is impossible to believe that they could ever have been part of the lateral masses.

Like Wilson and Mattocks (1897), this author has been able to detect thickenings in the ectoderm that extend rostrally and caudally from the otic vesicle. This author is not convinced, however, that what Landacre and he have recognized as a preauditory placode is the same structure that Wilson and Mattocks recognized. It is very likely that Wilson's branchial sense organ is the otic neuromast. If this is true, the otic neuromast and ganglion of the otic lateral line nerve arise from a lateral line placode in catfish embryos that is rostral to the ectodermal thickening that Landacre and this author have recognized as a preauditory placode. On the other hand, the structure termed the postauditory placode in different taxa (Wilson, 1891; Wilson and Mattocks, 1897; Landacre 1910; present account) is believed to be the same ectodermal thickening. It is possible that the postauditory placode is converted into a posterior lateral line placode. Its position appears to be compatible with such interpretation, but the series of catfish embryos in this author's laboratory are not so closely spaced that such a transformation can be inferred.

With the exception of catfishes, all the taxa in the early studies of lateral line development in teleosts lacked electroreceptors. Although ampullary organs were recognized very early in catfishes (Wright, 1884; Herrick 1901), and there was behavioral evidence that catfishes were electroreceptive (Parker and Van Heusen 1917), ampullary organs were not demonstrated to be the electroreceptors until much later (Roth 1968). Not surprisingly, Landacre (1910) did not comment on these receptors specifically, as he believed that all lateral line receptors in catfishes were induced from general ectoderm. The first description of the development of ampullary organs, then frequently termed small pit organs, in catfishes appears to have been that of Sato (1956). He noted that ampullary organ primordia can be recognized as distinct ectodermal cell masses, adjacent to the cephalic lateral lines, approximately 2.5 days after hatching. Approximately 60 hours later, the ampullary organ primordia have differentiated and opened to the surface as mature electroreceptors. Similar observations were reported by Srivastava and Seal (1981) regarding the development of ampullary organs in an Indian fresh water catfish, *Heteropneustes*.

Vischer (1989b) described the development of electroreceptors in the gymnotiform *Eigenmannia*. The first electroreceptors in this species develop after most neuromasts and initially form on the edges of the mechanoreceptive lateral lines. Vischer, however, was not able to determine whether the electroreceptor primordia arose from placodes or sensory ridges. In fact, his preparations did not allow him to determine whether even the neuromasts arose from sensory ridges (Vischer 1989a). In spite of this, Vischer (1989b) speculated that electroreceptors did arise from placodes. In the same year, however, Vischer et al. (1989) claimed to have injected "the preauditory placode" in developing *Eigenmannia* in order to trace the time course of the developing rami of the anterodorsal and anteroventral lateral line nerves. Because they did not label all rami in each of their injections, these authors concluded that different placodes must be responsible for the various branches of these lateral line nerves, and, furthermore, because their injections did not label any receptor primordia, that neither neuromasts nor electroreceptors arise from placodes but must be induced from general ectoderm. Given the time frame of these injections, however, it is

very likely that the authors were injecting either the ganglion of the anterodorsal or anteroventral lateral line nerves, not lateral line placodes per se, as their earliest injections labeled an already well developed superficial ophthalmic ramus of the anterodorsal lateral line nerve (their Fig. 4A). Assuming that *Eigenmannia* does have lateral line placodes, and that these placodes develop as in other fishes and amphibians, an extensive supraorbital sensory ridge would already have been present when these authors' earliest injections labeled the superficial ophthalmic ramus. If these injections failed to label most of the supraorbital sensory ridge, they would not have labeled electroreceptor or neuromast primordia. In a subsequent paper, Vischer (1995) described histological details of electroreceptor development in *Eigenmannia* and stressed that at least one unmyelinated fiber was always associated with even single-cell electroreceptive primordia. This observation further reinforced his belief that fibers of the lateral line nerves induce electroreceptor primordia, but he seemed less certain of his earlier conclusion that electroreceptor primordia arise from general ectoderm. In fact, he listed three hypotheses regarding the origin of electroreceptors and indicated that none of the three sources could be ruled out: general ectoderm, neural crest, and placodes.

Although no additional developmental studies of teleost electroreceptors have been published until the present account, a number of regeneration studies have also suggested that teleost electroreceptors are induced from general ectoderm by nerve fibers (Roth 1985, 1994; Zakon 1986; Teunis et al. 1991). Although these papers provide substantial evidence that teleost electroreceptor primordia are induced by nerve fibers, the argument that the primordia are induced from general ectoderm generally rests on the failure to observe either lateral line placodes or sensory ridges. This author's observations on the origin of ampullary organ primordia in channel catfish appear to refute this argument, as there is clear evidence that lateral line placodes and sensory ridges do exist and that initially, at least, all ampullary organ primordia arise within these sensory ridges. There is no question, however, that these observations must be experimentally confirmed. This can be done relatively easily by injecting the posterior lateral line placode in channel catfish embryos with a vital dye prior to the placode's beginning to migrate. If ampullary organ primordia on the trunk do arise from this placode, they should be labeled in addition to the neuromast primordia.

If future studies support this author's observations on the development of ampullary organs in channel catfish, then the out-group analysis of lateral line ontogenies proposed in the Introduction would indicate that the re-evolution of electroreceptors in teleosts, at least siluriform teleosts, did not occur by an ontogenetic change in the source or precursor of these receptors but must have involved a change in the patterning of the lateral line placodes and/or sensory ridges. At present, there is no information on the genetic changes that must have been responsible for the re-evolution of teleost electroreceptors, but they must have been more extensive than simple genetic derepression or piracy (utilization of one or more genes in an additional developmental pathway), because teleost electroreceptors have substantially altered histology, physiology and pattern of innervation (Bullock and Heiligenberg 1986).

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Congruence between chondrification and ossification sequences during caudal skeleton development: a *Moxostomatini* case study

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Abstract

Ontogeny can be studied either in terms of size and shape changes (i.e., traditional view of heterochrony) or developmental sequences. However, only a few studies have focused on the caudal skeleton of catostomid species and its developmental sequence value in systematics especially among closely related taxa. The early ontogenetic development of the caudal skeleton of the endangered catostomid copper redhorse (*Moxostoma hubbsi*, *Ostariophysii*, *Moxostomatini*) endemic to south eastern Canada and two closely related sympatric species, *M. anisurum* and *M. macrolepidotum*, was studied with cleared and double stained specimens. This study provides the first morphological description of the caudal skeleton of *M. hubbsi* as well as its developmental sequences (i.e., chondrification and ossification sequences). Congruence between chondrification and ossification sequences is tested intraspecifically and interspecifically for the three *Moxostoma* species. Intraspecific comparisons exhibit a weak congruence between both sequences for each *Moxostoma* species. There is a strong interspecific congruence among chondrification sequences and among ossification sequences. Phylogenetic and functional constraints might be responsible for the canalization of the chondrification and ossification sequences. Because of the weak intraspecific congruence between the two developmental sequences we suggest that further developmental studies integrate data derived from chondrification and ossification sequences. Acquisition of such ontogenetic data is of great significance in understanding how ontogeny and phylogeny interact to create morphological diversity.

Introduction

Morphological studies of the caudal skeleton of fossil and living teleosts are of great phylogenetic and evolutionary significance (Monod 1968; Patterson 1968; Nybelin 1973; Schultze and Arratia 1988, 1989; Arratia 1991; Arratia and Schultze 1992). In addition to systematic considerations, they are also pertinent in defining interrelationships between structure and function and how these relations change during ontogeny (Osse and van den Boogaart 1995). From a morphological standpoint, ontogenetic changes can be studied by reconstructing developmental sequences. Such sequences as ossification sequences express the order in which each element ossifies from cartilaginous precursor; for example, ossification sequences have been described in great detail for the cranial system (see Cubbage and Mabee 1996; Faustino and Power 2001; Wagemens and Vandewalle 2001).

During the past decade, the integration of ossification sequences within developmental patterns has gone beyond the simple description of morphology. Mabee (1993, 2000) and Smith (1996, 2001) improved the comprehension on the usefulness of such sequences in understanding the role of ontogeny in evolutionary changes. Müller (1991) and Rieppel (1993) have shown that sequences of chondrification (e.g., formation of cartilaginous structures) and ossification in the reptile limbs differ suggesting ontogenetic repatterning. Despite these fundamental insights, most recent developmental studies are still restricted to pattern of ossification ignoring the chondrification data.

The caudal skeleton has been described extensively for many taxa including otophysans (Weisel 1967; Monod 1968; Lundberg and Baskin 1969; Eastman 1980; Fink and Fink 1981). However, few authors have dealt with developmental sequences in particular in catostomid species with the exception of Weisel (1967).

The present paper deals with the description of chondrification and ossification sequences during the caudal development of three closely related species of catostomids, the copper redhorse (*Moxostoma hubbsi*), the shorthead redhorse (*M. macrolepidotum*) and the silver redhorse (*M. anisurum*). The primary objectives of this study are (1) to compare intraspecifically the congruence between the chondrification and ossification sequences, and (2) to compare patterns of chondrification and ossification among species.

Materials and Methods

Species examined. *Moxostoma hubbsi* (Ostariophysii, Catostomidae, Moxostomatini) is an endangered species, endemic to southwestern Quebec (Mongeau et al. 1992). Its range in Quebec is much more restricted than the range of the other redhorse species and had shrunk substantially since the discovery of the species in 1942. The only known self-sustaining population now occurs in the Richelieu River where it is sympatric with *M. anisurum*, *M. macrolepidotum*, the greater redhorse (*M. valenciennesi*) and the river redhorse (*M. carinatum*) (Mongeau et al. 1992). No particular conservation status is attributed to *M. anisurum* and *M. macrolepidotum*. Both species have a wide range of distribution, covering the eastern central part of North America (Lee et al. 1980).

Specimens examined. Ontogenetic series of *M. hubbsi*, *M. anisurum* and *M. macrolepidotum* were studied (Table 1). Specimens of *M. hubbsi* were reared artificially in 1990 following the protocol described by Branchaud and Gendron (1993). Specimens of *M. anisurum* and *M. macrolepidotum* were reared in 1994 following the same procedure. Genitors for the three species were captured in the Richelieu River (in the St. Lawrence lowlands) downstream to the St. Ours lock and the Chambly dam. The number of genitors used to induce the reproduction for each species was: 8 for *M. hubbsi*, 57 for *M. macrolepidotum* and 9 for *M. anisurum*. *Moxostoma* specimens belong to the biological collection of the Faune et Parcs Québec (FAPAQ), Direction de l'aménagement de la faune de Montréal, de Laval et de la Montérégie, Longueuil, Québec, Canada and the Biodôme de Montréal.

Specimen preparation. All the specimens were cleared and double stained with alizarin red S for bones and Alcian blue for cartilages following the method of Dingerkus and Uhler (1977) with the modifications of Potthoff (1984) for larval fishes. Some specimens of the three species did not stain with alizarin. This non-coloration could result either from (1) an excessive period of fixation in formaldehyde, (2) excessive concentration of the formaldehyde solution (Markle 1984), or (3) a poor stability of the alizarin (Laboratoire Mat inc., product number 06525CR, and colorant code 58005). Drawings and observations were done with a Leica MZ9.5 stereodissecting microscope with an attached camera lucida. In the drawings, the cartilaginous structures are dotted, whereas the ossified elements are not. Specimens were measured with a calibrated ocular micrometer to the nearest 0.1 mm. Notochord length (NL) was taken from the tip of the upper jaw to the posterior tip of the notochord. Standard length (SL) was taken between the anterior tip of the upper jaw and the posteroventral edge of the hypurals (Mabee 1993).

Terminology. Terminology of the caudal elements follows that of Monod (1968), Schultze and Arratia (1989), and Arratia and Schultze (1992); particular catostomid caudal structures were taken from Eastman (1980). Abbreviations of the elements are listed in Table 2. In this study, the two uroneurals present in *Moxostoma* spp. are referred to as the pleurostyle and the posterior uroneural. As the homologization of these elements is unclear we can not precise which one of the evolutionary series of uroneurals is present in *Moxostoma*. The terminology used for the pleurostyle follows Monod (1968) and corresponds to the "anterior uroneural" of Eastman (1980). We followed the nomenclature of Arratia and Schultze (1992) for the elements involved with the axial skeleton.

Table 1. Number and size range of examined specimens of *Moxostoma* species. The smallest size is given in terms of notochordal length (NL), whereas the largest size is given in terms of standard length (SL).

| | Number of specimens | Length (mm) | |
|---------------------------------|---------------------|-------------|------|
| | | NL | SL |
| <i>Moxostoma hubbsi</i> | 69 | 9.2 | 29.2 |
| <i>Moxostoma anisurum</i> | 57 | 11.4 | 25.2 |
| <i>Moxostoma macrolepidotum</i> | 83 | 10.9 | 30.3 |

Table 2. List of elements and abbreviations of caudal skeleton of *Moxostoma anisurum*, *M. hubbsi* and *M. macrolepidotum*. All the terms from Monod (1968), Schultze and Arratia (1989), Arratia and Schultze (1992).

¹ specific catostomid structures from Eastman (1980).

² elements integrated into the analysis of congruence between chondrification and ossification patterns.

| | |
|------------------------|---|
| ANS ¹ | accessory neural spine |
| arcPU1+U1 | fused ventral arcocentrum of preural centrum 1 and ural centrum 1 |
| CC | compound centrum |
| darcPU1-3 ² | dorsal arcocentra of preural centra 1-3 |
| darcU1 ² | dorsal arcocentrum of ural centrum 1 |
| E ² | cartilaginous or ossified epural |
| H1-6 ² | cartilaginous or ossified hypurals 1-6 |
| HS2-3 ² | cartilaginous or ossified haemal spines 2-3 |
| hy | hypurapophysis |
| nch | notochord |
| NS2-3 ² | cartilaginous or ossified neural spines of preural centra 2-3 |
| opc | opisthural cartilage |
| PH ² | parhypural |
| Pl ² | pleurostyle |
| pr | principal caudal rays (lepidotrichia) |
| pr.r | procurrent rays |
| PU1+U1 | chordacentrum of preural centrum 1 and ural centrum 1 |
| RNA ¹ | rudimentary neural arch |
| Unp ¹ | posterior uroneural |
| varc | ventral arcocentrum |
| varcPU1-3 | ventral arcocentra of preural centra 1-3 |
| vchcU2 | ventral chordacentrum of ural centrum 2 |

Analysis of congruence between chondrification and ossification sequences. A structure was considered to be forming or ossified when it uptakes the stain. Description of chondrification and ossification sequences involves all the elements posterior to preural centrum 3 inclusively. Chondrification and ossification sequences are defined in terms of successive events. A chondrification event corresponds to the appearance of a single cartilaginous precursor or the almost simultaneous appearances of cartilaginous precursors. An ossification event corresponds to the ossification of one or several cartilaginous precursors. The almost synchronous appearance of elements might be an artefact of coarse sampling. A rank value was attributed to each event in each sequence. Equal rank was readjusted according to the procedure of Dagnelie (1977). Spearman rank correlation coefficients are used in order to compare sequences. A non-parametric coefficient is most appropriate because the data (e.g., rank) do not follow a normal distribution. The formula for the Spearman correlation coefficient (r_s) is as follow (Dagnelie 1977):

$$r_s = 1 - \{(6 \sum d^2) / [n * (n^2 - 1)]\},$$

where “d” is the difference between rank values for each observation and “n” is the sample size corresponding to the number of elements involved in a complete sequence of chondrification or ossification. The degree of freedom (df) corresponds to n-2.

All the specimens of each ontogenetic series of the three *Moxostoma* species (see Table 1) were used to describe the sequences. Congruence between chondrification and ossification patterns was analysed (1) intraspecifically between chondrification and ossification patterns, and (2) interspecifically between chondrification patterns and between ossification patterns. In order to respect the homology between the structures, only those with a cartilaginous precursor and a corresponding ossified element were integrated into the analysis (Table 2). Among the three species, only the detailed sequences of *M. hubbsi* are provided because of the osteological similarity among the species and because this description is the first one on the anatomy of the endangered *M. hubbsi*.

Results

General morphology. The juvenile caudal skeleton of *M. hubbsi*, *M. anisurum* and *M. macrolepidotum* is similar. The caudal skeleton system is composed of the haemal spine of preural centrum 2, one parhypural, six hypurals (1-6), one epural, a pleurostyle, preural centrum 2 and a compound centrum (Fig. 1). Interspecific differences concern the length, the shape and the orientation of the hypurapophysis of parhypural. The general shape of the hypurapophysis is anvil-like. The base of the hypurapophysis is stout in *M. hubbsi* and *M. macrolepidotum* and gracile in *M. anisurum*. The hypurapophysis terminates in a bifurcating process either with spine like tips in *M. anisurum* and *M. macrolepidotum* or rounded tips in *M. hubbsi* (Fig. 1).

Haemal spine 2 articulates with preural centrum 2 by an arcocentrum; it is the only autogenous (*sensu* Monod 1968) haemal spine. The parhypural is fused proximally with hypural 1. Both elements articulate via the fused arcocentrum with a depression in the compound centrum. Hypural 2 is fused to the compound centrum (Fig. 1), whereas hypural 3 lies posteriorly to the compound centrum and shows no fusion with it. Hypurals 4-6 are free and lie posteriorly to the pleurostyle. The pleurostyle is located dorsally to the notochord and fused proximally to the posterodorsal part of the compound centrum. This elongated element expands dorsally almost to the posterior tip of the notochord. The posterior uroneural lies dorsally to the distal end of the pleurostyle. There are 18 principal rays of the caudal fin (Fig. 1); 9 principal rays in the hypochordal and 9 principal rays in the epichordal lobe of the caudal fin. Haemal spine 2 is the most anterior structure connected to a principal ray and is associated with only one principal ray. Each hypural is associated with 2-3 principal rays. Hypural 6 is the uppermost element associated with 1-2 principal rays (Fig. 1). After all rays are formed, they show a constant relation with haemal spine 2 and hypurals 1-6.

Sequence of chondrification of Moxostoma hubbsi. Most of the ventral cartilaginous elements appear earlier than the dorsal ones. In pre-flexion larvae, the first elements to appear are hypurals 1-3; they are present in specimens ca.11 mm NL (Fig. 2A). These elements are interpreted as hypurals because of the absence of the ventral arch which is characteristic of haemal spines. Based on the available series, these elements seem to appear almost synchronously (Fig. 2A). The next elements to form in early flexion larvae are the parhypural and hypural 4 (Fig. 2B). Their formation is achieved in specimens of at least 11.4 mm SL (Fig. 2B). Haemal spine 2 and hypural 5 are present in specimens between 11.5 and 11.7 mm SL (Fig. 2C). Following these structures, the first dorsal

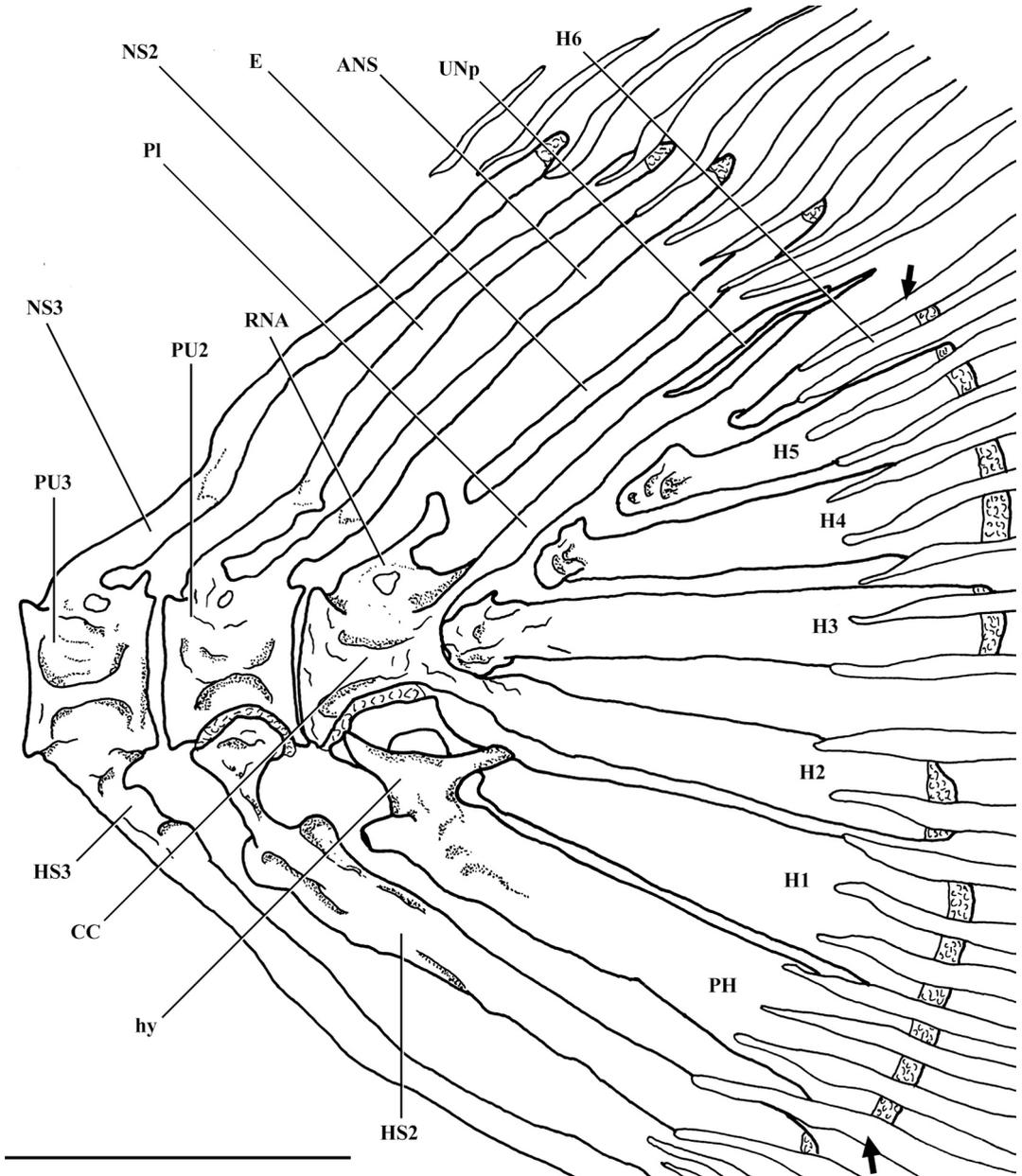


Figure 1. Caudal skeleton of *Moxostoma hubbsi*, 29.1 mm SL. Arrows indicate the first and last principal caudal rays. Scale bar = 1 mm.

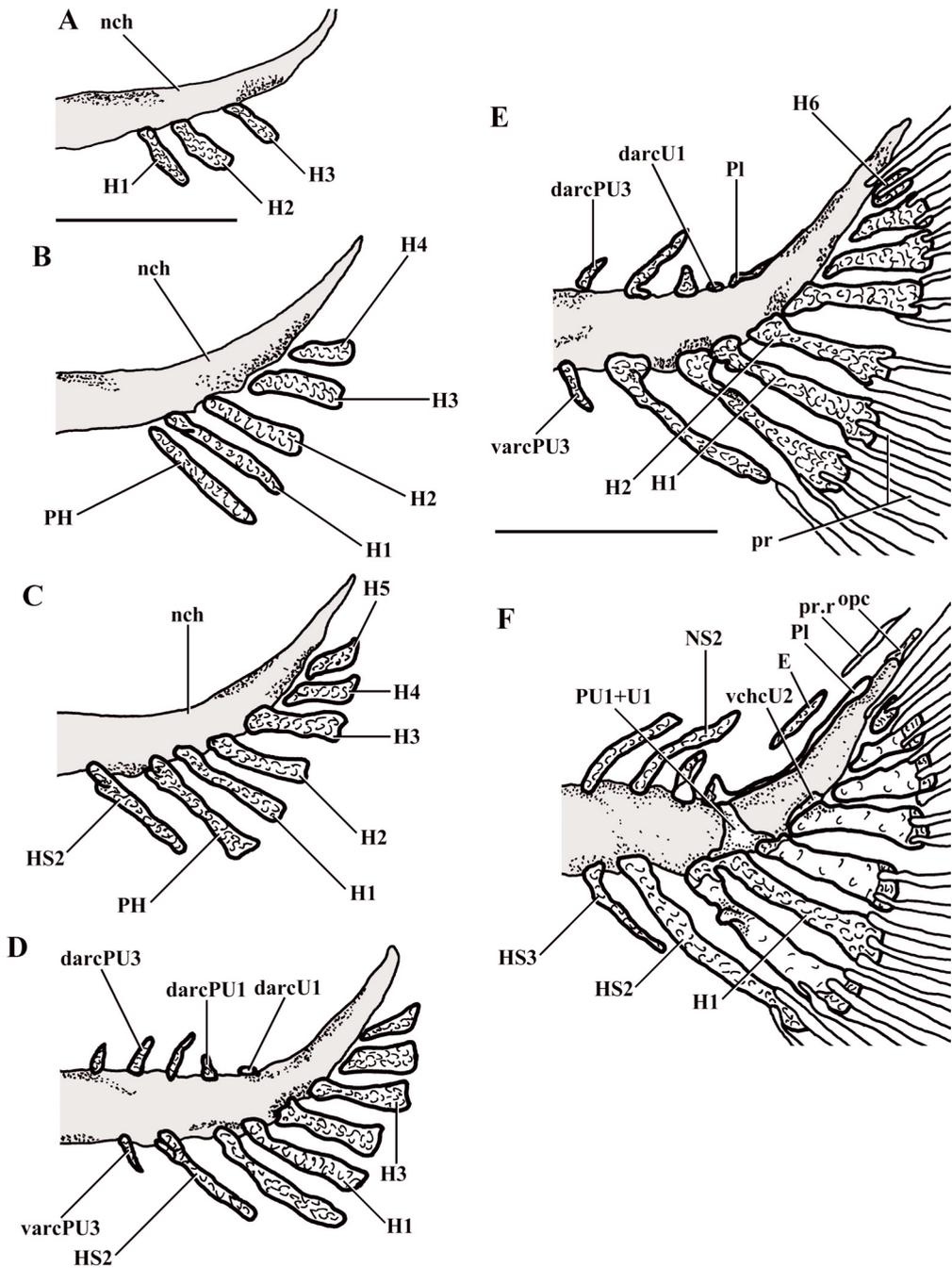


Figure 2. Ontogenetic series of caudal skeleton of young *Moxostoma hubbsi* specimens. A: Pre-flexion larva of 10.9 mm NL. Scale bar = 0.5 mm. B: Specimen of 11.4 mm SL. C: Specimen of 11.5 mm SL. D: Specimen of 11.6 mm SL. E: Specimen of 12.1 mm SL. Scale bar = 0.5 mm. F: Specimen of 12.2 mm SL. A-D, same scale and E-F, same scale.

structures form as small centers of chondrification. These structures include the dorsal arcocentrum of preural centra 1-3. Then the dorsal arcocentrum of ural centra 1 and the ventral arcocentrum of preural centrum 3 appear synchronously in specimens of 11.6 to 12 mm SL (Fig. 2D). A small thin cartilaginous pleurostyle appears dorsally to the notochord in specimens ca. 12.0 mm SL. At this stage, the pleurostyle is a splint-like element restricted to the level of hypural 2 and 3 (Fig. 2E). The pleurostyle forms at the same time as hypural 6. The epural forms in specimen ca. 12.2 mm SL (Fig. 2F). Simultaneously to the epural, a small opisthural cartilage appears at the tip of the notochord; the opisthural cartilage is not taken into account in the chondrification sequence analysis.

The sequence of chondrification of the ventral elements (i.e., haemal spines 2-3, parhypural and hypural 1-6) for the three *Moxostoma* species shows a general bidirectional pattern of development (Fig. 3). As described above, the first elements to form are hypurals 1-3 then the chondrification progresses bidirectionally, meaning anteriorly and posteriorly. In addition, the bidirectional pattern is fairly symmetrical and synchronous. On the other hand, the pattern changes from synchronous to an alternate bidirectional when considering haemal spine 3 and hypural 6 (Fig. 3). However, some discrepancies occur with respect to the order: (1) in some specimens of the three species, the parhypural appears earlier than hypural 4 and (2) haemal spine 2 appears earlier than hypural 5. These variations are responsible for the shift from a synchronous bidirectional pattern to an alternate bidirectional development of the ventral structures (Fig. 3).

Sequence of ossification of Moxostoma hubbsi. The principal caudal rays are the first structures to ossify but these elements are not taken into account in the ossification sequence. Following these structures, the pleurostyle and the dorsal arcocentrum of ural centra 1 ossify in specimens ca. 12.2 mm SL. Ossification of these structures is completed for specimens of 12.8 mm SL (Figs. 2F, 4A). Almost synchronously, two thin ventral independent chordacentra (i.e., the ventral chordacentra of preural centrum 1 fused with the ventral chordacentra of ural centrum 1 and ventral chordacentrum of ural centra 2) begin to form (Fig. 2F). Then the parhypural, hypurals 2-3 ossify and subsequently the dorsal arcocentrum of preural centra 1 and hypurals 4-5 ossify almost synchronously (Fig. 2F). The next structures to ossify are first; haemal spine 2 and hypural 1 then followed by haemal spine 3, neural spine 2 and neural spine 3 in specimens ca. 12.8 mm SL (Fig. 4A). A small splint-like posterior uroneural bone appears dorsoposteriorly to the pleurostyle in specimens of 12.8 mm SL (Fig. 4A). The posterior uroneural expands dorsally to the pleurostyle becoming a rod-like bone (Figs. 1, 4B). No cartilaginous precursor has been observed for this bone. Ossification of the dorsal arcocentra of preural centra 2-3 is achieved in a specimen of 17.2 mm SL. Synchronously, the chordacentra of preural centra 1 plus ural centra 1 and ural centra 2 become ring-shaped and expanded dorsally to surround the notochord. These structures precede the formation of the compound centrum (see Table 2 for abbreviations) and finally of hypural 6. The fusion of haemal spine 3 to preural centra 3 is achieved in specimens of 29.1 mm SL (Fig. 1). At this stage, the epural is ossified (Fig. 1).

Congruence between chondrification and ossification sequences in Moxostoma hubbsi. The complete sequences of chondrification and ossification are summarized in Tables 3 and 4. Both sequences are composed of 15 elements that can be regrouped in seven distinct events; for example, the first chondrification event corresponds to the simultaneous appearance of hypural 1, hypural 2 and

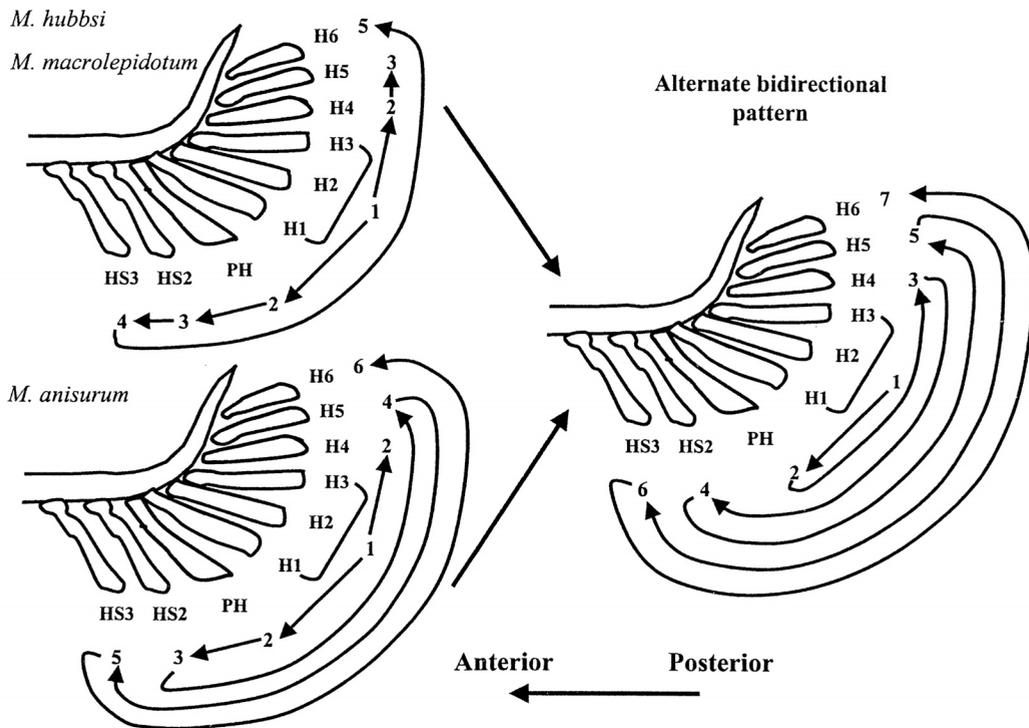


Figure 3. Diagrammatic representations of the bidirectional pattern of chondrification for the ventral element of the caudal skeleton of the three *Moxostoma* species. The pattern appears to be globally simultaneous bidirectional for the three *Moxostoma* species. For the three species this general pattern can shift to an alternate bidirectional pattern. Numbers correspond to the order of appearance of cartilaginous elements and arrows indicate the direction.

hypural 3, whereas the first ossification event includes the pleurostyle and the dorsal arcocentrum of ural centra 1. As shown in Table 4, the ossification does not follow the same sequence as the appearance of the cartilaginous precursors. There is no significant correlation between the order of formation of the elements ($r_s = 0.34, p > 0.05$). Thus, the two sequences are not congruent. This is mainly owing to a strong difference in rank value between both sequences for the pleurostyle, the dorsal arcocentrum of ural centra 1 and hypural 1. Indeed, the pleurostyle rank shifts from 13.5 in the chondrification sequence to 1.5 in the ossification sequence. When both sequences are compared without taking into account these three structures, there is a highly significant congruence between both sequences ($r_s = 0.93, p < 0.001$; Table 3).

Congruence between chondrification and ossification sequences of M. anisurum and M. macrolepidotum. Chondrification and ossification sequences of both species are also composed of seven distinct events (Table 4). As in *M. hubbsi* sequences, ossification of the different elements does not follow the same order as the chondrification sequence. However, there is a significant correlation between

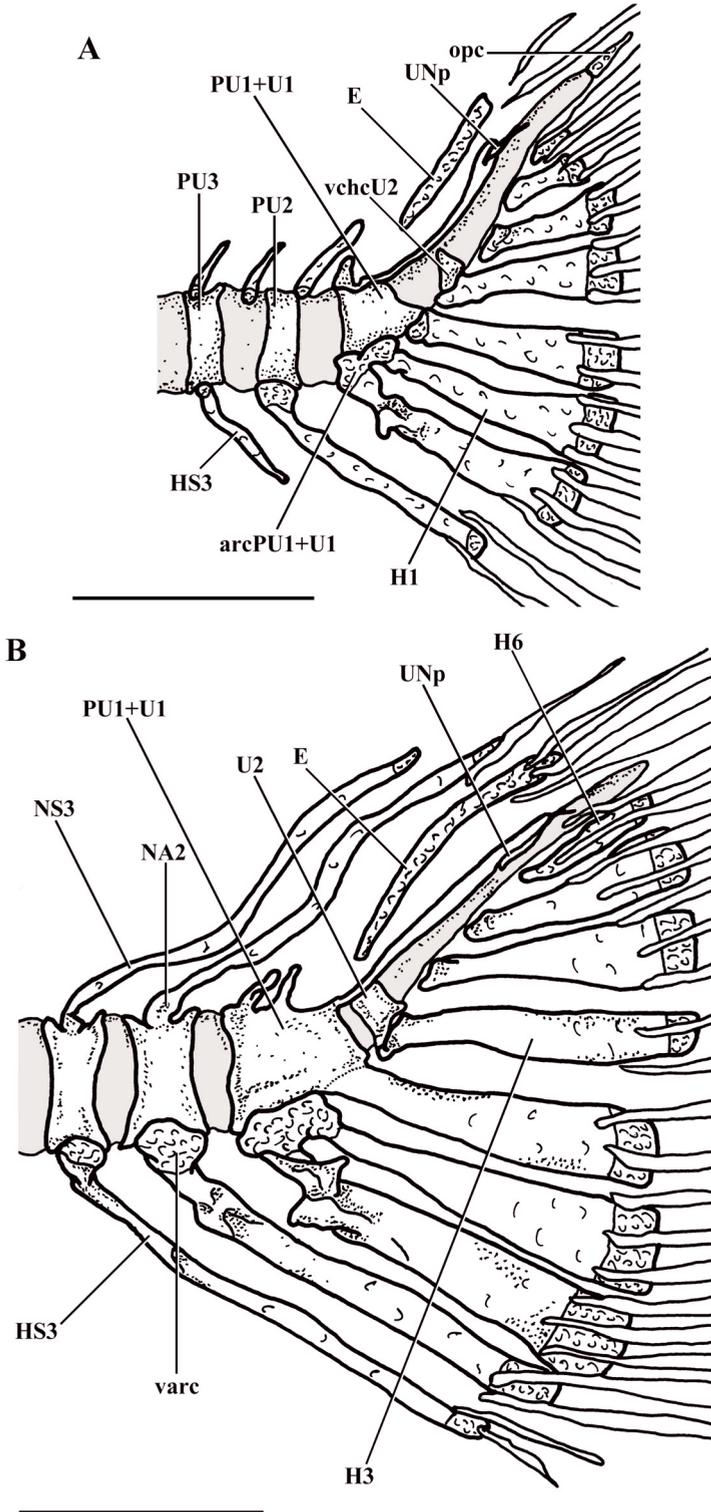


Figure 4. Caudal skeleton of young *Moxostoma hubbsi* specimens.

A: Young specimen of 12.8 mm SL.

Scale bar = 0.5 mm.

B: Specimen of 17.2 mm SL. Scale bar = 0.5 mm.

Table 3. Comparison and congruence test of *M. hubbsi* chondrification and ossification patterns with the complete sequence and without the pleurostyle, the dorsal arcocentrum of preural centra 1 and hypural 1 structures. Note that the major shifts in the complete sequence are in bold. See Table 2 for abbreviations.

| Complete sequence | | | | Sequence without Pl, darcU1, H1 | | | |
|-----------------------------|-------|-----------------------|-------|---------------------------------|-------|-----------------------|-------|
| Chondrification sequence | Ranks | Ossification sequence | Ranks | Chondrification sequence | Ranks | Ossification sequence | Ranks |
| H1 | 2 | PI | 1.5 | H2 | 1.5 | PH | 2 |
| H2 | 2 | darcU1 | 1.5 | H3 | 1.5 | H2 | 2 |
| H3 | 2 | PH | 4 | PH | 3.5 | H3 | 2 |
| PH | 4.5 | H2 | 4 | H4 | 3.5 | H4 | 5 |
| H4 | 4.5 | H3 | 4 | HS2 | 5.5 | H5 | 5 |
| HS2 | 6.5 | H4 | 7 | H5 | 5.5 | darcPU1 | 5 |
| H5 | 6.5 | H5 | 7 | darcPU1 | 8.5 | HS2 | 7 |
| darcPU1 | 9 | darcPU1 | 7 | NS2 | 8.5 | HS3 | 9 |
| NS2 | 9 | HS2 | 9.5 | NS3 | 8.5 | NS2 | 9 |
| NS3 | 9 | H1 | 9.5 | HS3 | 8.5 | NS3 | 9 |
| HS3 | 11.5 | HS3 | 12 | H6 | 11 | H6 | 11 |
| darcU1 | 11.5 | NS2 | 12 | E | 12 | E | 12 |
| PI | 13.5 | NS3 | 12 | | | | |
| H6 | 13.5 | H6 | 14 | | | | |
| E | 15 | E | 15 | | | | |
| Spearman coefficient | | | | | | | |
| $r_s = 0.34$ ($p > 0.05$) | | | | $r_s = 0.93$ ($p < 0.001$) | | | |

the order of chondrification and ossification of the elements for both species (*M. anisurum*: $r_s = 0.50$, $p < 0.05$; *M. macrolepidotum*: $r_s = 0.54$, $p < 0.05$). Although the two sequences for both species are congruent, the values are low. Despite the congruence between both sequences for *M. anisurum* and *M. macrolepidotum*, there is variability between both sequences (Table 5).

Intraspecific comparisons between chondrification and ossification patterns. Table 5 summarizes the Spearman correlation coefficients for the intra- and interspecific comparisons. Two intraspecific trends emerge from these results. First, there is no significant congruence between the two sequences for *M. hubbsi* ($r_s = 0.34$, $p > 0.05$). Second, there is a significant congruence between the sequences for *M. anisurum* ($r_s = 0.50$, $p < 0.05$) and *M. macrolepidotum* ($r_s = 0.54$, $p < 0.05$). The weak correlation between the sequences results partly from the pleurostyle position into the sequences that displays a shift of 12 ranks between the chondrification and ossification sequences.

Interspecific comparisons between chondrification and ossification patterns. Patterns of chondrification and ossification are congruent among species ($r_s = 0.99$, $p < 0.001$ for each comparison; Table 5). Minor changes in the relative rank of hypural 5, neural spine 3, dorsal arcocentrum of preural centra 1 and haemal spine 2-3 occur among species.

Table 4. Chondrification and ossification sequences of the three *Moxostoma* species. Arrows indicate the order of the sequence and “+” signs mean simultaneous formation of structures in an event.

| Events number | <i>Moxostoma hubbsi</i> | | <i>Moxostoma anisurum</i> | | <i>Moxostoma macrolepidotum</i> | |
|---------------|--------------------------|-----------------------|---------------------------|-----------------------|---------------------------------|-----------------------|
| | Chondrification sequence | Ossification sequence | Chondrification sequence | Ossification sequence | Chondrification sequence | Ossification sequence |
| 1 | H1+H2+H3 | Pl+darcU1 | H1+H2+H3 | Pl+darcU1 | H1+H2+H3 | Pl+darcU1 |
| | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| 2 | PH+H4 | PH+H2+H3 | PH+H4 | PH+H2+H3 | PH+H4 | PH+H2+H3 |
| | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| 3 | HS2+H5 | H4+H5+darcPU1 | HS2 | H4+H5 | HS2+H5 | H1+H4+H5 |
| | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| 4 | darcPU1+NS2+NS3 | HS2+H1 | H5+darcPU1+darcU1+NS2 | H1 | darcPU1+darcU1+NS2 | HS2 |
| | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| 5 | HS3+darcU1 | HS3+NS2+NS3 | HS3+NS3 | HS2+darcPU1 | HS3+NS3 | HS3+NS2+NS3+darcPU1 |
| | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| 6 | Pl+H6 | H6 | Pl+H6 | NS2+NS3+HS3+H6 | Pl+H6 | H6 |
| | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| 7 | E | E | E | E | E | E |

Table 5. Intraspecific and interspecific test of congruence between chondrification and ossification sequences. *Mohu* = *M. hubbsi*, *Moan* = *M. anisurum* and *Moma* = *M. macrolepidotum*. Note that $n = 15$ and $df = 13$.

| Intraspecific comparisons | | Mohu | Ossification Moan | Moma |
|---------------------------|-------------|------------------------------|------------------------------|-----------------------------|
| Chondrification | Mohu | $r_s = 0.34$ ($p > 0.05$) | | |
| | Moan | | $r_s = 0.50$ ($p < 0.05$) | |
| | Moma | | | $r_s = 0.54$ ($p < 0.05$) |
| Interspecific comparisons | | Chondrification | Ossification | |
| | Mohu - Moan | $r_s = 0.99$ ($p < 0.001$) | $r_s = 0.99$ ($p < 0.001$) | |
| | Moan - Moma | $r_s = 0.99$ ($p < 0.001$) | $r_s = 0.99$ ($p < 0.001$) | |
| | Moma - Mohu | $r_s = 0.99$ ($p < 0.001$) | $r_s = 0.99$ ($p < 0.001$) | |

Discussion

The juvenile and adult caudal skeleton of catostomid species is a stable morphological system. Eastman (1980) has studied the caudal skeleton of catostomids. According to his study, the interspecific pattern of the caudal skeleton for 20 species is similar in terms of the number and topography of the bony elements. Most of the variation concerns the orientation, size and shape of the hypurapophysis of the parhypural. Our results on *Moxostoma* corroborate (1) the stability of the catostomid caudal skeleton pattern and (2) that most of interspecific differences within *Moxostoma* also concern the hypurapophysis.

Based on a strong interspecific congruence between ossification sequences in poeciliids, Strauss (1990) pointed out the necessity to understand developmental and functional interrelationships among cartilaginous and ossified elements. Our results support Strauss' assessments concerning the importance to consider both chondrification and ossification sequences. Two ontogenetic trends emerge from the *Moxostoma* species. First, intraspecific comparisons demonstrate weak congruence between the chondrification and ossification sequences for *M. hubbsi*, *M. anisurum* and *M. macrolepidotum*. Second, interspecific comparisons among chondrification sequences and among ossification sequences suggest a strong congruence of the developmental pathways. According to Eastman (1980), the juvenile and adult morphology of the catostomid caudal skeleton is highly similar among species. For *Moxostoma*, not only the adult morphology is similar but also the process to achieve the terminal morphology. Thus, phylogenetic and functional constraints are potentially responsible for the developmental stability of the caudal skeleton.

Based on Smith's (1992) phylogenetic hypothesis, the three *Moxostoma* species are closely related. Therefore, a strong phylogenetic canalization of the development is potentially responsible for the stability of the developmental pathways as well as the highly similar morphology. Strauss (1990) pointed out that when considering the complexity of the inductive and morphogenetic interactions that control the development of complex structures, it is not surprising that ossification sequences among closely related taxa are highly conservative. In contrast to Strauss (1990), we can confirm that not only ossification sequences are conservative but chondrification sequences as well. These results are consistent with the extensive study of Mabee et al. (2000) on developmental timing and intraspecific variation within and among ossification sequences of fishes. As observed by Mabee et al. (2000) on the ossification of cranial bone in *Danio rerio*, *Betta splendens*, *Oryzias latipes* and *Barbus barbuis*, variation was observed among ossification sequences but also among chondrification sequences in each of the three *Moxostoma* species (see Fig. 3) but the general pattern of development is conserved. However, no analysis was conducted to quantify the intraspecific variation in both sequences. Further studies are needed to gather in the absence of such data.

Morphological structures and their development are strongly correlated with functional requirements (Weisel 1960, 1967; Strauss 1990; Mabee et al. 2000; Maglia et al. 2001). Hence, we can assume that functional needs canalized the developmental sequences. Bones implicated in early functional needs probably respond by earlier ossification (Strauss 1990; Mabee et al. 2000). For example, Weisel (1967) has shown that the pleurostyle (termed uroneural in his study) of *Catostomus macrocheilus* is one of the first bones to ossify; similar results are found for three *Moxostoma* species. This is also the case in *Danio rerio*, *Betta splendens*, *Oryzias latipes* and *Barbus*

barbus where bony elements of the opercular region and the cranial bones involved in feeding ossify earlier than other bones (Mabee et al. 2000). In the caudal fin of *Moxostoma*, the early ossification might be in response to mechanical stresses propagating by the thrust along the posterior part of the notochord after the flexion stage. Functional requirements probably shape also the pattern of cartilaginous structures. As shown in Figure 3, each one of the three species exhibits a bidirectional simultaneous or alternate pattern with minor shifts in the sequence of *M. anisurum*. This pattern results most likely from a uniform repartition of mechanical stresses during ontogeny in the hypochordal lobe of the notochord. Thus, it is likely that the interspecific congruence between developmental sequences as well as the weak intraspecific congruence between chondrification and ossification patterns are the result of functional requirements.

Other developmental constraints could also be responsible for differences in developmental sequences. In his study on crocodylian skeleton formation, Rieppel (1993) has shown that the sequences of chondrification and ossification differ owing to ontogenetic repatterning (Müller 1991). This difference was interpreted as a decoupling of two processes: (1) absence of ossification and (2) secondary fusion of primary elements. Rieppel's (1993) conclusions imply that two sequences cannot be deduced by the sole study of one sequence. With respect to *Moxostoma* spp., there is no evidence of the absence of ossification or of secondary fusion. The main differences between the chondrification and ossification sequences are characterized only by shifts of some elements (e.g., pleurostyle, dorsal arcocentrum of ural centra 1 and hypural 1) in the relative timing of the development. Heterochronic patterns (e.g., acceleration or retardation of ossification) that combine to differential functional requirements might be responsible for such changes in the order of sequences. However, additional studies are needed to understand the influence of heterochronic changes on developmental and functional interactions between chondrification and ossification sequences and also to test the level and types of variation within and among developmental sequences.

Conclusion

Ontogenetic studies in terms of chondrification and ossification sequences are of great interest to understand the evolution of morphological systems. Both sequences have the potential to be phylogenetically informative either to detect heterochronic patterns within an organism as a whole or at the structural level. Given the principle of total evidence (Kluge 1989), not only the terminal morphology of a system can be used as phylogenetic characters but also chondrification and ossification patterns have the potential to be also informative. On the other hand integration of data derived from both sequences may bring conflictual phylogenetic hypotheses. There is no a priori reason that the chondrification or the ossification sequence will be more or less informative. A complete set of developmental data will improve the robustness of phylogenetic hypotheses and the comprehension of the interaction between ontogeny and phylogeny. Further studies on chondrification and ossification sequences will be useful to understand how development and function interacts to influence a morphological program and create morphological diversity. Equal interest should be given to chondrification and ossification patterns. Phylogenetic studies of developmental sequences should integrate closely and distantly related taxa in order to understand the underlying ontogenetic processes of evolutionary changes.

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Bone development during metamorphosis of the Japanese flounder (*Paralichthys olivaceus*): differential responses to thyroid hormone

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Key words: *bone development, metamorphosis, thyroid hormone, Japanese flounder*

Abstract

The larvae of flatfish change their body structure during metamorphosis, including dramatic translocation of one of the eyes from one side of the body to the other. Such metamorphic processes are in general promoted by thyroid hormones (THs). This study focuses on the response of individual tissues to hormones, and morphological characteristics were examined in hormone-deficient larvae of the Japanese flounder (*Paralichthys olivaceus*). Treatment of flounder larvae with an inhibitor of thyroid hormone synthesis, thiourea (TU; 30 ppm, TU) inhibited translocation of the right eye, shortening of dorsal fin rays, and body pigmentation, as previously reported. Treatment also inhibited elongation of the pseudomesial bar (Pb), which is important for eye translocation, and formation of bones related to larval or juvenile characteristics – the actinost and the distal radial of the pectoral fin, and the pterygiophore of the anal fin. Bones other than these, which are similarly present in both larvae and juveniles, were unaffected. These results suggest the differential responsiveness of bones to thyroid hormone deficiency during early developmental stages, which seems to play a significant role in the morphological changes from larvae to juveniles. To examine the period of hormone responsiveness, thyroid hormone treatment with thyroxine (T4; 100 ppb for 2 weeks) was started at two different times for the fish receiving TU treatment. When T4 was given 2 weeks later than TU, the fish completely metamorphosed through the expected bone formation that had been suppressed by TU. However, when T4 treatment was started 4 weeks later than TU, body pigmentation did not occur, and the translocation of the right eye was not complete due to failure of Pb formation. It is suggested that individual tissues that change during metamorphosis have their own timing in response to thyroid hormones.

Introduction

A large number of teleosts undergo metamorphosis by changing their bodies from larval to juvenile forms so as to adapt to new habitats (Youson 1988). With regard to the study of dramatic changes in morphology, extensive research has been conducted on flatfish metamorphosis. For natural populations of the Japanese flounder (*Paralichthys olivaceus*), pelagic larvae migrate to shallow water and settle on the sea-bed, after which the body changes from a symmetrical to an asymmetrical form, which includes the relocation of one eye and pigmentation of one side (Minami 1982). As in amphibians, thyroid hormones (THs) induce flounder metamorphosis at the whole body level (Inui and Miwa 1985; Miwa et al. 1988), and at the individual tissue level - erythrocytes (Miwa and Inui 1991), skeletal muscle (Yamano et al. 1991), gastric glands (Miwa et al. 1992; Huang et al. 1998; Soffientino and Specker 2001), and chloride cells in the gill (Schreiber and Specker 2000). Therefore, eye migration, one of the most characteristic changes in flounder metamorphosis, is also expected to be under the control of THs.

In the Japanese flounder, it was revealed that the development of the pseudomesial bar (Pb), a bone present only in flatfish, is important for eye relocation (Okada et al. 2001). Changes in body proportion occur during flatfish metamorphosis (Seikai et al. 1986), and are also related to bone development (Ahlstrom et al. 1984). Although the role of thyroid hormones in skull development during amphibian metamorphosis has been studied (anurans, Hanken and Hall 1988; and urodeles, Rose 1995a,b), no research has been done on flatfish metamorphosis focusing primarily on bones and their control, despite the extreme morphological changes that occur.

The presence of a stage-specific response to TH is indicated at the whole body level during metamorphosis of the summer flounder (Schreiber and Specker 1998). Since the progress of metamorphosis was assessed using a stage index based on the location of the eyes, it is possible that some of the bones relating to eye relocation are directly controlled by THs and have specific response times. To clarify these points, the development of individual hard tissues were examined under TH-deficient conditions, and the extent of recovery was tested by THs supplied to the Japanese flounder at different times.

Materials and Methods

Experimental Animals. Fifty thousand fertilized eggs of the Japanese flounder were stocked in a 500 l tank at 15°C, with running seawater under a photoperiod of 11h:13h (L:D). More than 50% of the eggs hatched three days later, and the larvae that hatched on that day were fed on the rotifer *Brachionus rotundiformis* twice daily until the beginning of the experiments. Newly hatched *Artemia* nauplii of the Utah strain and an artificial diet were also provided from 12 to 39 days after hatching.

The classification of the developmental stages followed that of Minami (1982), in which right eye migration starts at stage E and finishes at stage I.

Experiment 1. On the 16th day post hatching (dph), 2 groups of 1000 larvae were transferred to 100 l transparent tanks with or without thiourea (30 ppm, TU, an inhibitor of thyroid hormone synthesis). Two-fifths of the seawater was changed every two days, and TU was added to keep the concentration constant.

For the analysis of thyroid hormones, larvae (1 g wet weight) were obtained from each tank every 5 days and frozen at -40°C. 3,5,3'-triiodothyronine (T3) and thyroxine (T4) were extracted from whole-body homogenate and measured by radioimmunoassay following the procedure of Tagawa and Hirano (1987, 1990).

The larvae on 16, 19, 22, 29, and 35 dph in each group were anesthetized in MS-222 solution and fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4). Body length, body height, and length of dorsal fin were then measured. Three to five samples were double-stained with alcian blue and alizarin red 'S', following the standard method described by Dingerkus and Uhler (1977). The bones and cartilages of the cranium and body were observed under a stereomicroscope. Bones and Cartilage were identified following the descriptions of Amaoka (1969) and Matsuoka (1985).

Differences in body length at each age in Experiment 1 were analyzed by *t*-test (Excel, Microsoft).

Experiment 2. Fish reared with TU in Experiment 1 were further divided into two groups ($n = 40$ per group) and transferred to 30 l tanks at 38 dph and 67 dph. The TU-pretreated and metamorphosis-retarded fish were further reared with only TU (30 ppm, control group), or with both TU and T4 (30 ppm and 100 ppb, respectively, T4 group). Two-fifths of the seawater was changed every two days, and TU and T4 were added to keep the concentrations constant. After 14 days of treatment, the fish were harvested and fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4). The external appearance was noted and the bones and cartilage were observed after undergoing the above-mentioned double staining method.

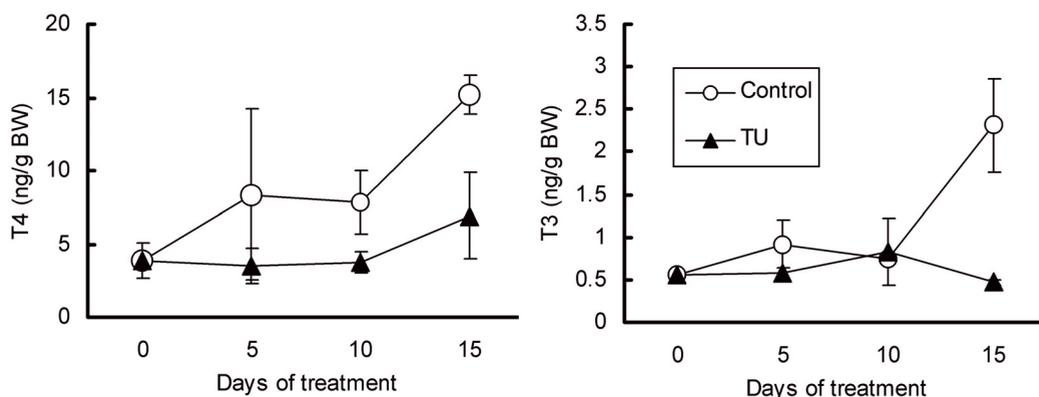


Figure 1. Changes in whole body thyroxine (T4) and 3,5,3'-triiodothyronine (T3) concentrations in *Paralichthys olivaceus* reared without (Control) or with thiourea (TU, 30 ppm). Vertical lines represent mean \pm SEM ($n=3$ pools).

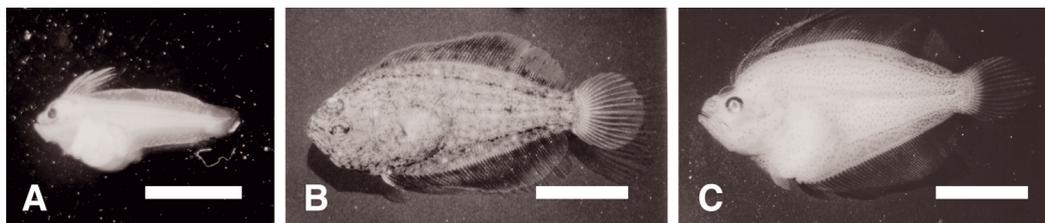


Figure 2. Effect of TU on external appearance of *Paralichthys olivaceus*. (A) initial larva of stage D (16 dph), (B) larva in control group reared in ordinary seawater (35 dph), (C) larva in TU group reared in 30 ppm TU for 20 days (35 dph). Bars = 5 mm.

Results

Experiment 1. Effect of thyroid hormone deficiency on development. Figure 1 shows the changes in tissue T4 and T3 levels of the TU-treated and the control fish. The T4 concentration in the control fish tended to increase during metamorphosis from 3.8 ng g^{-1} to 15.2 ng g^{-1} , while the T4 level in the TU fish remained lower during the first 10 days and increased slightly to 6.9 ng g^{-1} by day 15. The T3 level of the control fish was about $0.5 - 1.0 \text{ ng g}^{-1}$ during the first 10 days and increased to 2.5 ng g^{-1} by day 15. No increase in T3 concentration was observed in the TU-treated fish.

At the beginning of the experiment, at 16 dph, the fish had symmetrical bodies, elongated fin rays, and no pigmentation (stage D, Fig. 2A). After 20 days, the fish without TU were found to be completely metamorphosed (stage I, Fig. 2B), while eye migration, resorption of the dorsal fin rays, and asymmetrical pigmentation of the body in the TU-treated fish was prevented (Fig. 2C). As shown in Figure 3A, the body length of both groups increased similarly up to 29 dph, but the TU-treated fish were significantly larger at 35 dph (at the end of metamorphosis in the control group).

Figures 3B and 3C show the relative increases in body height, and dorsal fin length against body length. In both cases, changes in the relative growth were observed at body lengths of about 9 to 10 mm. The percentage of body height compared to body length was higher in the TU-treated fish after the flexion point of the relative growth curve (Fig. 3B). Resorption of the dorsal fin rays was prevented in the TU-treated fish (Fig. 3C).

Significant differences were found in several bones between the control and the TU-treated fish at 35 dph (Fig. 4). The pseudomesial bar (Pb) formed beside the relocated eye in the control fish (Fig. 4A), but was lacking in the TU-treated fish (Fig. 4B). No difference was observed in other parts of the cranium between the two groups. Development of the pectoral fins into juvenile type was also prevented by TU. The pectoral fin of the TU-treated fish did not shrink (Fig. 4C, D), and the actinost and the distal radials were not formed (Fig. 4E, F). The pterygiophore of the anal fin elongated in an anterior manner towards the pelvic fin and calcified in the control fish (Fig. 4C), while a weakly calcified pterygiophore was ventrally elongated in the TU-treated fish (Fig. 4D), resulting in a protruding abdomen for this group. No other notable differences were observed in hard tissues including vertebrae between the two groups.

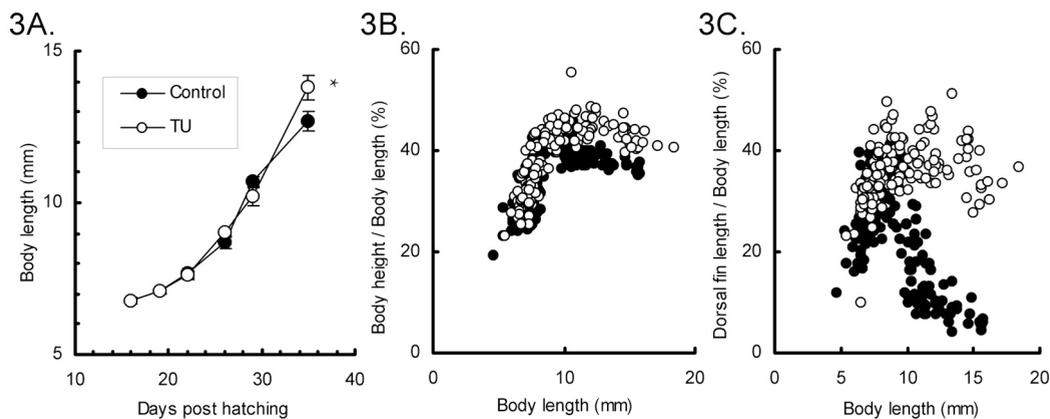


Figure 3. Growth of larvae and early juveniles of *Paralichthys olivaceus* reared without (control) or with thiourea (TU, 30 ppm). (A) body length. Vertical lines represent mean \pm SEM ($n > 28$), * indicates significant difference from control ($P < 0.05$ by t test). (B) Relative growth of body height against body length. (C) Relative growth of dorsal fin ray against body length.

Experiment 2. Response to T4 at different timings. The second experiment examined the difference in timing of T4 responsiveness among various tissues. External appearance is shown in Figure 5, and the internal skeleton is shown in Figure 6. At the beginning of the experiment, at 38 dph (Fig. 5A, 6A), the right eye was located on the right side, and pigmentation of the body was symmetrical, as in the TU-treated fish at 35 dph in Experiment 1.

At 51 dph, the fish receiving only TU treatment continued to grow larger. Although the right eye relocated slightly to the left side, there was no major differences from the fish at 38 dph (Figs. 5B, 6B). T4 treatment starting from 38 dph succeeded in inducing complete metamorphosis by 51 dph. The right eye was relocated completely, the dorsal fin rays were absorbed, and the body color was differentiated asymmetrically as in adults (Fig. 5C). The formation of the Pb, the actinost and the distal radials of the pectoral fin, and the pterygiophore of the anal fin were all complete (Fig. 6C).

At 81 dph, the fish receiving only TU continuously grew longer, without showing any remarkable changes in external and internal structure (Figs. 5D, 6D). The fish receiving T4 starting from 67 dph failed to produce juveniles of normal appearance (Fig. 5E). Resorption of the dorsal fin rays, as well as formation of the actinost and the distal radials of the pectoral fins, and the pterygiophore of the anal fin, were induced by T4 (Fig. 6E). However, the changes in body color were incomplete (Fig. 5E). Moreover, the right eye moved only slightly to the dorsal edge of the head where the cornea was visible from the left side of the fish (Fig. 5E). There was an absence of Pb, and the presence of the pterygiophore of the dorsal fin in the place where the right eye would pass through (Fig. 6E).

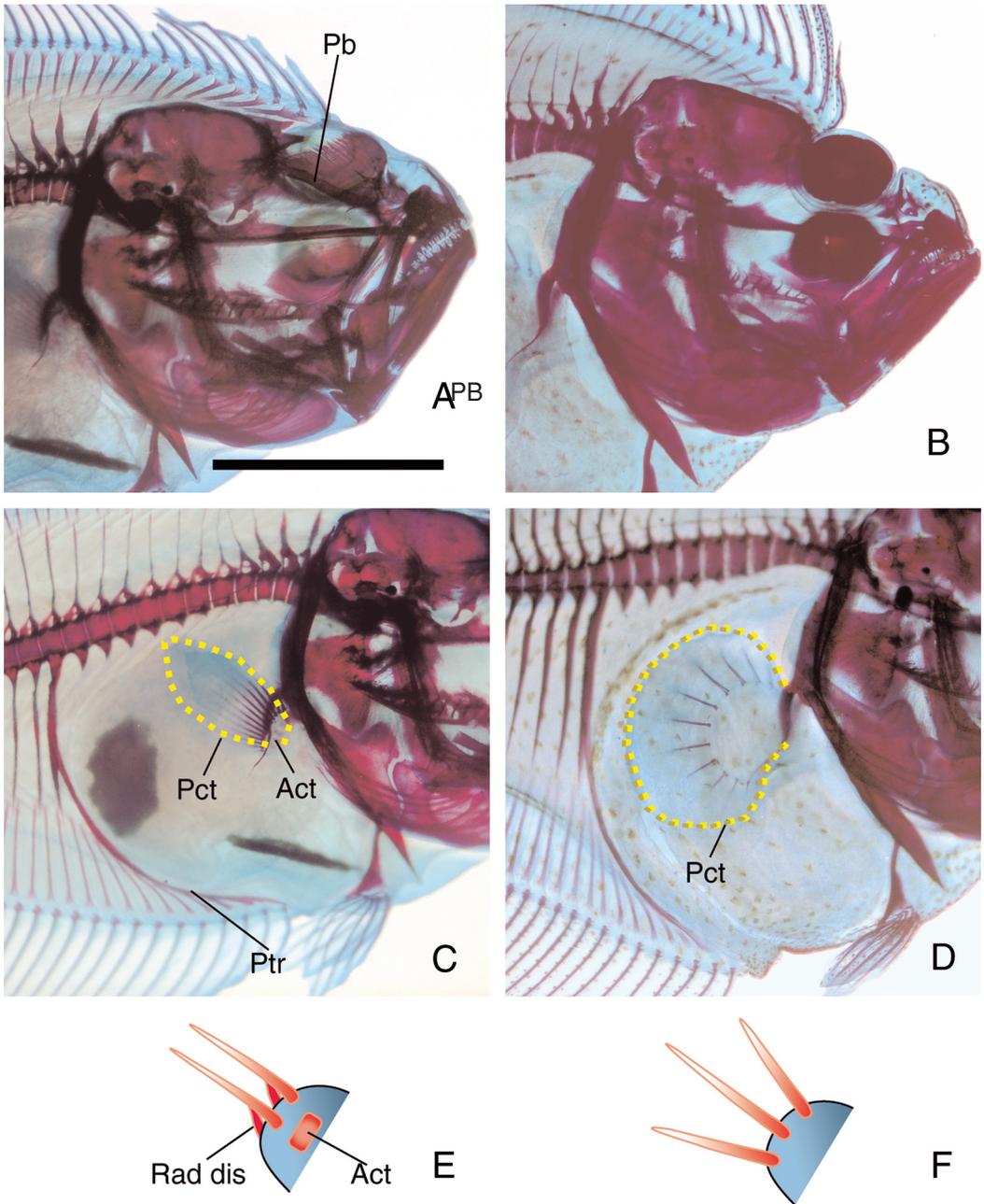


Figure 4. Anterior part of *Paralichthys olivaceus* at 35 dph reared 20 days; (A), (C) in ordinary seawater Control, and (B), (D) in 30 ppm TU. Schematic drawings of the pectoral fin of Control fish (E) and TU-treated fish (F). Photographs were taken on cleared and double stained samples from the blind side. The outline of the pectoral fin is indicated by a yellow broken line. Act, actinost; Pb, pseudomesial bar; Pct, pectoral fin; Ptr, pterygiophore of anal fin; Rad dis, distal radial. Bar 3 mm. This figure is reprinted as a colour plate in the book's appendix.

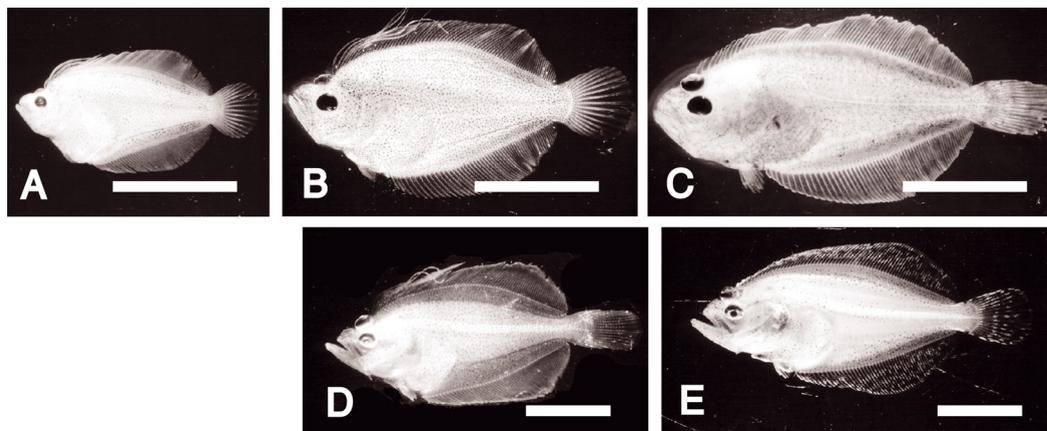


Figure 5. Differential responsiveness of external morphology of TU-treated fish to exogenous T4 provided at different timings. All the fish including control were reared under the presence of TU (30 ppm) from 22 dph. (A) 38 dph, initial control; (B) 51 dph, control; (C) 51 dph, T4 (100ppb, 38 to 51dph); (D) 81 dph, control; (E) 81 dph, T4 (100 ppb, 67 to 81 dph). Bars 1 cm.

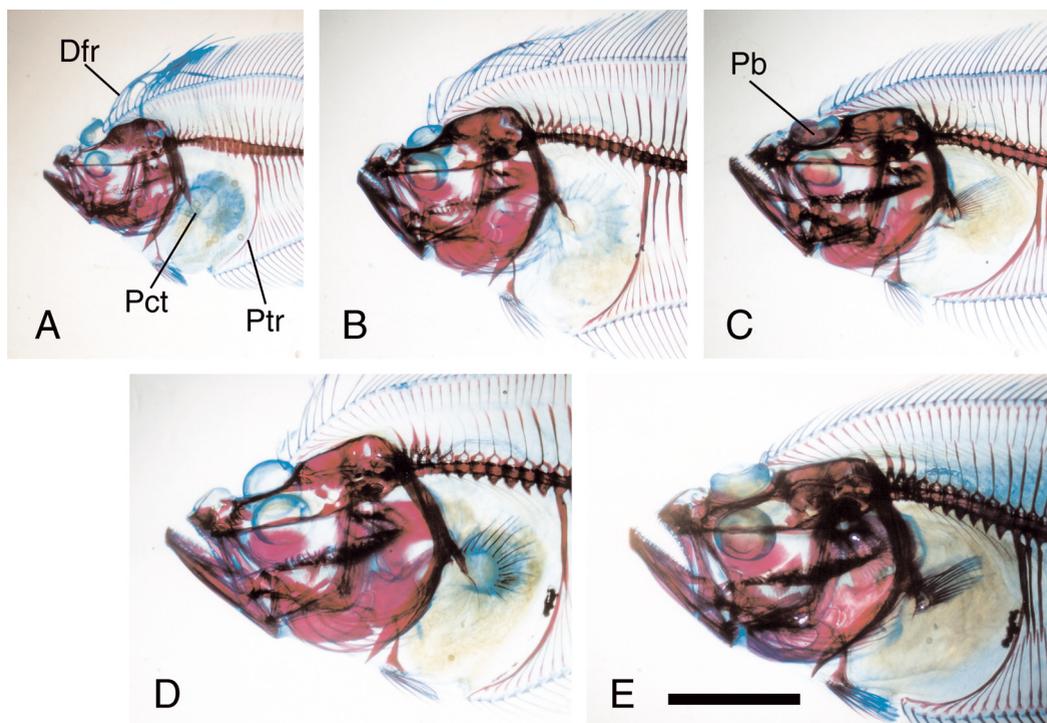


Figure 6. Differential responsiveness of internal morphology of TU-treated fish to exogenous T4 provided at different timings. All the fish including controls were reared under the presence of TU (30 ppm) from 22 dph. Photographs were taken on cleared and double stained samples. (A) 38 dph, initial control; (B) 51 dph, control; (C) 51 dph, T4 (100ppb, 38 to 51dph); (D) 81 dph, control; (E) 81 dph, T4 (100 ppb, 67 to 81 dph). Dfr, dorsal fin ray; Pb, pseudomesial bar; Pct, pectoral fin; Ptr, pterygiophore of anal fin. Bars = 1 cm. This figure is reprinted as a colour plate in the book's appendix.

Discussion

Thyroid hormones (THs) are one of the major regulators of bone development and remodeling in vertebrates (e.g., Mosekilde et al. 1990), including teleosts (LaRoche et al. 1966; Barrington and Rawdon 1967; Barder and Barrington 1972; Takagi et al. 1994). Since bone development and remodeling are essential aspects of morphological change that occurs during flatfish metamorphosis, particularly in eye relocation (Okada et al. 2001), the involvement of TH in the specific bones essential for metamorphic changes in flatfish has been implicated.

In the present study, thiourea (TU) treatment resulted in thyroid hormone deficiency in the larval flounder, as shown in Figure 1. Since the differences between the TU-treated and the control fish disappeared following exogenous T4 treatment provided at early stages (from 38 dph) (Figs. 5C, 6C), these differences were considered to be induced by hormone deficiency, not by the non-specific (toxic) effect of TU.

The effects of TH deficiency are summarized as follows:

- 1) Inhibition of pigmentation on the left side of the body (Fig. 2C);
- 2) Inhibition of Pb formation (Fig. 4B) and right eye relocation (Fig. 2C);
- 3) Inhibition of the development of the anal fin pterygiophore (Fig. 4D) and inhibition of body height reduction (Fig. 3B);
- 4) Inhibition of the absorption of the dorsal fin ray (Fig. 3C);
- 5) Inhibition of the formation of actinost and distal radials of the pectoral fin, and inhibition of pectoral fin shrinkage (Fig. 4D, F).

Increase in body length, however, was unaffected (Fig. 3A), suggesting little or no effect of thyroid hormone deficiency on the growth of vertebrae. Consequently, there is a differential response to TH among the bones, at least at the early stages of development in the Japanese flounder. Therefore, we have tentatively classified the bones into two types; TH-dependent and TH-independent during early developmental stages.

Predictably, the TH-dependent bones were all related to the functional and morphological changes that occur during metamorphosis. TH-dependent changes were observed in the elongated dorsal fin rays and large pectoral fins, as well as in a tall body height, which helped the larvae to float easily during the pelagic period and are only required in the larval period, disappearing during metamorphosis. One of the TH-dependent bones, the pseudomesial bar (Pb), is an important bone for eye relocation during metamorphosis (Okada et al. 2001). This finding explains the suspension of metamorphosis by TU treatment at specific stages; at stage G (Japanese flounder, in this study) and from prometamorphosis to early climax of metamorphosis in the summer flounder (Schreiber and Specker 1998), the last stages before the drastic relocation of one eye. On the other hand, the growth of vertebrae is TH-independent, at least during the early stages. Although other bones were not compared in detail, most of the bones other than the above-mentioned TH-dependent bones are considered unaffected by TH deficiency at the early stages. In addition, in the summer flounder, the inhibitory effect of TU appeared at 40 dph for metamorphosis, but did not appear before 53 dph for growth (Schreiber and Specker 1998). Ordinary body growth during

early development may be on a “steady state” time scale (degree days, for example), and the metamorphosis is on a time scale determined by an event-specific trigger (other than absolute growth) mediated by THs. The larvae migrate to the near-shore shallow areas to settle passively or actively (Tanaka et al. 1989a), and it is well known that there is size variation in the Japanese flounder on settling (Tanaka et al. 1989b). The possible presence of two time scales, one for growth and one for metamorphosis, may explain, at least partially, size variation at the time of metamorphosis and settlement.

TH is known to activate osteoblasts in teleosts (Takagi et al. 1994) and to accelerate osteoblastic differentiation in mammals (Klaushofer et al. 1995). It is possible that bones or their rudiments are originally only responsive to THs at a specific stage during bone formation, and therefore the bones categorized as TH-independent are just “mature” bones and no longer require THs for growth when TU treatment is given.

In the second experiment, the effect of the timing of T4 supplementation was examined first in TU-induced developmentally inhibited larvae. As shown in Figures 5 and 6, supplementation of T4 from 38 dph induced complete metamorphosis, but supplementation from 67 dph failed to induce pigmentation and the formation of Pb, resulting in juveniles with abnormal coloring and eye location. Therefore, it is clear that there is a critical time point between 38 and 67 dph when the TH responsiveness of the pigment cells and Pb (or their rudiments) expires. On the other hand, the dorsal fin rays or pectoral fin-related bones are considered to maintain responsiveness until 67 dph. Even among tissues that are modified during metamorphosis by THs, each tissue seems to have a different timing of TH responsiveness. Concerning skull development during anuran metamorphosis, T3 responsiveness is expressed earlier in chondrogenesis and later in ossification (Hanken and Summers 1988; Hanken and Hall 1988), indicating a different timing for the commencement of the response among different tissues. The present study showed that bones which are important for morphological changes during flounder metamorphosis require TH, and a specific expiration time of TH responsiveness was suggested for each bone. It is expected that the different expression of thyroid hormone receptors (TRs) in each tissue is the fundamental phenomenon concerning different responsiveness to TH. In the Japanese flounder, TRs have been identified (Yamano et al. 1994; Yamano and Inui 1995) and TR transcripts shown in most of the tissues (Yamano and Miwa 1998). As the definition of metamorphosis is complicated in teleosts (Youson 1988), it may be possible to determine the individual “metamorphic change” from the TH responsiveness of larval fish.

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Development of the immune system and use of immunostimulants in Senegalese sole (*Solea senegalensis*)

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Key words: larval development; immune system; lymphoid cells; histology; immunostimulants; sole

Abstract

The development of the immune system was examined in Senegalese sole (*Solea senegalensis*) during the early life stages and until day 121 after hatching. Haemopoietic cells in the kidney and the appearance of the spleen were first observed on day 6 after hatching, whereas the thymus was first observed on day 9 after hatching. The effect of two immunostimulants, beta-glucane and mannuronic acid polymer, was determined on the survival and growth of Senegalese sole during a rearing period of 121 days. No effect was observed on either parameter during the first feeding period. An outbreak of disease occurred on day 44 after hatching, accompanied by high mortality rates, skin lesions and haemorrhages in internal organs. No significant differences in mortality rates were observed between the different treatments ($p>0.05$).

Introduction

The development of lymphoid tissues has been poorly studied in the early life stages of marine fish species. Senegalese sole is considered as an interesting new species for aquaculture due to its high growth potential and the low mortalities during the first feeding period. The comparatively high rates of survival during first feeding period could be attributed to a relatively accelerated development of the immune system during the early stages. A first target of this study was to examine the

timing of appearance of the lymphoid organs during the early developmental stages. The determination of the timing of immuno-competence of the larvae may also prove important in relation to the use of vaccines. The immune system depends mainly on the non-specific immune defences during the first stages of development (Vadstein 1997). During the rearing of marine fish larvae high mortalities are commonly observed, so substances that may stimulate the non-specific immune defences may be important to improve the larval survival. The activity of cellular immune defence system was enhanced by use of beta-glucane in the feed of salmonids and turbot (Sakai 1999), while mannuronic acid polymer is one of the few immunostimulants tested during the rearing of marine fish larvae (Conceicao et al. 2001). A second target of this study was to determine the effect of two immunostimulants, beta-glucane and mannuronic acid, on the survival and growth of Senegalese sole larvae and juveniles.

Materials and Methods

Sole larvae were reared during the first feeding period in 90 l tanks; tanks contained individual biofilters and about 8000 larvae. Three treatments were used in this experiment and three tanks were included in each treatment. The first treatment (G) received beta-glucane, the second (F) mannuronic acid, whereas the third treatment (C) received no immunostimulants and was used as a control. The immunostimulants were bioencapsulated in live food and added to the fish on day 2, 3 and 4 days after hatching (DAH) via rotifers, and on day 14, 15, and 16 DAH via *Artemia*. Mannuronic acid beads were prepared at the SINTEF Group of Fisheries and Aquaculture, Norway, while beta-glucane was kindly supplied by BIOTEC, ASA, Norway. The immunostimulants were bioencapsulated in the live food during a 30 min incubation in seawater to which had been added immunostimulants at high concentrations. During the bioencapsulation of beta-glucane in *Artemia*, however, efficient bioencapsulation was accomplished after incubation in seawater to which had been added immunostimulants for a 24-hour period. On day 13 after hatching, two groups from each treatment were transferred to 4 l trays. The number of larvae that was transferred to each tray was 300-310 larvae. The larvae were fed *Artemia* until day 40 and artificial diet thereafter.

Samples for histological sections were taken at 0, 3, 6, 9, 12, 13, 16, 19, 22, 25, 28, 31, 36, 39, 44, 49, 59, 74, 89, and 121 days after hatching (DAH) and were fixed in Bouin's fluid (Hopwood 1990). The samples were embedded in paraffin and the sections (about 5 mm) were stained with hematoxylin and eosin.

ANOVA tests were used to compare results from the different treatments. Survival data in percentages were arcsin-transformed, and data on the growth of the larvae were log-transformed before running the statistical tests.

Results and Discussion

No effect, on growth nor on survival was observed during the early larval period. The kidney was first observed 3 DAH, the spleen and the haemopoietic tissue of the kidney 6 DAH, and the thymus 9 DAH (Figure 1).

The first structures of the kidney observed were the renal tubules 3 DAH. However, the haemopoietic tissue of the kidney appeared three days later, on day 6. The kidney developed along the notochord forming two distinct zones, an anterior part (pronephros) and a posterior part (mesonephros). The spleen was observed 6 DAH, in a position adjacent to the gut, in the vicinity of the pancreas and the gut. The distinction between red and white pulp became apparent 28 DAH. The thymus was observed in the base of the fourth branchial arch, in close contact to the opercular cavity epithelium. As the samples were not taken on a daily basis, it is impossible from these results to determine the exact timing of appearance of the lymphoid organs in Senegalese sole. The lymphoid organs appeared in the following sequence: kidney, spleen, and thymus, as it has been earlier observed in the case of other flatfish like turbot (*Scophthalmus maximus*) and Japanese flounder (*Paralichthys olivaceus*) (Chantanachookhin et al. 1991; Padrós and Crespo 1996). On day 121, large melanomacrophage centers and lymphoid tissue surrounding blood vessels were observed. The abundance of melanomacrophage centers in the spleen was more distinct in the beta-glucane treatment added, compared with the other two treatments.

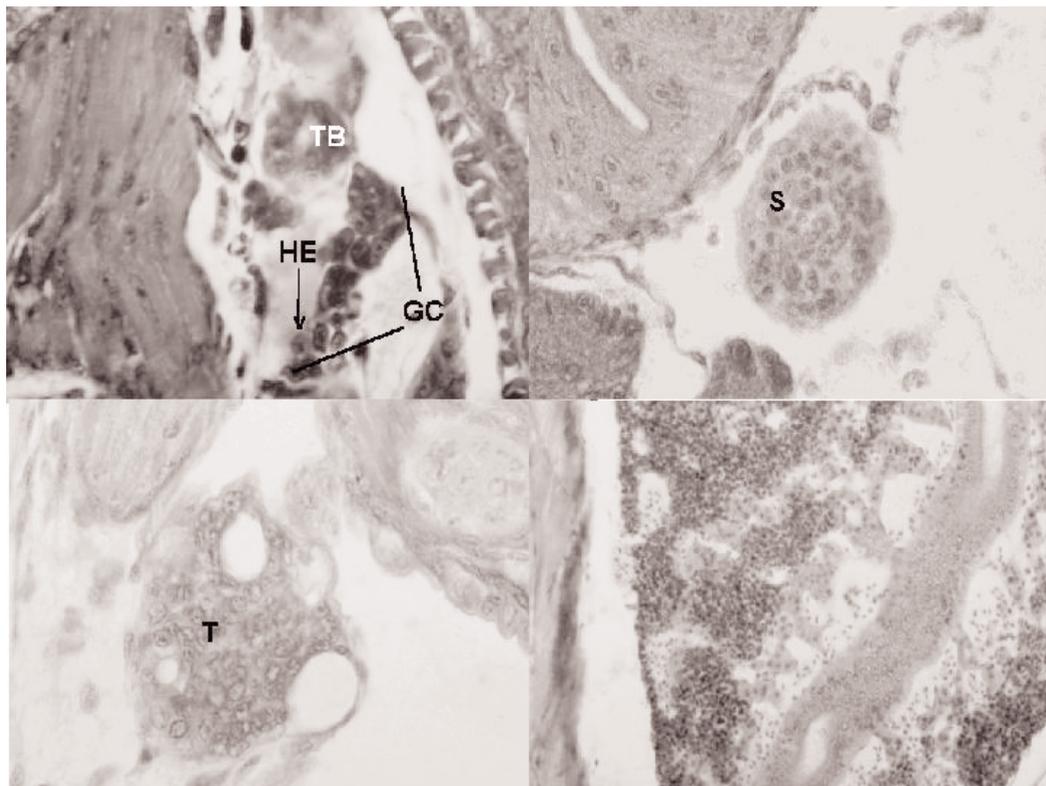


Figure 1. Upper left. Haematopoietic cells in the kidney of a 6-day old larva, HE: Haematopoietic cell, GC: Group of cells, TB: Renal tubules. Lower left. Thymus in a 9-day old larva, T: Thymus. Upper right. Spleen in a 9-day old larva, S: Spleen. Lower right. Hemorrhages in kidney of a 44-day old larva from control treatment. Haematoxylin-Eosin staining and amplification 1000x in all sections.

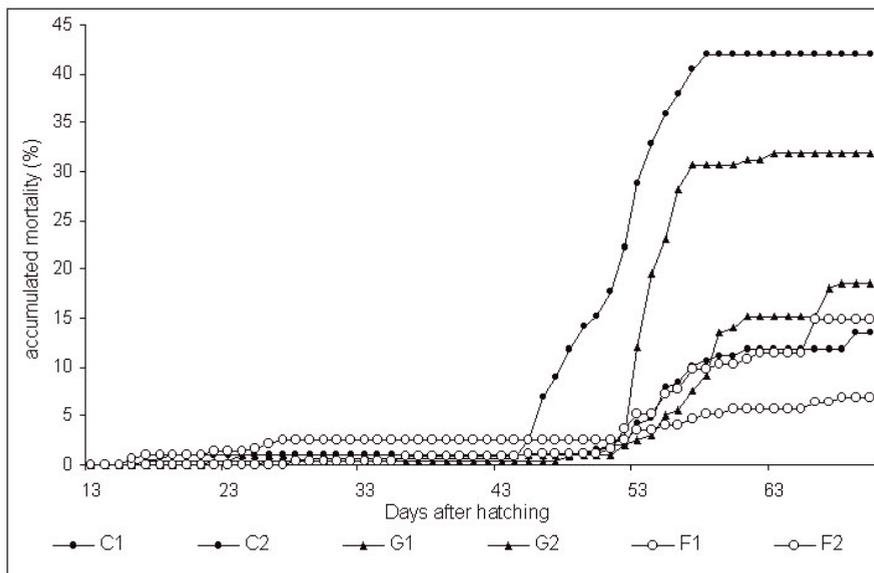


Figure 2. Percentages of accumulated mortality in the three groups of sole (*Solea senegalensis*) between day 13 and 70 after hatching. C1, C2 control replicates, whereas G1, G2, and F1, F2, replicates from beta-glucane, and manuronic acid treatment, respectively.

An accidental outbreak of disease occurred on day 44 after hatching, accompanied by high mortality rates, skin lesions and haemorrhages in internal organs. The differences in mortalities between the treatments were not significant (Figure 2).

The immunostimulants were added for the last time about a month before the outbreak of the disease, so it could be concluded that more frequent addition of immunostimulants is necessary to have a significant effect on the immune system of the postlarvae. Further studies need to be done to determine the effect of immunostimulants when using more frequent addition of immunostimulants.

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Yolk resorption in developing plaice (*Pleuronectes platessa*)

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Key words: *yolk syncytial layer, free amino acids, yolk proteins, ammonia*

Abstract

Embryonic and yolk-sac larval development of plaice (*Pleuronectes platessa*; 7 °C) were investigated in order to correlate physiological and anatomical events during the resorption of the endogenous yolk. Emphasis was placed on nitrogenous metabolites (proteins, free amino acids [FAA] and ammonium ions [NH₄⁺]) and the yolk syncytial layer (YSL). Four distinct developmental phases were found in relation to yolk resorption: The first phase, from fertilization to gastrulation and YSL formation, was characterized by constant levels of FAA, protein, NH₄⁺, and P_i. The second phase, lasting from gastrulation to hatch, was characterized by a rapid decrease in FAA, an accumulation of NH₄⁺, and an increase in egg protein, while the liver developed as a compact unvascularized anlage. During this phase, there was low pinocytotic activity in the inner YSL membrane and the splanchnic lateral plate mesoderm covered only the dorsal part of the yolk. During these first two phases the egg wet mass, dry mass and hence the relative water content did not show significant variation. After hatch, entering the third phase, the larvae showed a rapid decrease in yolk volume, NH₄⁺, and FAA pool concomitantly with a continued increase in body length and muscle growth. The YSL now showed pinocytotic activity and contained high amounts of rough endoplasmic reticulum. During this phase, the splanchnic mesoderm covered the dorsal and lateral parts of the yolk, the mouth opened, the liver became vascularized, and the digestive tract remained as a straight tube. The fourth phase commenced when maximum larval length and body mass were attained on day 24-26 post fertilization. The FAA and NH₄⁺ remained low, and body protein, wet mass, dry mass, and relative water content decreased. Organogenesis advanced even with the lack of exogenous food. Electrophoresis of dissected yolk from lyophilized larvae revealed that a high molecular weight protein (95 kD) was degraded during the second pre-hatch phase concomitant with a temporal increase of protein bands of lower molecular weights stretching into the third post-hatch phase. The study suggests an instrumental role of the YSL in the developing plaice for the sequential uptake of yolk amino acids, first from the free pool, then from the yolk proteins.

Introduction

The majority of marine teleosts are oviparous and reproduce via external fertilization of post-ovulatory broadcast eggs. Once ovulated, such eggs must contain all necessary components and factors required to initiate and maintain metabolism and development prior to exogenous feeding. In terms of quantity, it is the yolk that comprises by far the greatest fraction of the egg at spawning, and it is thus the yolk that will sustain the embryo and larva until it has developed the functional ability to feed upon exogenous prey.

Earlier studies of yolk resorption in teleosts examined either the physiology or anatomy of the developing embryos. For example nitrogenous metabolites present in the yolk, particularly free amino acids (FAA) and proteins have been shown to be sequentially utilized during development (Finn et al. 1995a; b; 1996; Sivaloganathan et al. 1998; Rønnestad et al. 1999; Thomsen 2000; Wright and Fyhn 2001; Hiramatsu et al. 2002a; b; Ohkubo and Matsubara 2002), while anatomical studies have suggested that endocytosis followed by intracellular digestion in the yolk syncytial layer (YSL) was the major mechanism by which yolk was resorbed (Walzer and Schönenberger 1979a; b; Heming and Buddington 1988; Trinkaus 1992; 1993). The YSL is an extra-embryonic structure that forms when marginal blastomeres collapse into the yolk cell during the mid-blastula transition (Kimmel and Law 1985; Sakaguchi et al. 2002) and it is a major signalling layer for the induction of mesoderm and endoderm (Kimmel and Schier 2002). All nutrients from the yolk must pass through the YSL to reach the embryo. Few studies have examined yolk substrate utilization in relation to the development of the YSL and the functional anatomy of marine fish embryos. Furthermore, apart from the studies of Morrison (1993) and Poupard et al. (2000) there is an almost complete lack of anatomical studies of the yolk resorption of teleosts that spawn pelagic eggs.

We therefore conducted this study of yolk resorption in a species of marine teleost that spawns pelagic eggs with emphasis on nitrogenous metabolites (proteins, FAA, and NH_4^+) as physiological indicators of yolk resorption. These metabolites are related to the anatomical status, in particular the YSL, as determined by light and electron microscopy during embryonic and yolk-sac larval development.

Materials and methods

Mature specimens of male and female plaice (*Pleuronectes platessa*) were caught by gill netting on the spawning grounds near Bergen, Norway, in March 2000. Mature eggs were stripped from single females and fertilized with milt from single males. Two series of fertilized eggs were obtained from different females (A: 1800g; B: 550g). Eggs were incubated in an aquarium ($7^\circ\text{C} \pm 0.5^\circ\text{C}$) with a seawater salinity of 34.5ppt in a cold room, close to its optimal temperature. About three-quarters of the seawater was exchanged every second day and the yolk-sac larvae did not receive any external feed during the experiment. Samples of egg and larval material were collected every second day from fertilization to 28 days post fertilization (DPF). The stages of development were observed on live eggs and larvae.

Biometry. Thirty eggs were measured for egg diameter and yolk length (rostral-caudal) and height (dorso-ventral) from fertilization until hatching. After hatching 20 larvae were measured according to standard length, total length, muscle height, as well as yolk height and length. Measurements were conducted using a dissecting scope with a calibrated ocular micrometer. Egg and yolk volumes were calculated from the measured diameters using the formula of an ellipsoid, assuming that the yolk length and height represented the major and minor axes, respectively.

Sampling for physiological studies. Four replicates of 10 pooled eggs and 4 replicates of 3 pooled larvae were collected every second day from fertilization until the end of the experiments. The eggs and larvae were lightly blotted on filter paper and placed in pre-weighed Eppendorf tubes. Wet mass was determined on a Sartorius top balance (accuracy ± 0.1 mg). In addition, samples of 15–30 larvae were placed on Teflon weigh boats for lyophilization and subsequent separation of yolk from somatic tissues by dissection. All samples were collected in a cold room (6–8 °C) and stored at -80 °C until further analyses. Dry mass was determined, after lyophilization for 48 h, by weighing on a Cahn Model 25 Automatic Electrobalance (accuracy ± 1 μ g).

Analytical procedures. Samples for the biochemical analyses were extracted in 6% trichloroacetic acid (TCA) under rotation for 48 h in a cold room (6–8 °C). Extracted samples were centrifuged (10 min, 4 °C, 10 000 g) and the supernatant used for quantitation of FAA (series A and B), NH_4^+ and inorganic phosphate (P_i , series B only). Analyses of FAA, NH_4^+ , and P_i were performed as described by Finn et al. (2002a). The precipitate was washed once with 6% TCA, before being solubilised in 1 M NaOH, and analyzed for total protein content (series A and B) as described by Finn et al. (2002b). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for visualisation and estimation of the molecular weight of yolk proteins, in accordance with the principles of Rosenberg. Lyophilized whole eggs and dissected yolk from larvae were homogenized directly in reduced loading buffer (2.5% β -mercapto-ethanol, 0.0625M Tris, 10% glycerol, 2% SDS, 0.01% bromophenol blue, pH 6.8) to give an approximate soluble protein concentration of 1 μ g/ μ L. The samples were boiled for 5 min. and applied to duplicate 7.5% T (total acrylamide and bis-acrylamide concentration), 3.3% C (percentage of bis-acrylamide of the total) homogeneous acrylamide/bis-acrylamide separating gels (0.75mm) using the Tris-Tricine buffer system of Schagger and von Jagow (1987). The gel buffer consisted of 3M Tris-HCl with a pH of 8.45. The stacking gel consisted of 4% T, and 3.3% C acrylamide/bis-acrylamide. Samples were electrophoresed in a Bio-Rad Protean II cell at 95 V for 80 min, and the protein bands were visualized with Coomassie brilliant blue G-250. For estimation of the apparent molecular weight of the visualized protein bands, Bio-Rad precision pre-stained markers at 250, 150, 100, 75, 50, 37, 25, 15, and 10 kD (Cat # 161-0372) were applied to both sides of the gel. The gels were scanned with an Epson GT-9600 scanner using transmitted light, and the relative mobility of the standards and yolk proteins measured digitally at 160x magnification.

Microscopy. Histological materials for light microscopy (LM) and transmission electron microscopy (TEM) were prepared every second day between day 7 and day 28 post fertilization.

Six eggs or 6 larvae (series A only) were fixed in a teleost fixative (Totland et al. 1996) and stored at 4°C until further preparation. The chorion of each egg was sliced open using two razorblades to ensure proper infiltration. For LM studies, one sample from each day was dehydrated through a graded ethanol series and prepared using Technovit 7100 according to the procedures of Kulzer Histo- Technik, Germany. Saggital sections (transverse sections for 7 and 9 DPF) of eggs and larvae were obtained by making semi-thin serial sections (1 μm) on a microtome (Leica, model # RM 2155) and stained in toluidine blue as described by Philpott (1966). For TEM studies, one sample from each day was investigated. The embryo (eggs without chorion) and the larvae were cut in three parts and the part containing the liver and yolk-sac was prepared for further examination. Samples were prepared and stained according to Grotmol et al. (1997) and observed in a JEOL 100S transmission electron microscope.

Statistical treatment. Statistical analyses were performed using data analysis in Microsoft Excel (version 97). Student's *t*-test was used for comparing two independent samples assuming equal variance. Differences were considered to be significant at the 5% level.

Results

Biometric and gravimetric measurements. Egg volumes did not change significantly between fertilization and hatching. The yolk volume, however, declined in a sigmoidal fashion (Fig. 1). During the egg stage there was low utilization of the yolk reserves. Hatching occurred over an extended period in both series between day 13.5 and 17.5 PF. After hatch, rates of yolk resorption increased, and final yolk resorption occurred on 28 DPF (series A, Fig. 1) and 21 DPF (series B, data not shown). The muscle height and standard length of the body were only measured after hatching (Fig. 1). The larval body increased rapidly in length until maximum values were attained on 24 DPF for both series. During subsequent development, however, standard length significantly decreased for series B (*t*-test; $p < 0.01$), while for series A the decrease was borderline significant ($p = 0.056$). Muscle height increased to about 21 DPF for both series ($p < 0.0001$), but thereafter, it stabilized for series A, while series B showed a significant decrease towards the end of experiment ($p < 0.0001$).

Egg wet mass was stable around 3.7 $\text{mg} \cdot \text{ind}^{-1}$ in series A (Fig. 2) and around 2.2 $\text{mg} \cdot \text{ind}^{-1}$ in series B (data not shown). Due to the loss of chorion and perivitelline fluids at hatch, there was a significant reduction in wet mass of about 24 – 38% in series A, and 40% in series B. The lower drop in wet mass during the early hatching period for series A was due to the high wet mass of the hatched larvae on 13.5 DPF. Once hatch was completed at 17.5 DPF, the wet mass of both series decreased steadily until the end of the experiments.

Egg dry mass of both series was maintained at an approximately constant level until the time of hatching (Series A: $319 \pm 14 \mu\text{g} \cdot \text{ind}^{-1}$ [Fig. 2] and series B: $178 \pm 7 \mu\text{g} \cdot \text{ind}^{-1}$). At hatching there was a significant decrease of about 40–45% of dry mass due to the loss of chorion and perivitelline colloids. The larvae of both series showed a slow but progressive decrease in the dry mass throughout development. The total reduction in dry mass was low compared to wet mass reduction. Only 4%

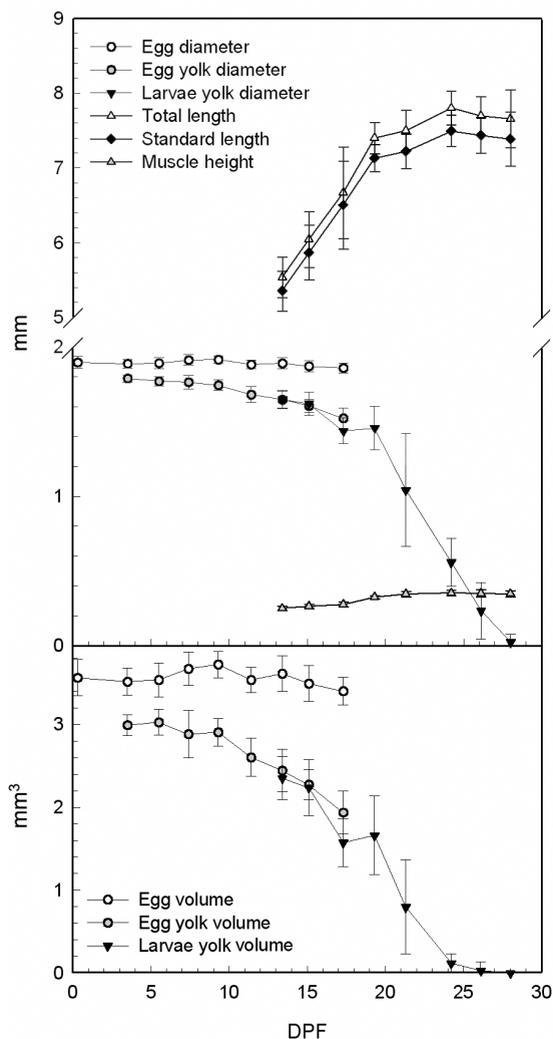


Figure 1. Metric (mm) and volumetric (mm³) measurements of plaice (*Pleuronectes platessa*, series A) eggs and yolk-sac larvae. Development is given as days post fertilization (DPF). The hatching period was from 13.5 to 17.5 DPF. The data points are given as mean \pm SD, $N = 30$ for eggs and $N = 20$ for larvae. The yolk diameter is the mean value of major and minor diameter. Both eggs and larvae were measured during the hatching period, but length measurements only on hatched larvae.

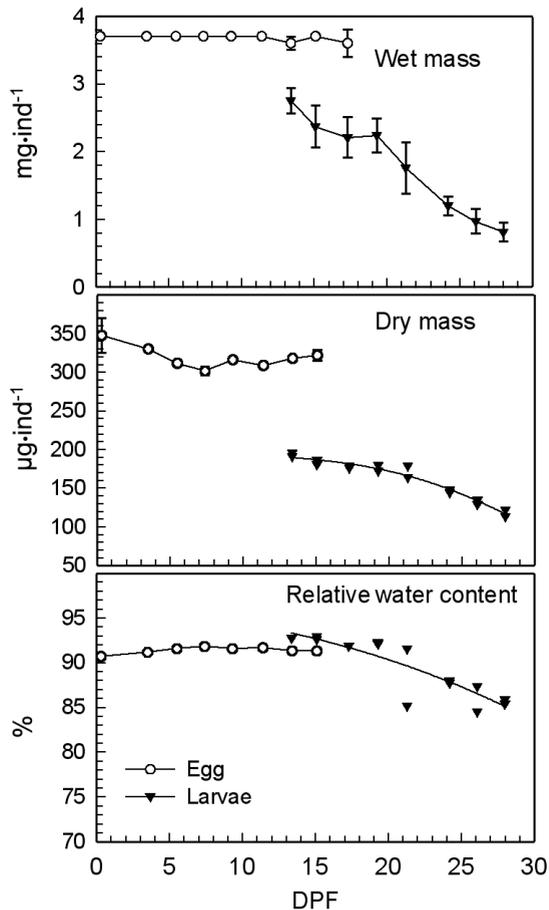


Figure 2. Individual wet mass (mg·ind⁻¹), dry mass (μ g·ind⁻¹) and relative water content (%) of developing eggs and yolk-sac larvae of plaice (*Pleuronectes platessa*, series A). Development is given as days post fertilization (DPF). The hatching period was from 13.5 to 17.5 DPF. The data points are given as mean \pm SD, for wet mass, $N = 8$ groups of 10 pooled eggs and 4 groups of 3 pooled larvae. For dry mass and water content, $N = 4$ groups of 10 pooled eggs and individual values of 2 groups of 3 pooled larvae. The curve of larvae data points is smoothed using a SigmaPlot sigmoid regression line.

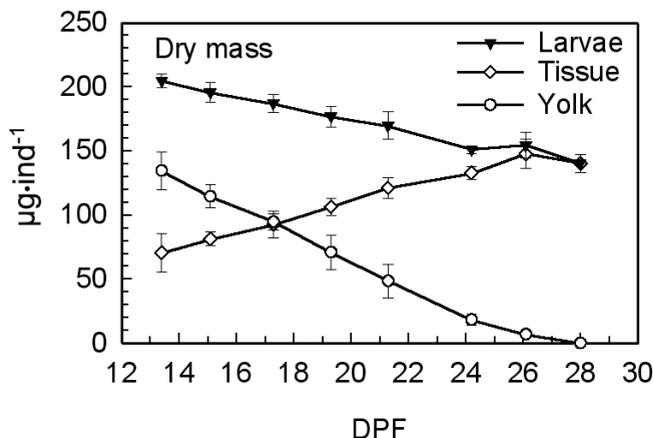


Figure 3. Dry mass ($\mu\text{g}\cdot\text{ind}^{-1}$) of whole larvae and dissected body and yolk compartments from lyophilized larvae of plaice (*Pleuronectes platessa*, series A). Development is given as days post fertilization (DPF). The data are given as mean \pm SD, $N = 6$.

(series B: 3%) of the drop in wet mass in series A was due to dry mass reduction, the remaining 96% (series B: 97%) was due to water loss.

The eggs of both series showed a stable relative water content (mean 91.6 (0.4%) from fertilization until hatching (Fig. 2). The newly hatched larvae seemed to maintain this level of hydration but, during post-hatch development, there was a steady and significant ($p < 0.05$) decrease in the relative water content from about 92% to about 86%.

Dissection of the yolk from the somatic tissues revealed that the body constituted about 35% of whole larval dry mass at early hatching (13.5 DPF) but increased with development so that on 17 DPF the yolk and the body made up equal amounts of dry mass (Fig. 3). Maximum tissue mass occurred on 26 DPF. For hatched larvae between 13 and 26 DPF the amount of yolk dry mass conserved to form larval body dry mass was 61%.

Free amino acids (FAA). The total content of FAA showed a sigmoidal pattern of decrease during development until 24 DPF both in series A (Fig. 4) and series B (data not shown). There were no significant changes in the FAA content of either series prior to 4 DPF. Commencing after 5 DPF, both the non-essential (NEAA) and essential (EAA) amino acids decreased with development (Fig. 5). The most abundant NEAA in the eggs were serine, alanine, glutamine, glutamic acid, and glycine, while leucine, valine, and isoleucine were the most dominant among the EAA. There was no sparing of EAA versus NEAA. Following hatch, the larvae had low or decreasing levels of each FAA, with the exception of taurine, which was maintained at a stable level.

Total protein. Eggs and larvae of the two experimental series had different total protein contents ($123.4 \pm 12.6 \mu\text{g}\cdot\text{ind}^{-1}$: series A [Fig. 4]; $104.2 \pm 6.9 \mu\text{g}\cdot\text{ind}^{-1}$: series B) reflecting their difference in size. Both series, however, showed similar trends of increasing protein content towards hatch. After hatch the yolk-sac larval protein content remained stable until 21 DPF (series A) and 24 DPF (series B), but declined significantly thereafter.

Ions (NH_4^+ and P_i). No significant change in the NH_4^+ content was observed until 4 DPF, but thereafter and until hatch, there was a progressive accumulation (Fig. 4). The amount increased steadily from 64 to 102 $\text{nmol}\cdot\text{ind}^{-1}$. At hatch the NH_4^+ content decreased rapidly until it stabilized at a low level of about 4 $\text{nmol}\cdot\text{ind}^{-1}$ from 20 DPF until the end of the experiments. In contrast to NH_4^+ , the P_i content decreased from 21.5 to 2.2 $\text{nmol}\cdot\text{ind}^{-1}$ during the embryonic phase of development (Fig. 4). It remained stable at levels of 6–11 $\text{nmol}\cdot\text{ind}^{-1}$ in the larvae. Compared to the egg, hatched yolk-sac larvae had significantly ($p < 0.001$) higher P_i contents at the same developmental age.

SDS- PAGE gel electrophoresis. The electrophoretic profiles of protein homogenates from whole eggs and dissected yolks from the developing larvae revealed three major bands in the newly fertilized eggs: a high molecular weight (Mw) protein band of 95 kD and two low Mw protein bands of 22 and 15 kD (Fig. 6). During the egg stage, the high Mw protein band became faint and finally disappeared prior to hatch, and a new band of 66 kD appeared while the intensity of the 22 kD increased. Throughout the larval stages, the 66, 22, and 15 kD bands disappeared, while intermediate bands at 45 and 17 kD became apparent at 19 DPF. At 26 DPF, there was only 6 μg yolk left (Fig. 3) and all the protein bands were weak.

Histological studies. The lateral plate splanchnic mesoderm, which forms the inner wall of the coelom, was observed as a simple squamous epithelium over the dorsal part of the YSL at 7 and 9 DPF (data not shown). By 15 DPF, in the caudal and lateral area of the yolk-sac, the splanchnic mesoderm epibolized ven-

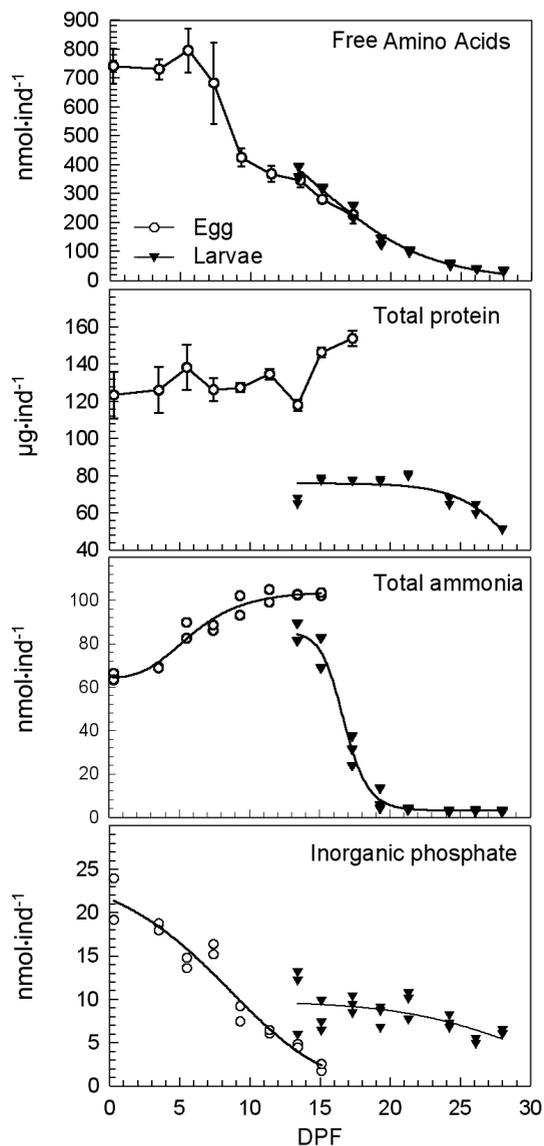


Figure 4. Free amino acids (FAA, series A), total protein (series A), ammonium ions (NH_4^+ , series B) and inorganic phosphate (P_i , series B) of plaice (*Pleuronectes platessa*) eggs and yolk-sac larvae. Development is given as days post fertilization (DPF). The hatching period was from 13.5 to 17.5 DPF. The data points of FAA and total protein are given as mean \pm SD, $N = 4$ groups of 10 eggs and as individual values where $N < 4$ for larvae. The curve of individual data points is smoothed using a SigmaPlot sigmoid regression line.

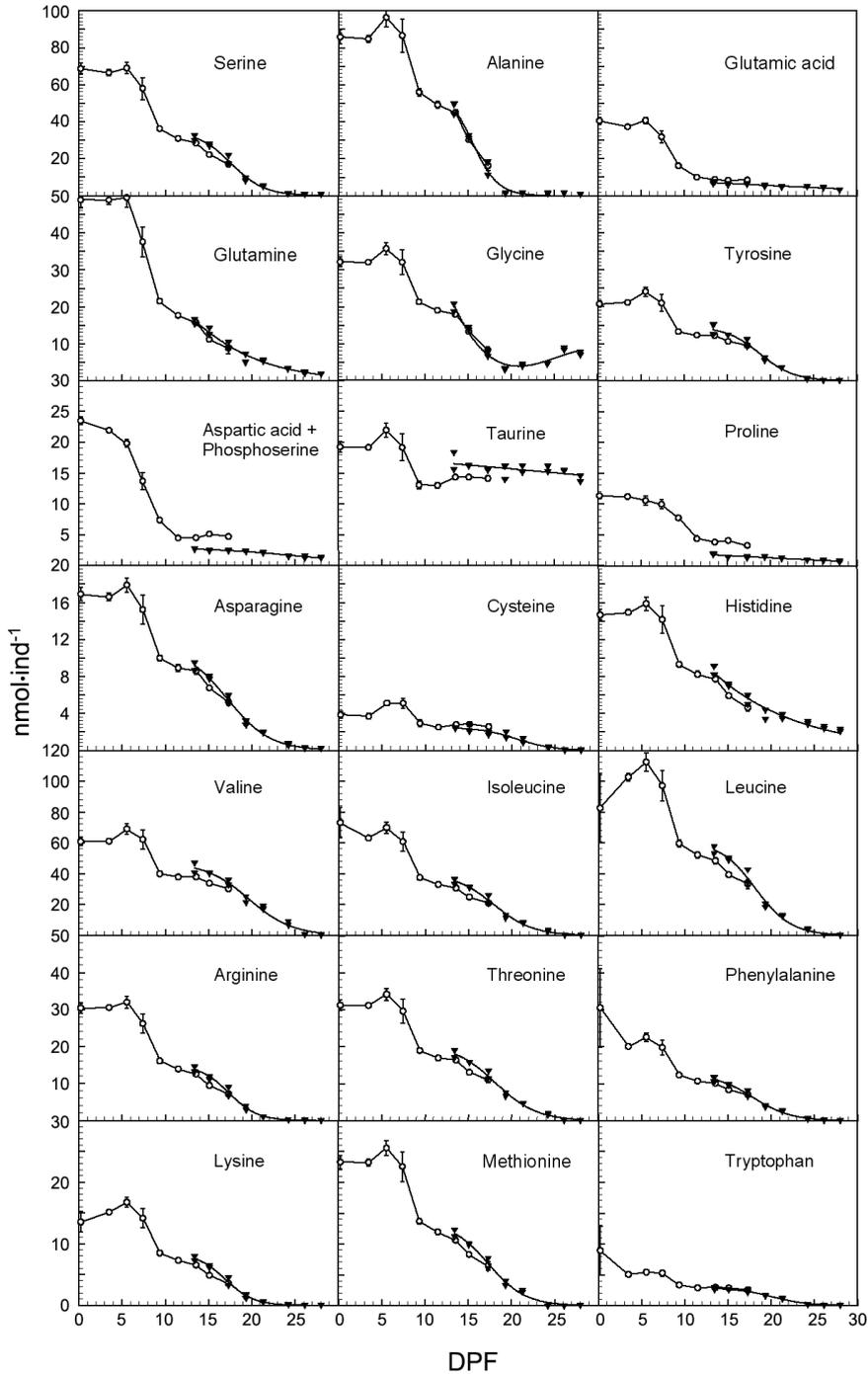


Figure 5. Non-essential (NEAA; alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, phosphoserine, proline, serine, taurine, and tyrosine) and essential (EAA; arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) free amino acids (FAA) of developing eggs and yolk-sac larvae of plaice (*Pleuronectes platessa*, series A). Development is given as days post fertilization (DPF). The hatching period was from 13.5 to 17.5 DPF. The data points are given as mean \pm SD, $N = 4$ groups of 10 pooled eggs and individual values of 2 groups of 3 pooled larvae. The curves fitted to the data for larvae are smoothed using a SigmaPlot sigmoid regression line for all FAA except glycine, which was smoothed using a SigmaPlot polynomial cubic regression line.

trally without contacting the epidermis, and gradually encircled the yolk as development proceeded (Fig. 7A, B). At 20 DPF, the splanchnic mesoderm extended cranially to the somatic mesoderm, which underlied the epidermis and constituted the outer wall of the coelom (Fig. 7C). At this stage, the splanchnic mesoderm was observed in close contact with the YSL. By the end of the yolk stage, the splanchnic mesoderm covered the entire surface of the YSL.

The earliest sections taken (7 DPF) revealed that the YSL covered the whole yolk compartment. The dorso-lateral part of the YSL was electron dense with abundant endoplasmic reticulum (ER), free ribosomes, and small (0.5-1 μ m) mitochondria (Fig. 8A). Nuclei were rare, and mitochondria had only a few cristae. The dorsal part of YSL had large and slender nuclei (data not shown), surrounded by a network of rough ER (rER). A few cell extensions of the outer YSL membrane were observed at 7 DPF.

At 15 DPF, the YSL of newly hatched larvae had a homogeneously developed cytoplasmic content surrounding the yolk (Fig. 8B). The YSL consisted of large cell nuclei (data not shown), which were located in the middle of the syncytium, surrounded by rER and mitochondria, while thin peripheral zones were largely devoid of organelles. At this stage, the YSL had different cell extensions on the inner and outer membranes. The inner membrane extensions, termed blebs (Morrison 1993), were spherical with a narrow connection to the YSL. The outer YSL membrane had areas with numerous long extensions, while other areas were rather smooth. Small vesicles were seen underneath the inner membrane of the YSL. Most of the YSL was filled with rER and mitochondria by 19 DPF during the phase of maximal yolk resorption. At this stage, numerous deep invaginations were observed in the inner membrane of the YSL (data not shown).

At 26 DPF no yolk could be seen in the YSL of the TEM slices examined (Fig. 8C). The mesodermal membrane surrounding the YSL was highly folded and the YSL had numerous long cell extensions (data not shown). The cell extensions were in close contact with the mesodermal membranes.

The digestive tract initially extended as a straight tube from the stomodeum and terminated posterior to the yolk-sac. On 15 DPF, the mouth of hatched larvae was open. Prior to hatch, the intestinal wall constituted a single layer of simple columnar epithelium cells at 9 DPF, and differentiated into a multicellular layer with microvilli by 15 DPF. At 26 DPF, the intestine had looped in a cranial direction. The hepatic diverticulum extended from the foregut into the surrounding mesenchyme and was observed at 11 DPF. The liver was first evident as a compact organ, but became infiltrated with sinuses on 19 DPF (Fig. 7C).

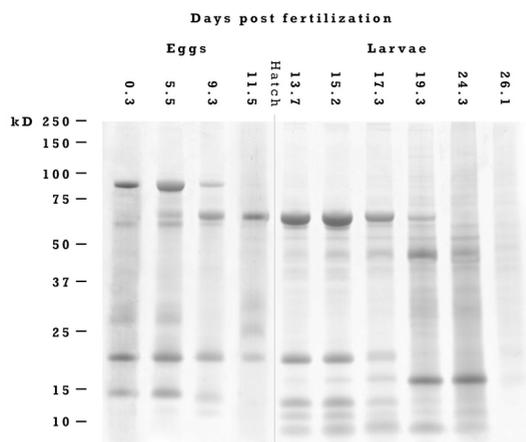


Figure 6. Coomassie Blue G-250 stained SDS-PAGE gels (7.5% homogenous) of yolk proteins from lyophilized whole eggs and dissected yolks from larvae of plaice (*Pleuronectes platessa*, series A). Development is given as days post fertilization (DPF). Molecular weight standards (kiloDaltons [kD]) are shown at the left.

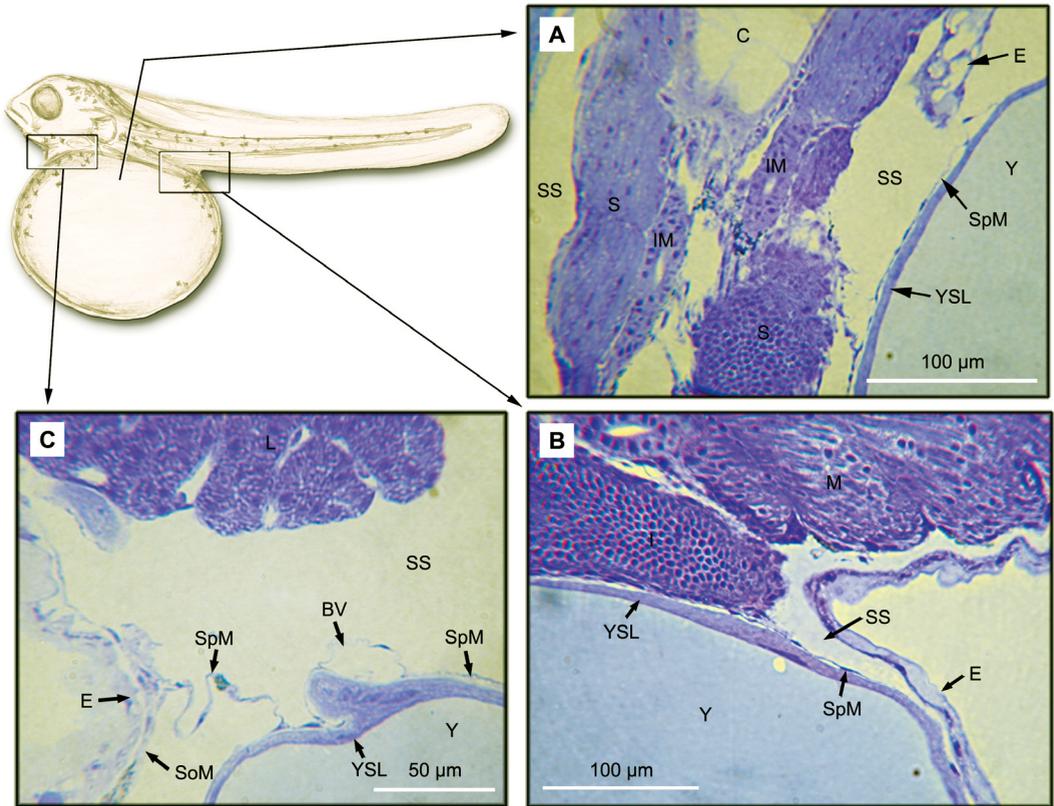


Figure 7. Histological sections stained with toluidine blue showing the fate of the splanchnic mesoderm and yolk syncytial layer of yolk-sac larvae of plaice (*Pleuronectes platessa*, series A). **A:** Transverse section of an embryo (15 DPF). The splanchnic mesoderm covers the dorsal part of the yolk syncytial layer and the yolk. Note that the splanchnic mesoderm ended lateral to the embryonic tissue, and does not continue around the entire yolk-sac. **B:** Saggital section of a larva (15 DPF, days post fertilization). The splanchnic mesoderm covers the dorsal part of the yolk syncytial layer and the yolk, but ended caudal as a loose cell layer close to the anus. **C:** Saggital section of anterior region of a larva (20 DPF). Splanchnic mesoderm attached to the somatic mesoderm which underlies the epidermis in the cranial area of the yolk-sac. Note the blood vessel evident in the splanchnic mesoderm. BV: blood vessels, C: Chorda, E: epidermis, IM: intermediate mesoderm, I: intestine, L: liver, M: muscles, SoM: somatic mesoderm, S: somite, SpM: splanchnic mesoderm, SS: subdermal space, Y: yolk, YSL: yolk syncytial layer. This figure is reprinted as a colour plate in the book's appendix.

Stages of development. The development stages of plaice from fertilization until the end of the yolk-sac period are given in Fig 9. At 7°C, the cleavage period was completed 2 DPF as the enveloping layer of the blastodisc started to epibolize over the yolk compartment. Gastrulation was considered terminated when the blastopore closed on 5 DPF. At 6 DPF the eyes became pigmented. On 9 DPF, the heart started to beat, melanocytes were observed in the skin, and trunk muscle contractions occurred. While still encapsulated as an egg, the embryo was protected by the

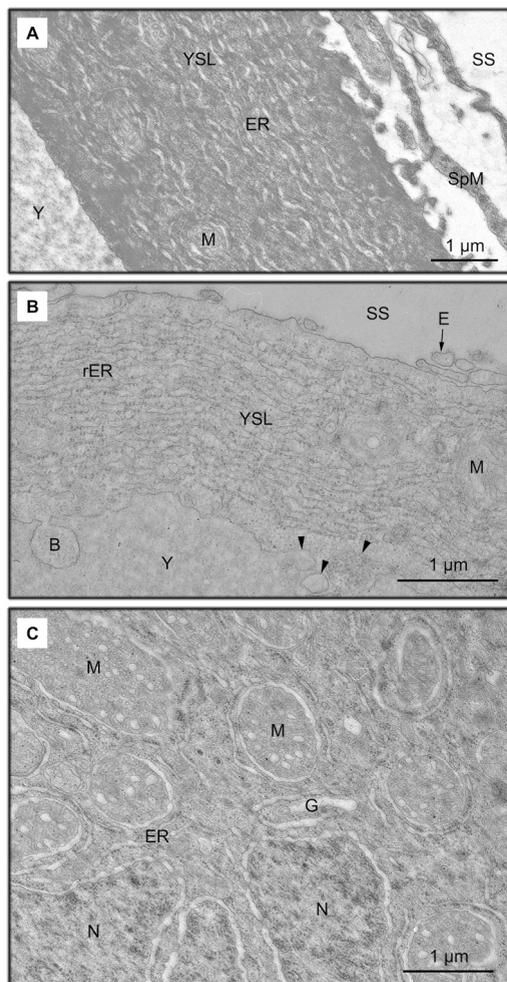


Figure 8. Electron-microscopic observation, of the yolk syncytial layer (stained with uranyl acetate and lead citrate) during the yolk mass resorption of developing embryos and yolk-sac larvae of plaice (*Pleuronectes platessa*, series A). **A:** The dorso-lateral part of the yolk syncytial layer surrounding the yolk containing endoplasmic reticulum, and early stages of mitochondria (7 DPF, days post fertilization). **B:** The yolk syncytial layer containing rough endoplasmic reticulum and mitochondria (15 DPF). Blebs are observed as protrusions from the inner yolk syncytial layer membrane facing the homogenous yolk and cell extensions are observed at the outer membrane entering the subdermal space. Pinocytotic vesicles (arrow-heads) are evident underneath the inner membrane. **C:** The remains of the yolk syncytial layer (26 DPF). Numerous mitochondria are observed, the nuclei are smaller and darker than earlier stages, and remains of endoplasmic reticulum and Golgi apparatus can still be seen. **B:** bleb, **E:** cell extension, **ER:** endoplasmic reticulum, **G:** Golgi apparatus, **M:** mitochondrion, **N:** nucleus, **rER:** rough endoplasmic reticulum, **SpM:** splanchnic mesoderm, **SS:** subdermal space, **Y:** yolk, **YSL:** yolk syncytial layer.

chorion, such that the epidermis was exposed to the perivitelline fluids.

When the embryo hatched (13.5–17.5 DPF), it entered the free swimming yolk-sac stage. The larvae initially swam with the yolk upward until the yolk had been reduced to about 65% of its original volume (Fig. 1). Approximately 20 and 25% of the yolk was consumed prior to

hatch in series A and B, respectively, while the remaining yolk was consumed after hatch when the larvae became more active. Despite the size difference in the two egg groups, the pattern and rate of yolk resorption was similar for both series, although the larvae from the smaller eggs (Series B) consumed their yolk reserves quicker than larvae from the larger eggs (Series A).

Discussion

Development and growth. Heming and Buddington (1988) and Mani-Ponset et al. (1996) have proposed three phases of yolk resorption in marine teleosts. However in the present study it is suggested that in fact four phases exist (Fig. 9). As the rate of resorption increases after epiboly and YSL formation, the endotrophic pre-hatch phase was divided into two separate phases. The first pre-

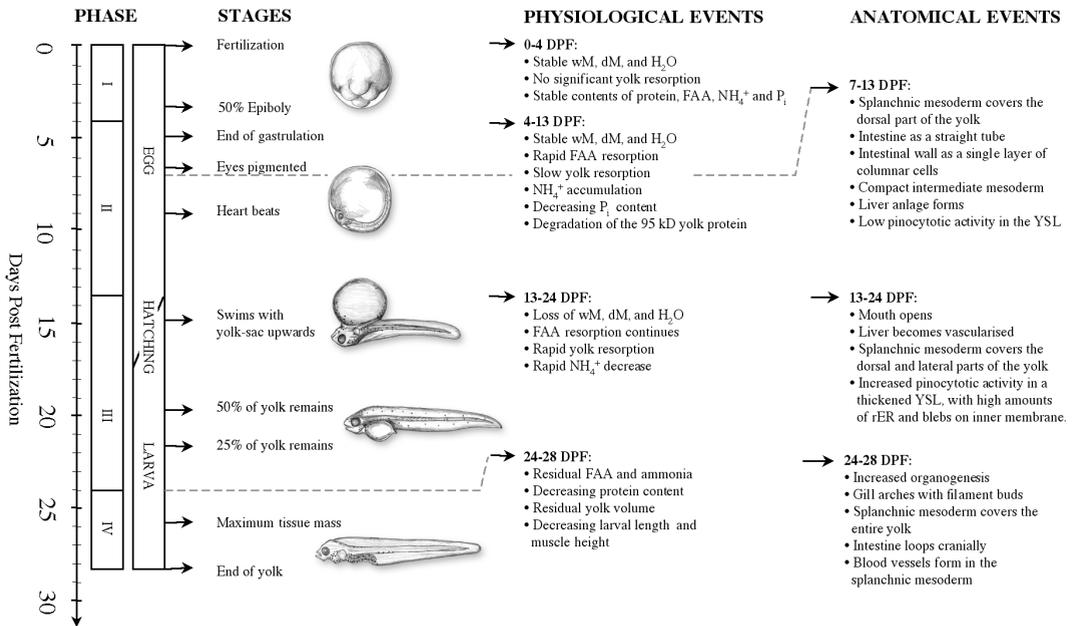


Figure 9. Summary of the physiological and anatomical events during the yolk resorption of developing plaice (*Pleuronectes platessa*). The hatching period was from 13.5 to 17.5 DPF, and the post-hatch stages refer to the early hatched larvae of series A. Drawings of the plaice embryos and larvae were made from live samples and are not drawn to scale. DPF: days post fertilization, wM: wet mass, dM: dry mass, H₂O: relative water content, FAA: free amino acids, rER: rough endoplasmic reticulum, YSL: yolk syncytial layer.

hatch phase, lasting from fertilization to gastrulation, was characterized by insignificant yolk consumption. This was due partly to a non-functional YSL as noted by for *Fundulus* and partly due to low metabolic activity as previously shown for plaice (De Silva and Tytler 1973) and other pelagic marine fish embryos (Rønnestad et al. 1992a, b; Finn et al. 1991 1995a, c; 1996; Sivaloganathan et al. 1998). During this phase, neither the FAA nor the NH₄⁺ ion levels were seen to change for plaice. It has been argued for turbot that cytosolic glycogen is the only fuel during this phase. The current data support this notion. The second pre-hatch phase started after the completion of the YSL during epiboly and lasted until hatching. The yolk volume started to reduce rather slowly, while the FAA consumption showed a more rapid decrease. However, contrary to expectation (Heming and Buddington 1988), the YSL showed a low pinocytotic activity. After hatch, the third phase of yolk resorption was characterised by a rapid resorption of yolk. This phase was correlated to a clear pinocytotic activity and a high translational activity seen in the YSL as evidenced from the great volumes of rER found in the present study. The mouth opened approximately two days after hatch, and, under natural conditions, the yolk-sac larvae would enter a mixed endo-exotrophic period. The terminal fourth phase commenced when maximum larval length and body mass were attained on 24 – 26 DPF. The yolk reserves reached their finite levels and the rate of con-

sumption gradually decreased. This latter phase marked the end of the mixed endo-exotrophic period when the larvae would normally rely solely on exotrophic nutrients.

Although the egg diameter was quite different between the two series, both series were in accordance with earlier reports of the size range of plaice eggs: 1.66 – 2.17 mm (Russel 1976; Rainuzzo et al. 1993; Thorsen et al. 1993). When normalised with respect to dry mass, however, the larvae had the same relative protein contents of 33 – 35%, and similar egg FAA contents of 21 – 27%. Further, the relative water contents were not significantly different with a mean of $91.6 \pm 0.4\%$. Thus, despite the size difference of the eggs, the pattern and timing of yolk resorption was very similar for the two series. During the embryonic phases of development, the stable wet and dry masses indicated that there was a good transfer of yolk directly to the body and hence little energy lost due to metabolism. An estimate of the yolk conservation during the embryonic phases can be made by subtracting the difference between dry masses of the egg and newly hatched larvae (i.e. chorion and colloids) from the dry mass of the egg near fertilization. Such a calculation will provide a reasonable estimate of the yolk mass, assuming that the mass of the germinal cytoplasm was negligible. Using this approach, the conservation of yolk between fertilization and hatch (13.5 DPF) was 79%, while the conservation of yolk from hatch to maximum tissue mass was 61%. The lower yolk conservation of the larvae was likely due to more energy being dissipated by swimming than the embryos. Hence, in terms of dry mass transfer, the transformation of yolk substance to body tissue, was 68% during development. This value is in good agreement with the general conservation of yolk reported for other marine species with pelagic eggs (Ronnestad et al. 1993; Finn 1994).

Resorption of yolk across the yolk syncytial layer (YSL). The YSL surrounding the yolk of plaice had a uniform thickness throughout development. This is contrary to observations on turbot (*Scophthalmus maximus*), where there was a thickening at the anterior and posterior part of the yolk compartment, while the YSL in front of the liver and the intestine remained thin. Similarly, the thickness of the YSL of sea bream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*), and pike perch (*Stizostedion lucioperca*) has been shown to vary around the yolk (Mani-Ponset et al. 1996). For plaice, only the width of the YSL seemed to change without altering its uniformity as the yolk volume decreased. This has also been noted for trout (*Salmo fario trutta* L.). At 7 DPF, the YSL covered the whole yolk in the present material. The short cell extensions of the YSL outer membrane at 7 DPF correlate with the slow consumption of yolk. The dorsal section seemed to be more developed, possibly due to earlier settlement of the nuclei at the dorsal area of the YSL. The nuclei are suggested to enter the yolk periphery when marginal blastomeres collapse during the mid-blastula phase, and their mitotic divisions are arrested prior to epiboly (Trinkaus 1993; Kimmel and Law 1985; Kimmel et al. 1995; Sakaguchi et al. 2002). Similar to other species of marine teleost (Mani-Ponset et al. 1994; 1996; Poupard et al. 2000), cytoplasmic organelles filled the YSL of the plaice in the present study and were evenly distributed except for the nuclei, which were mostly concentrated in the center of the YSL. For the freshwater brown trout (*Salmo fario trutta*), there were two zones of organelles in the YSL, an inner vitellolysis zone (containing numerous yolk platelets) and an outer cytoplasmic zone (where yolk platelets were rare). Such zonation was not found in the YSL of turbot, Atlantic cod (*Gadus morhua*) (Morrison 1993), or in the present investigation of plaice.

The rate at which yolk reserves are depleted is thought to be a function of the surface area and the activity of the hydrolytic enzymes enhancing the digestion of the yolk in the YSL. The YSL developed an extensive network of microvillar extensions during development, greatly increasing the membrane surface area. Similar networks were also found in the YSL of three species of cichlids. The pinocytotic vesicles observed in the present experiment are believed to contain absorbed yolk (Fig. 8B). The larger vesicles probably contain lipoproteins, as similar vesicles as well as the apolipoprotein E expression has been observed in the YSL of turbot.

The pinocytotic activity at the inner membrane of the YSL was low prior to hatch and it is therefore suggested that contrary to the proposals of Heming and Buddington (1988), pinocytosis does not play a major role in resorption of yolk during early plaice ontogeny. Rather, other membrane transport mechanisms for the uptake of yolk nutrients to the YSL must be active. During this second pre-hatch phase of yolk resorption, a low pinocytotic activity was observed at the same time as the FAA consumption increased. This may be associated with observations that the YSL expresses endodermal genetic characteristics and that the intestine has transport proteins that absorb the FAA from the intestinal lumen to the bloodstream (Elliot and Elliot 1997).

The early utilization of FAA by plaice embryos is at variance with the general assumption that amino acids are primarily deaminated in the liver. The plaice embryo at this stage of development lacks a vascularized liver, indicating limited functionality. Other cells in the embryo (e.g. presumptive skeletal muscles or YSL) may be responsible for oxidation of FAA. Furthermore, it is noteworthy that the decline in FAA seemed to occur more rapidly than the decline in yolk volume (Figs. 4 and 1, respectively). This was contrary to the findings for lemon sole (*Microstomus kitt*), where a linear correlation between yolk volume resorption and FAA decrease was found (Rønnestad et al. 1992b).

For plaice, once the YSL had formed, a clear and rapid decline in FAA and P_i content was noted in the second pre-hatch phase of yolk resorption, while the total protein and NH_4^+ ion content increased. This pattern of decreasing FAA and P_i together with an accretion of protein and NH_4^+ ions seems to be a general trend among the pelagic eggs of marine teleosts. The depletion of the FAA pool in the present study co-occurred with the disappearance of a 95 kD yolk protein and the temporal emergence of protein bands of lower Mw (67, 45, 22, 17, and 15 kD; Fig. 6). The 95 kD protein may be homologous to the 92 kD yolk protein found in barfin flounder (*Verasper moseri*), or the 97 kD yolk protein in haddock (*Melanogrammus aeglefinus*) (Reith et al. 2001). For barfin flounder, the FAA resorption coincided with the utilization of the 92 kD yolk protein, and the appearance of protein bands at 67 and 23 kD. These studies suggest that amino acids are recruited from a high molecular weight yolk protein (92 – 97 kD) when the FAA pool no longer fulfils the nutritional requirements of the pelagic teleost embryo.

The degradation of the 95 kD band in plaice in the present study is intriguing since it implies a specific intravitelline degradation mechanism. A range of acid hydrolases, such as cathepsins, are important for yolk degradation, and have been found in the eggs of seabass (*Dicentrarchus labrax*) (Carnevali et al. 1999; 2001), and salmonids. The degradation of yolk proteins is especially interesting since it implies differential recognition by hydrolytic enzymes, although no mechanism to explain the differential processing of the yolk proteins has been found. Differential yolk protein processing has been found during early development of salmonids (Olin and von der Decken 1990; Thomsen 2000, Hiramatsu et al. 2002a), perch (*Perca fluviatilis*) (Krieger and Fleig 1999),

seabass (Carnevali et al. 2001), winter flounder (*Pleuronectes americanus*) (Hartling and Kunkel 1999), and barfin flounder (Ohkubo and Matsubara 2002), and also in an ancient Osteichthyan hybrid sturgeon (Hiramatsu et al. 2002c). However, no such yolk protein processing was noted in other ancient Osteichthyans such as shovelnose sturgeon (*Scaphyrhynchus platorhynchus*) and alligator gar (*Atractosteus spatula*) (Finn et al. 2002c; d) and the differential processing of yolk proteins may be associated with the specific activities of the YSL and the meroblastic teleolecithal nature of teleost eggs.

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Biochemical and molecular studies of the polyunsaturated fatty acid desaturation pathway in fish

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Abstract

Fish have an absolute dietary requirement for certain polyunsaturated fatty acids (PUFA) termed “essential fatty acids” (EFA) that include members of both the n-6 and n-3 series typified by linoleic acid, 18:2n-6, and α -linolenic acid, 18:3n-3. However, the biologically active forms of EFA are generally the C₂₀ and C₂₂ metabolites of 18:2n-6 and 18:3n-3, viz. 20:4n-6, 20:5n-3 and 22:6n-3. Some fish species can convert C₁₈ PUFA to the C₂₀ and C₂₂ PUFA through a series of alternating desaturation and chain elongation reactions mediated by microsomal systems containing elongases and Δ 6 and Δ 5 fatty acid desaturases. In species that cannot perform these conversions, the C₂₀ and C₂₂ PUFA themselves are dietary EFA and their C₁₈ homologues do not satisfy EFA requirements. The extent to which the foregoing statements apply quantitatively to a given fish species varies widely. Therefore, a vital area in lipid nutrition in fish is the provision of sufficient amounts of the correct EFA to satisfy the requirements for normal growth and development, requirements that can vary quantitatively during the life of the fish and are particularly important factors in larval marine fish. This paper reviews the work on defining and characterising the fatty acid desaturation and elongation pathway in fish. Biochemical studies have been advanced by the use of cell cultures which have elucidated key parts of the pathway. Thus, the presence of the so-called Sprecher shunt, where 22:6n-3 is produced from 20:5n-3 through two successive elongations and a Δ 6 desaturase followed by peroxisomal chain shortening, was demonstrated in trout. Similarly, the block in the pathway in marine and/or piscivorous fish could be due to either a deficiency of C₁₈₋₂₀ elongase or Δ 5 desaturase and this varies between different marine species. Recent work has focussed on the molecular biology of the pathway with the cloning of fatty acid desaturases and elongases from a variety of fish species. Zebrafish have been used as a model species and a unique desaturase possessing both Δ 6 and Δ 5 activity along with an elongase with very high C₁₈₋₂₀ activity have been cloned and characterised. Understanding this pathway is of increased importance due to the current dependence of salmonid and marine fish aquaculture on fish oil, the supply of which is becoming increasingly limited and unsustainable, necessitating the use in fish feeds of sustainable plant oils, rich in C₁₈ PUFA, but devoid of C₂₀ and C₂₂ PUFA.

Introduction

Lipid nutrition of fish is a subject that has received enormous attention in the last 10 years (see Sargent et al. 2002). In particular, much work has focussed on the optimal requirements and functional roles of polyunsaturated fatty acids (PUFA) during larval and early developmental stages of marine fish (Sargent et al. 1999). However, the study of lipid and fatty acid biochemistry of larval fish, especially marine larval fish, is hampered by their very small size. This can place a significant limitation on the amount of material available for study. Of course, the small size of larvae can be compensated, in some instances by numbers, particularly if the enzymes and/or metabolic pathways can be effectively studied in homogenates or some other similar preparation of whole animals. However, it is often far more illuminating to study specific organ, tissue or subcellular fractions and in these cases the considerable practical problems of dissecting large numbers of very small animals through a binocular microscope can be prohibitive. One alternative is to use larger animals. This usually requires the use of older animals such as juveniles and this can be acceptable in some circumstances where the developmental stage of the fish or the ontogeny of the enzyme systems or metabolic pathways is not a major issue. However, a further alternative is to go even smaller, by studying the pathways at a cellular or molecular level.

This paper describes the utilization of both cell culture systems and molecular techniques in the study of the genes, enzymes and metabolic pathways of lipid and fatty acid metabolism in fish. The advantages (and disadvantages) of utilizing cell culture systems in metabolic studies are described and the types of data that can be obtained are illustrated through studies performed in our own laboratory over the last 5-6 years. The aims of these studies were to elucidate the PUFA desaturation and elongation pathway in salmonids, and the nature of the deficiency in the pathway in marine fish, and the metabolic pathway behind the metabolism of 18:5n-3 in fish. Recently, molecular studies have begun to elucidate the genetics of these processes through the cloning and characterisation of the genes involved which will enable further studies of their expression and regulation.

Cell culture studies. Fish cell culture is long-established and many cell lines are available commercially and from various research laboratories around the world. Fish cell culture has mainly been developed over the years as a diagnostic tool in pathology particularly in the area of virology where the cell lines offer a range of host cells for diagnosis, characterisation and research into therapies. However, cell lines have been used extensively and very successfully in metabolic studies in the mammalian field. Similarly, several years ago, we decided to utilize a variety of cell culture systems, including established cell lines as model systems in our studies investigating lipid and especially fatty acid metabolism in fish.

Advantages of cell cultures. In these studies, cell culture systems offered three main advantages over studies employing whole fish. These can be summarised as control, containment and cost. Firstly, environmental conditions can be controlled easily and very precisely in cell culture systems. Temperature can be controlled simply by adjusting the temperature controller of the incubator and/or by having incubators at different temperatures. Thus, studies investigating both acute and chronic

effects of temperature can be performed very easily and in a variety of ways (Tocher and Sargent 1990a). Similarly, the osmolality of the medium can be adjusted easily, at least in the case of increased salinity, by the addition of appropriate amounts of sodium chloride to the medium, as may be required with cell cultures from marine fish (Tocher et al. 1988). We have performed studies in this way to investigate the effects of increasing salinity changes on lipid and fatty acid compositions in an Atlantic salmon (*Salmo salar*) cell line (AS) (Tocher et al. 1994, 1995a). Osmolality below the normal level found in most commercial media preparations ($\sim 300 \text{ mOsm.kg}^{-1}$) is a little more difficult but possibly of much less interest in any case. However, the medium and associated supplements supply all the nutrition to the cells, and so studies into the effects of nutrients can also be performed with relative ease. There are a considerable number of different media formulations and supplements commercially available from which to choose. As with salinity above, it is easier to look at additional nutrient supplements to the cells and these can be added in high purity and in various forms and concentrations. Removal of specific nutrients may be more difficult if they are normal components of cell culture media formulations although it is entirely possible, albeit slightly more time consuming, to formulate your own medium.

Cell cultures also offer the advantage of containment. This could include the use of radioisotopes for metabolic tracer studies, potentially hazardous or toxic chemicals such as carcinogens in toxicology studies, and pathogenic or infectious micro-organisms. Containment is primarily achieved through the use of tissue culture flasks that offer sufficient protection even if used vented, but can be used unvented if an appropriate medium such as Leibovitz L-15, which does not contain bicarbonate buffer and thus does not require exposure to a CO_2 atmosphere, is utilized. To list cost as an advantage of cell culture may be surprising to some but this is certainly a major factor to include. Some capital expenditure is required but this can be tailored somewhat to both specific requirements and budget. Ideally, a dedicated cell culture laboratory with sealed floors and walls, single purpose sink areas, air conditioning and separate areas for media preparation, primary culture preparation and subculture would be desirable but not essential. A vertical laminar air flow cabinet, a cooled incubator, an inverted microscope and a dedicated fridge-freezer set aside in a dedicated area of a larger laboratory are probably the minimum requirements. This represents no more than moderate capital expenditure. Consumables, including media, sera, other reagents and disposable plasticware (flasks, pipettes, centrifuge tubes and vials/containers) are not cheap but save considerable time, a vital factor when man-power is the single most expensive item in the research budget. Perhaps the most important factor in assessing the cost-effectiveness of cell culture is the huge cost of the alternative. Studies with fish require aquaria, with all the associated costs of water supply and purification, fish and feed costs and, of course, husbandry staff. In addition, some studies would be very much more difficult to perform with fish. Studies on temperature effects require aquaria to be maintained at non-ambient temperatures and thus require heating or cooling of the water and/or the room. Work with radioisotopes is extremely difficult with whole fish particularly when ^{14}C is used due to the possibility of production and release of $^{14}\text{CO}_2$ into the atmosphere. Containment is similarly a problem when using toxins or pathogens and in all these cases it adds to the costs of performing experiments with fish.

Problems of using fish cell cultures in metabolic studies. The use of cell cultures is not, however, without its own problems. The first of these is temperature. For the majority of fish cell lines the optimum growth temperature is in the 20 - 25°C range. These include cell lines from Atlantic salmon (AS), rainbow trout (*Oncorhynchus mykiss*) (RTG-2, RTH) and turbot (*Scophthalmus maximus*) (TF) which are all routinely cultured at 22°C. However, the normal ambient temperature in U.K. waters for these species of fish would rarely exceed 15°C, a temperature we routinely use as a “holding” temperature, to slow the growth of the cells during periods when they are not being actively used in experiments. Culture at 10°C or below usually results in unacceptably low growth rates even in cell lines from these cooler water fish. Therefore, fish cell lines such as those above are being cultured at a temperature higher than normal, a situation that does not occur in mammalian cell culture. In contrast, other fish species, such as Mediterranean fish including gilt-head sea bream (*Sparus aurata*), would normally experience water temperatures in the low 20’s and thus cells derived from them (SAF-1) would not be at an unusually high temperature when cultured at 20 – 25°C. These are particularly important points to be aware of in relation to temperature adaptation/acclimation studies where the lower temperature, say 10°C, actually represents a more normal temperature for some cell lines and 22°C could be regarded as a stressed temperature, whereas in other cell lines the opposite would be true.

A second problem with the use of fish cell cultures is one of particular importance in relation to lipid and fatty acid studies. Cell culture media are normally devoid of fatty acids and so cells in culture generally derive all their lipid and fatty acids from the lipid contained in the serum supplement, which is an almost ubiquitous supplement due to its various properties including promotion of attachment, growth and proliferation of the cultured cells. Fetal bovine serum (FBS), the predominant serum supplement used in cell culture including fish cell culture, is relatively rich in PUFA and for mammalian cells, FBS provides a sufficient amount and balance of n-6 and n-3PUFA. In contrast, although the total amount of PUFA is adequate, fish cells grown in FBS display lower percentages of n-3PUFA and are enriched in n-6PUFA in comparison with fish tissues (Tocher et al. 1988). This has important consequences when cultured fish cells are used in studies of fatty acid metabolism. We have used two approaches to solve this problem. Firstly, we investigated the possibility of producing fish cell lines that can grow and proliferate in the absence of serum. To date, we have found one cell line, EPC-EFAD, derived from the carp (*Cyprinus carpio*) epithelial papilloma line, EPC, that can survive and proliferate in essential fatty acid-deficient (EFAD) medium (Tocher et al. 1995b). The EPC-EFAD line has now been growing continually in EFAD medium for over 7 years and 130 passages although the rate of proliferation is lower than the parent EPC line. This cell line is virtually devoid of n-6 and n-3PUFA but contains appreciable amounts of n-9PUFA (Tocher and Dick 2001) and thus does not represent a model system for fish normally although they have been useful in studies on the effects of EFA deficiency on fatty acid metabolism in freshwater fish (Tocher and Dick 1999, 2000, 2001). An alternative solution is to reduce the serum added to the medium and to supplement with a mix of pure fatty acids designed to restore the fatty acid composition of the cells to that of the original tissue in the fish. For instance, primary cultures of turbot brain astroglial cells established in medium containing FBS contained increased proportions of 18:1(n-9), and total n-9 and n-6 PUFA, and greatly reduced n-3PUFA in comparison with turbot brain. Supplementation with a mixture of 5 µM 20:5n-3 and

25 μM 22:6n-3 acids for 4 days significantly increased the percentages of these acids in total cellular lipid of trout and turbot astrocytes and restored the n-3PUFA composition of the cells to that found in brain (Bell et al. 1994; Tocher et al. 1996).

A final caveat to the use of cell cultures in metabolic studies relates to interpretation and extrapolation of the results. It is obvious that cell cultures are not whole animals. Many factors important in controlling and regulating metabolism are simply not replicated in the cell culture systems. Complex multi-cell type organ structure is difficult to replicate in cell culture and even most tissue specific features such as 3D-structure, orientation and sidedness are lost in culture and, in addition, the cells themselves may be dedifferentiated (as in cell lines) and of changed morphology. Nonetheless, many features of inherent intracellular biochemistry and metabolism will be retained by cells in culture and provided the researcher is aware of the limitations then cell cultures provide a very useful additional experimental tool. Cautious extrapolation to the whole animal is possible particularly when the cell data are entirely consistent with other available data and, particularly, whole animal data, but ultimately whole animal studies are required for final confirmation.

Types of cell culture systems. Different types of cultured cell systems can be utilized to fit the particular requirements of the studies. In our own studies we have used three types, the first of which is short-term cultures, where the cells are attached to the substrate (plastic), but there is no growth or division over the time-course of the experiment, around 2 – 24 h (Buzzi et al 1996, 1997). The major benefit of these cultures is that the cells retain their differentiated phenotype. The retention of differentiated phenotype is also the aim with primary cultures that are attached, and grow and divide over a much longer period of time, ranging from days to weeks (Tocher and Sargent 1990b). Depending upon the cell type, some limited subculture of primary cultures may be possible but not always. Established cell lines are immortal, growing and dividing at infinitum with routine subculture necessary to maintain the cells in optimum condition (Tocher et al. 1988). The down side of cell lines being that they are usually de-differentiated, possessing either fibroblast or epithelial morphology. The following sections describe the use of some of these cell cultures as model systems to investigate specific aspects of fatty acid metabolism in fish.

Determining the PUFA desaturation/elongation pathway in trout. All vertebrates, including fish, lack $\Delta 12$ and $\Delta 15$ ($\omega 3$) desaturases and so cannot form 18:2n-6 and 18:3n-3 from 18:1n-9. Therefore, 18:2n-6 and 18:3n-3 are essential fatty acids in the diets of vertebrates. These dietary essential fatty acids can be further desaturated and elongated to form the physiologically essential C_{20} and C_{22} PUFA, 20:4n-6, 20:5n-3 and 22:6n-3 (Fig.1). With one exception the reactions occur in the microsomal fraction of the liver and the same enzymes act on the n-3 and the n-6 fatty acid series. Originally the insertion of the last, $\Delta 4$, double bond in 22:6n-3 was assumed to occur through direct $\Delta 4$ desaturation of its immediate precursor 22:5n-3. However, Howard Sprecher and coworkers showed that in rat liver, the 22:5n-3, is further chain elongated to 24:5n-3 which is then converted by $\Delta 6$ desaturation to 24:6n-3 which is then converted, by a chain shortening reaction in the peroxisomes, to 22:6n-3 (Sprecher 1992; Sprecher et al. 1995).

Whether the production of 22:6n-3 in fish involved $\Delta 4$ desaturation of 22:5n-3 or $\Delta 6$ desaturation of 24:5n-3 with chain shortening of the resultant 24:6n-3 to 22:6n-3 was investigated in our laboratory by Buzzi et al. (1996, 1997). The cell system chosen was primary hepatocytes prepared by collagenase perfusion of intact, isolated liver from rainbow trout fed a n-3PUFA-deficient (olive oil) diet to stimulate the PUFA desaturation pathway. These cells were maintained in short-term culture for up to 24h. Incubation of hepatocytes for 3h with [1- 14 C]18:3n-3 or [1- 14 C]20:5n-3, added as complexes with fatty acid-free bovine serum albumin, resulted in the recovery of large amounts of radioactivity as 22:6n-3 with only traces of radioactivity recovered in C₂₄ PUFA (Table 1). In contrast, when liver microsomes were incubated for 3h with the same radioactive fatty acids, no radioactivity was recovered in 22:6n-3, but substantial amounts of radioactivity were recovered in 24:5n-3 and 24:6n-3 (Table 1). These data suggested that the pathway as proposed by Sprecher for rat liver also occurred in trout liver. Incubation of the trout hepatocytes with [1- 14 C]24:5n-3 resulted in radioactivity being recovered in both 22:6n-3 and 24:6n-3 (Table 2). Similarly, incubation of trout hepatocytes with [1- 14 C]24:6n-3 resulted in the recovery of radioactivity in 22:6n-3 (Table 2). Thus, the experiments with primary hepatocytes prepared from rainbow trout had provided data consistent with the fact that the production of 22:6n-3 in trout occurred through the so-called "Sprecher shunt". Thus, 20:5n-3 is elongated by two sequential steps to 24:5n-3 which is then desaturated by a $\Delta 6$ desaturase to 24:6n-3, all in the microsomes, and that this intermediate is then chain shortened to 22:6n-3 at an extra-microsomal site, presumably peroxisomes (Buzzi et al. 1996, 1997). While all the steps in the pathway from 18:3n-3 to 22:6n-3 in Fig. 1. have so far been established for fish only in rainbow trout hepatocytes, there is accumulating evidence that the same pathway occurs in primary hepatocytes from Atlantic salmon (Tocher et al. 1997), Arctic charr (*Salvelinus alpinus*), brown trout (*Salmo trutta*) (Tocher et al. 2001a), zebrafish (*Danio rerio*),

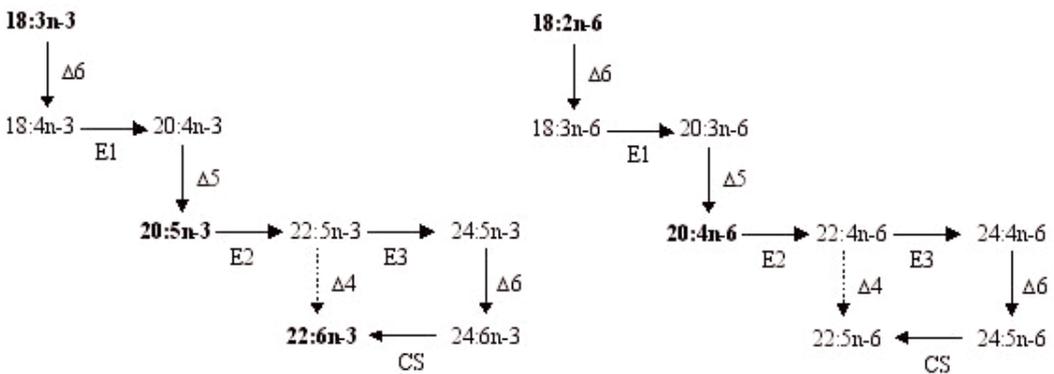


Figure 1. Pathways for the biosynthesis of C₂₀ and C₂₂ PUFA from 18:3n-3 and 18:2n-6 showing the two possible routes for the production of 22:6n-3 from 20:5n-3 (and 22:5n-6 from 20:4n-6). $\Delta 6$, $\Delta 5$ and $\Delta 4$ represent microsomal fatty acyl desaturase activities, E1, E2 and E3 denote microsomal fatty acyl elongase activities and CS denotes peroxisomal chain shortening. The dotted lines indicate pathways for which there is no direct evidence in fish.

Table 1. Desaturation of [$1-^{14}\text{C}$]18:3n-3 and [$1-^{14}\text{C}$]20:5n-3 by hepatocytes and liver microsomes from rainbow trout fed an (n-3)-deficient diet. Results are expressed as a percentage of total radioactivity recovered in specific fatty acids in polar lipids and are means \pm SD (n=3). Based on data taken from Buzzi et al. (1996).

| Fatty acid | Hepatocytes | Microsomes |
|------------------------------|----------------|----------------|
| [$1-^{14}\text{C}$]18:3n-3 | | |
| 18:3 | 22.5 \pm 0.8 | 52.7 \pm 4.5 |
| 20:3 | 3.7 \pm 0.5 | 19.0 \pm 4.9 |
| 22:3 | 1.1 \pm 0.1 | 3.0 \pm 1.0 |
| 24:3 | 1.2 \pm 0.1 | 1.9 \pm 0.2 |
| 18:4 | 8.9 \pm 0.7 | 9.0 \pm 0.3 |
| 20:4 | 4.6 \pm 1.7 | 0.3 \pm 0.0 |
| 22:4 | 1.5 \pm 0.2 | 1.6 \pm 0.2 |
| 20:5 | 15.3 \pm 3.6 | 6.0 \pm 1.1 |
| 22:5 | 4.8 \pm 1.0 | 1.5 \pm 0.1 |
| 24:5 | trace | 2.7 \pm 0.4 |
| 22:6 | 36.4 \pm 7.5 | trace |
| 24:6 | trace | 2.4 \pm 0.6 |
| [$1-^{14}\text{C}$]20:5n-3 | | |
| 20:5 | 39.7 \pm 0.7 | 57.8 \pm 1.0 |
| 22:5 | 10.1 \pm 0.5 | 13.8 \pm 2.1 |
| 24:5 | 4.3 \pm 0.4 | 4.7 \pm 0.1 |
| 22:6 | 45.9 \pm 0.7 | trace |
| 24:6 | trace | 23.6 \pm 1.2 |

Table 2. Metabolism of [$1-^{14}\text{C}$]24:5n-3 and [$1-^{14}\text{C}$]24:6n-3 by hepatocytes from rainbow trout fed an (n-3)-deficient diet. Results are means \pm SD (n=3). Based on data taken from Buzzi et al. (1997).

| Fatty acid | Radioactivity recovered in specific fatty acid fractions in total polar lipid (percentage) |
|------------------------------|--|
| [$1-^{14}\text{C}$]24:5n-3 | |
| 20:5 | trace |
| 22:5 | 1.4 \pm 0.1 |
| 24:5 | 56.6 \pm 9.9 |
| 22:6 | 23.1 \pm 6.2 |
| 24:6 | 18.9 \pm 5.2 |
| [$1-^{14}\text{C}$]24:6n-3 | |
| 20:5/22:5 | 11.5 \pm 1.5 |
| 22:6 | 28.1 \pm 4.8 |
| 24:6 | 60.4 \pm 3.6 |

tilapia (*Oreochromis niloticus*) (Tocher et al. 2001b), and carp cells in culture (Tocher and Dick 1999). Cell studies were unable to resolve whether the same $\Delta 6$ fatty acid desaturase catalysed each of these steps or whether different $\Delta 6$ desaturases (isoenzymes) were involved for the C_{18} and C_{24} PUFA (see later).

Determining the deficiency in the PUFA desaturation/elongation pathway in marine fish. It had been known for some time that the EFA requirements of freshwater and marine fish are qualitatively different, as in rainbow trout 18:3n-3 alone can satisfy the EFA requirement, with 18:2n-6 only required for optimal growth, whereas in the most studied marine species, turbot, the longer chain PUFA 20:5n-3 and 22:6n-3 are required. This suggested a difference in the fatty acid desaturase/elongase activities, and it was subsequently shown that this *in vivo* difference was also present in cultured cell lines (Tocher et al. 1989). Initial studies involving supplementation of turbot cells (TF) in culture, compared to both rainbow trout cells (RTG-2) and Atlantic salmon cells (AS), with various n-3 and n-6 PUFA had shown that the apparent deficiency in the desaturase/elongase pathway in turbot was either in the C₁₈ to C₂₀ elongase (C₁₈₋₂₀ elongase) multi-enzyme complex or the the fatty acyl Δ5 desaturase step (Tocher et al. 1989). Defective C₁₈₋₂₀ elongase appeared the more likely of the two alternative based on (i) the ability of turbot cells to produce 20:4n-6 when supplemented with 20:3n-6, which bypasses the elongase and indicated the presence of some Δ5 desaturase activity, (ii) the accumulation of 18:4n-3 and 18:3n-6 in cells supplemented with 18:3n-3 and 18:2n-6, respectively, and (iii) the accumulation of 18:2n-9, and not 20:2n-9 or 20:3n-9, in cells cultivated in the absence of EFA. However, results from *in vivo* injection studies with other marine fish species such as gilthead sea bream were more consistent with a deficiency in Δ5 desaturase activity (Mourente and Tocher 1994). Therefore, as the situation in marine fish was unclear, and as a deficiency in the fatty acid elongase activity responsible for the conversion of C₁₈ to C₂₀ PUFA had not been reported in any other animal or cell line, we aimed to establish unequivocally the location of the defect in the desaturase/elongase pathway in marine fish using the established cell lines, AS, TF and SAF-1. Each of these cell lines was incubated for 4 days with various ¹⁴C-labelled n-3PUFA that were the direct substrates for individual enzymic steps in the desaturation/elongation pathway (Ghioni et al. 1999; Tocher and Ghioni 1999). Thus, 18:3n-3 was the direct substrate for Δ6 desaturase, 18:4n-3 was the direct substrate for C₁₈₋₂₀ elongase, 20:4n-3 was the substrate for Δ5 desaturase and 20:5n-3 was the substrate for C₂₀₋₂₂ elongase (Table 3). The data in Table 3 show the percentage of radioactivity recovered as the products of each enzymic step. Thus, the results showed that all three cell lines had substantial Δ6 activity as 76%, 82% and 66% of radioactivity from [1-¹⁴C]18:3n-3 was recovered as Δ6 desaturated products in AS, TF and SAF-1 cells, respectively. However, both marine cell lines showed very reduced C₁₈₋₂₀ elongase activity compared with AS cells. However, whereas the SAF-1 cell line showed virtually no Δ5 desaturase activity, the TF cell line showed considerable Δ5 activity (Table 3). All cell lines showed similar levels of C₂₀₋₂₂ activity. Thus the primary deficiency in the PUFA desaturation/elongation pathway in gilthead sea bream cells was established to be at the level of Δ5 desaturase whereas the only deficiency observed in the TF cells was at the C₁₈₋₂₀ elongase. The SAF-1 cell line may also show a deficiency in C₁₈₋₂₀ elongase but it is possible that the virtual absence of Δ5 activity results in the accumulation of 20:4n-3 which inhibits C₁₈₋₂₀ elongase through a feedback mechanism. Irrespective of which enzyme step was deficient, the cell line data were entirely consistent with earlier feeding studies and *in vivo* studies indicating that marine fish were unable to produce significant amounts of 20:5n-3 and 22:6n-3 from 18:3n-3.

Determining the metabolism of 18:5n-3 in fish. Octadecapentaenoic acid (all-*cis* 18:5n-3) is a fatty acid characteristically present in certain algal groups in marine phytoplankton, including dinoflagellates, haptophytes and prasinophytes, all of which have important roles in the marine ecosystem (Sargent et al. 1995). 18:5n-3 is usually co-associated in these organisms with 22:6n-3. Given that biosynthesis of 22:6n-3 involves peroxisomal chain shortening of its precursor 24:6n-3, it is possible that 18:5n-3 is biosynthesized by chain shortening of 20:5n-3. However, marine zooplankton and fish ingesting phytoplankton contain little or no 18:5n-3 demonstrating that this fatty acid is readily metabolized by marine animals. It could be completely catabolized by marine animals by β -oxidation but it may also be directly chain elongated to 20:5n-3.

In this study, [U-¹⁴C]18:4n-3 and [U-¹⁴C]18:5n-3 were prepared from the haptophycean alga *Isochrysis galbana* cultured in sodium ¹⁴C-bicarbonate, and their metabolism studied in cultured cells from turbot (TF), sea bream (SAF-1) and Atlantic salmon (AS) that differ in their abilities to perform C₁₈ to C₂₀ elongation reactions. The rationale being that the TF cell line's deficiency in C₁₈ to C₂₀ fatty acid elongase would perhaps help to differentiate between the two possible pathways for the metabolism of 18:5n-3 in fish as suggested above. Incubation of the cell lines with both labelled 18:4 and 18:5 showed two remarkable features (Table 4). Firstly, no radiolabelled 18:5 was ever detected in any of the three cell lines, even when labelled 18:5n-3 was incubated with the cells and even in short incubations of less than 1h. Secondly, the pattern of distribution of radioactivity was identical for both fatty acids, that is the recovery of radioactivity in different fatty acid fractions after incubation with [U-¹⁴C]18:5 was identical to the distribution of radioactivity after incubation with [U-¹⁴C]18:4 (Table 4). Indeed, the pattern only varied between the cell lines based upon the differences in their PUFA desaturation/ elongation pathways. The one difference between incubation with 18:4 and 18:5 was that the quantitative recovery of radioactivity was significantly lower with 18:5n-3. These results showed that 18:5n-3 was not metabolised in fish cells by chain elongation to 20:5n-3. In retrospect, this was perhaps unsurprising as, unlike 18:4n-3, 18:5n-3 is not a normal intermediate in the desaturation/elongation pathway (Fig.1). However, 18:5n-3 is a normal intermediate in the pathway for the β -oxidation of 20:5n-3 (Fig.2). In contrast, 18:4n-3 is not an intermediate in the PUFA β -oxidation pathway although the first step in the β -oxidation of 18:4n-3, dehydrogenation, results in the formation of *trans* Δ 2, all-*cis* Δ 6,9,12,15-18:5 (2-*trans* 18:5n-3) (Fig.2). The 2-*trans* 18:5n-3 intermediate is also produced by the action of a Δ^3 ,

Table 3. Apparent activities of enzymes of the PUFA desaturation and elongation pathway in Atlantic salmon (AS), turbot (TF) and sea bream (SAF-1) cell lines. Data represents the percentage of total radioactivity recovered as products of each enzymic step. n.d., not detected. Based on data recalculated from Ghioni et al. (1999) and Tocher and Ghioni (1999).

| Substrate | Δ 6 desaturase | | | C ₁₈₋₂₀ elongase | | | Δ 5 desaturase | | | C ₂₀₋₂₂ elongase | | |
|-----------------------------|-----------------------|------|-------|-----------------------------|------|-------|-----------------------|------|-------|-----------------------------|------|-------|
| | AS | TF | SAF-1 | AS | TF | SAF-1 | AS | TF | SAF-1 | AS | TF | SAF-1 |
| [1- ¹⁴ C]18:3n-3 | 76.0 | 81.9 | 66.1 | 60.3 | 18.5 | 25.2 | 38.7 | 11.2 | n.d. | 4.9 | 3.2 | n.d. |
| [U- ¹⁴ C]18:4n-3 | – | – | – | 81.2 | 25.9 | 19.0 | 56.4 | 19.5 | 0.7 | 9.2 | 5.1 | n.d. |
| [U- ¹⁴ C]20:4n-3 | – | – | – | – | – | – | 38.8 | 62.3 | 0.7 | 7.8 | 17.8 | n.d. |
| [1- ¹⁴ C]20:5n-3 | – | – | – | – | – | – | – | – | – | 12.1 | 12.8 | 10.9 |



Figure 2. Section of the β -oxidation pathway for *n*-3PUFA showing the position of 2-*trans* 18:5n-3 as a common intermediate in the β -oxidation of 18:5n-3 and 18:4n-3.

Δ^2 -enoyl-CoA-isomerase acting on 18:5n-3, this enzyme being the next step in the β -oxidation pathway after the production of 18:5n-3. Thus, 2-*trans* 18:5n-3 is a common intermediate in the β -oxidation of both 18:4n-3 and 18:5n-3. It appeared therefore that 18:5n-3 incorporated into the fish cells was treated as a β -oxidation intermediate by the fish cell lines resulting in the production of 2-*trans* 18:5n-3 in amounts which probably exceeded the capacity of the β -oxidation pathway. This resulted in the reversal of the dehydrogenase step and production of labelled 18:4n-3 (Fig.2) which was then metabolised as normal via the desaturation/elongation pathway producing labelled 20:4n-3 and 20:5n-3 (Fig.1). A proportion of the 2-*trans* 18:5n-3 proceeded down the β -oxidation pathway resulting in the overall lower recovery of radioactivity when the cells were incubated with 18:5 compared to cells incubated with 18:4. To further test this hypothesis, cells were also incubated with either 18:5n-3 or 2-*trans* 18:5n-3, and similar mass increases of 18:4n-3 and its

Table 4. Recovery of radioactivity in specific fatty acids after incubation of Atlantic salmon (AS), turbot (TF) and gilthead sea bream (SAF-1) cell lines with [U - ^{14}C]18:4n-3 and [U - ^{14}C]18:5n-3. Data represent the percentage of total radioactivity recovered. n.d., not detected. Based on data taken from Ghioni et al. (2001).

| Fatty acid | AS | | TF | | SAF-1 | |
|------------|------|------|------|------|-------|------|
| | 18:4 | 18:5 | 18:4 | 18:5 | 18:4 | 18:5 |
| 18:4n-3 | 18.8 | 24.0 | 74.1 | 76.7 | 81.0 | 82.6 |
| 20:4n-3 | 23.6 | 23.2 | 4.4 | 4.5 | 13.2 | 10.3 |
| 22:4n-3 | 1.2 | 1.1 | 0.8 | 1.1 | 5.1 | 6.0 |
| 18:5n-3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 20:5n-3 | 48.4 | 46.1 | 16.4 | 14.8 | 0.7 | 1.1 |
| 22:5n-3 | 4.5 | 3.3 | 1.6 | 1.2 | n.d. | n.d. |
| 22:6n-3 | 1.7 | n.d. | n.d. | n.d. | n.d. | n.d. |

elongation and further desaturation products occurred in cells incubated with 18:5n-3 or 2-*trans* 18:5n-3. We therefore concluded that 18:5n-3 was readily converted biochemically to 18:4n-3 via a 2-*trans* 18:5n-3 intermediate generated by a Δ^3 , Δ^2 -enoyl-CoA-isomerase acting on 18:5n-3 and, therefore, that 2-*trans* 18:5n-3 was implicated as a common intermediate in the β -oxidation of both 18:5n-3 and 18:4n-3 (Ghioni et al. 2001).

Molecular studies. Very recently, molecular biological and genetic techniques have begun to be applied to lipid and fatty acid metabolism in fish in order to elucidate the genetics of the above pathways through the cloning and characterisation of the genes involved enabling further studies on the expression and regulation of the genes. These techniques have particular advantages when applied to larvae. Firstly, the small size of fish larvae presents no problem in the preparation of RNA and/or cDNA even if tissue-specific RNA is required as relatively little tissue is required. The larval RNA/cDNA can not only be used in routine gene expression studies through conventional Northern blotting or real-time PCR but can also be used for cloning genes expressed specifically in larvae. In addition, modern in-situ hybridisation techniques can also be used to locate organ- and tissue-specific gene expression and are equally, or indeed more, able to be applied to larvae as to larger fish. The above cell culture studies have demonstrated the great significance of PUFA desaturase and elongase enzymes in fish. Several questions still remained though including a) was there one or two different $\Delta 6$ desaturases (isoenzymes) for the desaturation of C_{18} and C_{24} PUFA, and b) what were the precise defects in $\Delta 5$ desaturase and C_{18-20} elongase in marine fish (Tocher et al. 1998). The following sections describe our current studies aimed at cloning and characterising PUFA desaturase and elongase genes in fish.

Cloning and characterisation of PUFA desaturase genes in fish. A zebrafish EST sequence (Genbank accession no. AI497337) was identified that displayed high homology to mammalian $\Delta 5$ and $\Delta 6$ desaturase genes. Thus, cDNA was synthesized from zebrafish liver total RNA using reverse transcriptase and a portion of this cDNA was then subjected to PCR amplification with appropriate primers predicted from the zebrafish EST sequence. The products were cloned into the pYES2

plasmid, and nucleotide sequences determined. The 1590 bp open reading frame of the zebrafish cDNA encoded a protein with substantial similarity to vertebrate $\Delta 6$ desaturases. Overall amino acid identities were 64% to human $\Delta 6$ desaturase and 58% to human $\Delta 5$ desaturase (Hastings et al. 2001). In addition, the zebrafish protein contained a similar N-terminal cytochrome b_5 -like domain and the three catalytically important histidine boxes conserved in all members of the desaturase gene family and believed to be involved in catalysis. When the zebrafish cDNA was expressed in the non PUFA-producing yeast *Saccharomyces cerevisiae* it conferred the ability to convert linoleic acid (18:2n-6) and α -linolenic acid (18:3n-3) to their corresponding $\Delta 6$ desaturated products, 18:3n-6 and 18:4n-3 (Table 5). However, in addition, it conferred on the yeast the ability to convert di-homo- γ -linolenic acid (20:3n-6) and eicosatetraenoic acid (20:4n-3) to arachidonic acid (20:4n-6) and eicosapentanoic acid (20:5n-3), respectively, indicating that the zebrafish gene encoded an enzyme having both $\Delta 6$ and $\Delta 5$ desaturase activities (Table 5). The enzyme was more active towards n-3 and $\Delta 6$ substrates compared to n-6 and $\Delta 5$ substrates. This was the first report of a functionally characterized polyunsaturated fatty acid desaturase enzyme of fish, and the first report of a fatty acid desaturase in any species with both $\Delta 6$ and $\Delta 5$ activities. Recently, we have shown that the zebrafish desaturase has no $\Delta 4$ desaturase activity but was able to desaturate 24:5n-3 to 24:6n-3 suggesting that a single $\Delta 6$ desaturase may be responsible for the desaturation of both C_{18} and C_{24} substrates (Table 5).

Further PUFA desaturase genes with homology to the zebrafish desaturase and vertebrate $\Delta 6$ desaturase genes in general have been cloned from fish. Genes from carp, Atlantic salmon and cod have been cloned in our own laboratory and other putative desaturase genes have been cloned from cherry salmon (*Oncorhynchus masou*), tilapia, sea bream and rainbow trout (Seilez et al., 2001). Most of these genes remain to be functionally characterised but preliminary data has suggested that the Atlantic salmon gene also has both $\Delta 6$ and $\Delta 5$ activities with the latter being greater. Phylogenetic analysis indicated that, with respect to other functionally characterized genes, the ze-

Table 5. Desaturase activities associated with the zebrafish PUFA desaturase gene expressed in the yeast *Saccharomyces cerevisiae*. Results are expressed as the percentage of substrate fatty acid converted to the product fatty acid. n.d., not detected.

| Substrate fatty acid | Product fatty acid | Substrate desaturated (percentage) | Desaturase activity |
|----------------------|--------------------|------------------------------------|---------------------|
| 18:3n-3 | 18:4n-3 | 29.4 | $\Delta 6$ |
| 18:2n-6 | 18:3n-6 | 11.7 | $\Delta 6$ |
| 20:4n-3 | 20:5n-3 | 20.4 | $\Delta 5$ |
| 20:3n-6 | 20:4n-6 | 8.3 | $\Delta 5$ |
| 22:5n-3 | 22:6n-3 | n.d. | $\Delta 4$ |
| 22:4n-6 | 22:5n-6 | n.d. | $\Delta 4$ |
| 24:5n-3 | 24:6n-3 | ~5-10% | $\Delta 6$ |
| 24:4n-6 | 24:5n-6 | 2-5% | $\Delta 6$ |

brafish sequence had highest homology with mammalian $\Delta 6$ desaturases, with human $\Delta 5$ desaturase appearing to be distinct from the $\Delta 6$ desaturase sequences (Fig.3). All the fish genes clustered together. Although more fatty acid desaturase genes may be found in zebrafish, salmon and mammals, it is conceivable that the bi-functional desaturase described here is a component of a prototypic vertebrate PUFA biosynthetic pathway that has persisted in freshwater fish species. That humans and other mammals have two distinct enzymes for $\Delta 5$ and $\Delta 6$ desaturation may be an adaptation to a terrestrial diet providing lower amounts of pre-formed C_{20} and C_{22} PUFA than the diets of a vertebrate ancestor that they share with freshwater fish. Functional divergence of the products of a putative ancient gene duplication event is a possible mechanism underlying adaptation to such a dietary change.

Cloning and characterisation of PUFA elongase genes in fish. Fatty acid elongation, the addition of 2-carbon units, is effected in four steps each catalysed by a specific enzyme. The first step is a condensation reaction of the precursor fatty acyl chain with malonyl-CoA to produce a β -ketoacyl

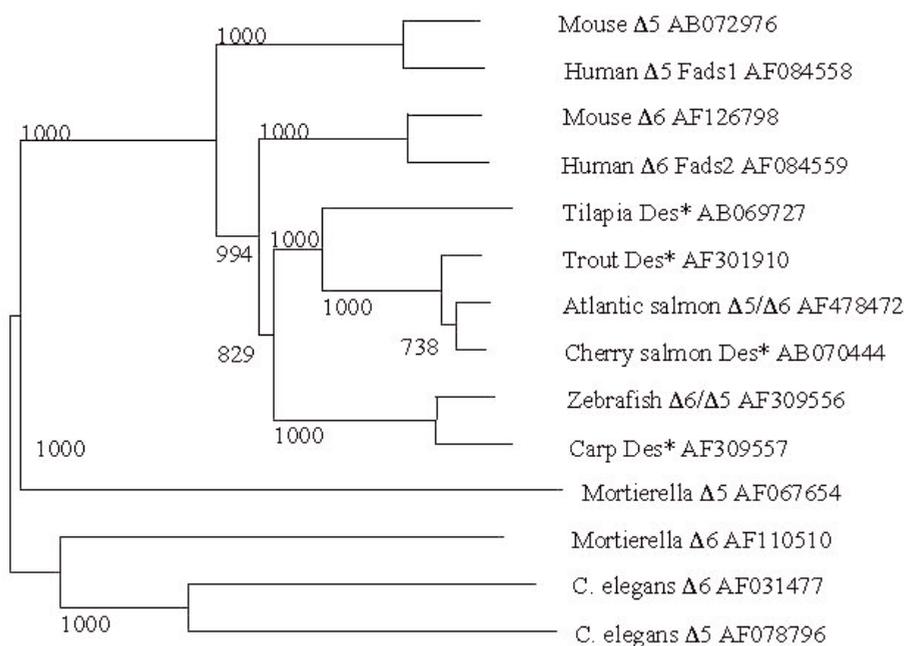


Figure 3. Phylogeny of desaturase deduced amino acid sequences. Sequences marked with an asterisk are not functionally characterized. Data base accession numbers for the nucleic acid sequences are indicated. Deduced amino acid sequences were aligned using ClustalX and sequence phylogenies were predicted using the Neighbour Joining method of Saitou and Nei (1987). Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping the data through 1000 iterations with the numbers representing the frequencies with which the tree topology presented here was replicated after the iterations. Horizontal branch lengths are proportional to the number of amino acid replacements per position, the scale bar indicating this value.

chain that is then hydrogenated in three successive steps. The condensation step is widely regarded as the “elongase”, and the one that determines the substrate specificity and is rate limiting. *Mortierella alpina* elongase (GLELO) amino acid sequence cDNA encoding a PUFA elongase was used to probe *in silico* for related sequences in the Genbank EST database. This identified mammalian, chicken, *Xenopus* and zebrafish ESTs. Consensus PCR primers were designed in conserved motifs and used to isolate full length cDNA from livers of several fish species using the rapid amplification of cDNA ends (3' and 5' RACE) strategies to clone full length elongase cDNAs of zebrafish, carp, salmon and turbot (AF465520). The amplified cDNAs encoded putative open reading frames (ORFs) of 291-295 amino acids whose sequences were highly conserved among the fish species and with other vertebrate elongases. The fish elongase polypeptides have up to 7 predicted transmembrane (TM) domains, a canonical endoplasmic reticulum retention signal, and several potential phosphorylation sites which may be important in regulation of enzyme function. Expression of the zebrafish gene in the yeast *S. cerevisiae* demonstrated that the ORF encoded a fatty acid elongase with substrate specificity ranging from the monounsaturated fatty acid palmitoleic acid (16:1n-7) to the long chain highly unsaturated fatty acid, 22:5n-3. The zebrafish elongase activity was in the rank order $C_{18-20} > C_{20-22} > C_{22-24}$ and was more active towards n-3 substrates than n-6 substrates (Table 6). Recently, functional characterisation of the salmon and turbot elongases has revealed that they have similar specificities to the zebrafish enzyme with the rank order for overall activity being zebrafish > salmon > turbot. The turbot enzyme was relatively more active towards the C_{20} substrates than C_{18} substrates compared to the zebrafish and salmon enzymes. However, it was particularly interesting that the turbot gene coded for a functionally active protein. This was not contradictory to the cell culture data as, although the deficiency in the desaturation/elongation pathway appeared to be at the C_{18-20} elongase step in TF cells, there was activity present. The sequence data suggested another possibility for low C_{18-20} elongase activity in TF cells as the Kozak sequence (which marks the following methionine codon as the start codon) in the turbot cDNA is a poor signal for initiation of translation and turbot elongase was less efficient than zebrafish and salmon elongases particularly for C_{18} substrates.

Table 6. Elongase activities associated with the zebrafish PUFA elongase gene expressed in the yeast *Saccharomyces cerevisiae*. Results are expressed as the percentage of substrate fatty acid converted to the product fatty acid. n.d. not detected.

| Substrate fatty acid | Product fatty acid | Substrate elongated (percentage) | Elongase activity |
|----------------------|--------------------|----------------------------------|-------------------|
| 18:4n-3 | 20:4n-3 | 85.4 | C_{18-20} |
| 18:3n-6 | 20:3n-6 | 70.7 | C_{18-20} |
| 20:5n-3 | 22:5n-3 | 46.4 | C_{20-22} |
| 20:4n-6 | 22:4n-6 | 25.6 | C_{20-22} |
| 22:5n-3 | 24:5n-3 | 4.9 | C_{22-24} |
| 22:4n-6 | 24:4n-6 | trace | C_{22-24} |

Conclusions

The use of a variety of cell culture systems has greatly advanced biochemical studies which have in turn elucidated key parts of the PUFA desaturation and elongation pathway in fish. The presence of the so-called Sprecher shunt, where 22:6n-3 is produced from 20:5n-3 through two successive elongations and a $\Delta 6$ desaturase followed by peroxisomal chain shortening, was demonstrated in primary hepatocytes isolated from trout. Similarly, studies on established cell lines revealed that the block in the pathway in marine and/or piscivorous fish was due to either a deficiency of C_{18-20} elongase or $\Delta 5$ desaturase and this varied between different marine species. Current work is focussing on the molecular biology of the pathway with the cloning of fatty acid desaturases and elongases from a variety of fish species. Zebrafish have been used as a model species and a unique desaturase possessing both $\Delta 6$ and $\Delta 5$ activity and an elongase with very high C_{18-20} activity have been cloned and characterised. The zebrafish desaturase was capable of desaturating both C_{18} and C_{24} $\Delta 6$ substrates. Understanding this pathway is of increased importance due to the current dependence of salmonid and marine fish aquaculture on fish oil, the supply of which is becoming increasingly limited and unsustainable, necessitating the use in fish feeds of sustainable plant oils, rich in C_{18} PUFA, but devoid of C_{20} and C_{22} PUFA (Sargent et al. 2002).

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In vivo assays of docosahexaenoic acid biosynthesis in fish

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Abbreviations: docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), fatty acid ethyl ester (FAEE), fish oil (FO), polyunsaturated fatty acids (PUFA), vegetable oil (VO).

Key words: *docosahexaenoic acid, biosynthesis, trout*

Abstract

A method was developed for measuring the synthesis of docosahexaenoic acid from linolenic acid in vivo. Rainbow trout (*Oncorhynchus mykiss*) were fed a pulse of diet containing deuterated (D₅) (17,17,18,18,18)-18:3n-3 ethyl ester and 21:4n-6 ethyl ester and sampled at intervals post-dose. The appearance of D₅-22:6n-3 in different tissues was determined by gas chromatography-negative chemical ionisation mass spectrometry of the pentafluorobenzyl esters. In trout of circa 5 g weight acclimated onto a diet containing 11% vegetable oil, sufficient to meet the essential fatty acid requirement for n-3 and n-6 fatty acids, and 5% fishmeal, the whole body accumulation of D₅-22:6n-3 was linear over 7 days corresponding to a rate of 0.54 ± 0.12 ug g fish⁻¹ mg D₅-18:3n-3 eaten⁻¹ day⁻¹. The amount of D₅-22:6n-3 peaked in liver at day 7, in the whole carcass at day 14 and in brain and eyes at day 24. Trout reared on a diet lacking fish oil and fish meal, and therefore completely lacking 20:5n-3 and 22:6n-3, gave a similar rate of 22:6n-3 synthesis whereas fish reared on a diet containing 11% fish oil gave a rate of approximately one tenth those fish reared on vegetable oil. Smaller trout (0.8 g) fed a diet containing no fish meal or fish oil gave a rate of 22:6n-3 synthesis almost 10 fold higher than that of 6 g fish. A further series of experiments showed that caeca as well as liver were a site of 22:6n-3 synthesis. At early time points (2 days post-feeding) over 5 times more D₅-22:6n-3 was found in caeca than in liver on a per mg protein basis and this decreased towards unity by day 15. The method is sensitive (threshold circa 1 pg fatty acid) and permits identification and quantitative analysis of all the pathway intermediates (D₅-18:4n-3, D₅-20:4n-3, D₅-20:5n-3, D₅-22:5n-3, D₅-24:5n-3, D₅-24:6n-3) if required.

Introduction

It is well established that marine fish and their larvae are unable to synthesise long-chain n-3 polyunsaturated fatty acids (PUFA) from linolenic acid (18:3n-3) and therefore have an absolute dietary requirement for long-chain n-3 PUFA, specifically eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Sargent et al. 1999). Since the larvae of marine fish are small they require a diet of very small particle size and in mariculture this is met by live prey such as zooplankton, rotifers and *Artemia*. However, because rotifers and *Artemia* have a poor PUFA profile (Leger et al. 1986), a great deal of effort has been expended in developing systems for enriching rotifers and *Artemia* with PUFA concentrates to ensure the correct dietary amounts and ratios of the different PUFAs are supplied (e.g. McEvoy et al. 1996).

In contrast freshwater fish, including salmonids, are capable of further desaturation and elongation of dietary 18:3n-3 and 18:2n-6 to the functionally essential C20 and C22 PUFA, 22:6n-3 and 20:4n-6 (Henderson and Tocher 1987). Salmonid larvae are considerably larger than the larvae of most marine fish and can take artificial diets based on fish meal and fish oil from first feeding so their PUFA biosynthetic ability is not required under normal commercial feeding regimes. The decline in global catch fisheries, and therefore a shrinking supply of marine fish meal and oil, together with a rapidly expanding world aquaculture industry, means it is increasingly desirable to spare this resource by substituting alternative meals and oils and to enhance the production of DHA from 18:3n-3 in those species possessing the DHA biosynthetic pathway (Sargent and Tacon 1999).

Docosahexaenoic acid is a functionally essential fatty acid in all vertebrates (Lauritzen et al. 2001) including fish (Bell et al. 1995). In spite of this, there is little quantitative information available on rates of formation in any species or on the bioequivalence of 18:3n-3 and 22:6n-3. The use of stable isotope tracers and GC-MS have allowed this problem to be addressed at the whole animal level for the first time (reviewed by Emken 2001). Here we describe an *in vivo* assay for DHA biosynthesis which uses deuterated linolenic acid (D_5 -17,17,18,18,18)-18:3n-3 as substrate with quantitation by GC-negative chemical ionisation MS of the pentafluorobenzyl esters and selective ion scanning for product D_5 -fatty acids. The method was developed in small trout (4-6 g) (Bell et al. 2001) but in principle is applicable to any species from first-feeding. The aim of this paper is to show the type of information which can be gained from this approach, specifically the effects of dietary oils and fish size on the rate of DHA synthesis, and the tissue distribution of newly synthesised DHA and intermediate fatty acids.

Materials and Methods

Chemicals. Chloroform, methanol, ethanol, isohexane and diethyl ether were HPLC grade from Fisher (Loughborough, Leicestershire, UK). Diisopropylamine, anhydrous acetonitrile and pentafluorobenzyl bromide were obtained from Aldrich (Gillingham, Dorset, UK). D_5 (17,17,18,18,18)-linolenic acid was purchased from Cambridge Isotope Laboratories, Andover, MA as the fatty acid ethyl ester. Linseed oil was from ICN (Basingstoke, Hampshire, UK) and refined olive oil from Tesco supermarkets. High oleic acid sunflower oil was a gift from Croda Chemicals, Goole, UK. Fish meal was from Biomar, Grangemouth, UK. All other chemicals were from Sigma (Poole, Dorset, UK).

Synthesis of 21:4n-6 and 24:6n-3. Heneicosatetraenoic acid ($\Delta 6,9,12,15-21:4$) ethyl ester was prepared by a one carbon addition to 20:4n-6 fatty acid (Rodriguez et al. 1997). The product was obtained in 51.2% yield and was 98.9% pure by GC and GC-MS of the fatty acid methyl ester. Tetraosaheptaenoic acid ($\Delta 6,9,12,15,18,21-24:6$) ethyl ester was similarly prepared by two sequential one carbon additions to 22:6n-3 and was obtained in 30.2% yield, 94.0% pure.

Fish and diets. Rainbow trout were obtained from a commercial hatchery and kept in a running freshwater aquarium at ambient temperature (3.5 to 16.5°C) on a 14 h/10 h light/dark cycle. Fish were fed a diet based on casein and a blend of vegetable oil containing predominantly oleic acid with 18:2n-6 and 18:3n-3 at approximately 1% each, to maximise 22:6n-3 synthesis and satisfy the fish's essential fatty acid requirements. The full composition of the diet was (g.Kg⁻¹): vitamin-free casein 480, starch 150, fish meal 50, mineral mix 47, vitamin mix 10, arginine 4, methionine 3, cystine 2, leucine 4, orange G 1, α -cellulose 138.6, blended vegetable oil 110, antioxidant mix 0.4. The composition of the mineral mix, vitamin mix and antioxidant mix were described previously (Tocher et al. 1996). The final diet provided 50% crude protein and 11% oil blended to give 0.99% 18:2n-6, 1.02% 18:3n-3 and 0.12% highly unsaturated fatty acids (20:5n-3 and 22:6n-3) from the fish meal, which was added to make the diet palatable and readily accepted by the fish. The remaining fatty acids were predominantly 16:0 (1.02%) and 18:1n-9 (7.18%). Later experiments substituted all the fish meal for casein and replaced 9% of the casein with 9% gelatin. The diets were gelled as described by Castell et al. (1972) and fed wet. Fish were fed the experimental diets for at least 8 weeks before starting the experiments.

Preparation of labelled diet. A small portion of diet containing D₅-18:3n-3 fatty acid ethyl ester (FAEE) and 21:4n-6 FAEE was prepared as follows. An oil sample containing 10 mg D₅-18:3n-3 FAEE, 2.5 mg 21:4n-6 FAEE, 153 mg of high oleic acid sunflower oil and 61 μ g antioxidant was dissolved in 0.82 ml isohexane and 1.335 g of dry diet mix added. The isohexane was then removed at 37°C under nitrogen and the diet desiccated in vacuo for 18 h. The diet was mixed thoroughly, 0.95 ml water added and mixed to a stiff paste. This was extruded through a 1 ml disposable syringe, dried at room temperature for 2-3 h and cut into pieces appropriate for the size of fish. The diet was stored under argon at -20°C and was used within 3 days.

Experimental protocol. Groups of 10 to 26 fish were acclimated in 15, 40 or 100 litre tanks, depending on their size, with running water for at least 4 days before starting an experiment. They were then fed the labelled diet. For the larger fish (> 2 g weight) all the diet was observed to be eaten. With smaller fish any uneaten diet was recovered from the tank after 2 h and the lipids extracted and analysed to determine how much tracer had been ingested. The fish were then fed the normal unlabelled diet and sampled at intervals thereafter. The water temperature ranged from 9.5 to 12.5°C for all groups of fish assayed.

Fish were terminally anaesthetised with MS 222 (ethyl 3-aminobenzoate methane sulphonate) and weighed. Individual tissues were dissected for analysis as described earlier (Bell et al. 2001). Caeca were the section of mid gut containing the caeca. As much adipose tissue as possible was removed from the gut tissues. Portions of liver and caeca were taken for protein determination by

the method of Lowry et al. (1951). Samples were homogenised in chloroform/methanol 2:1 (by vol.) using a Potter or ultraturrax homogeniser and a Folch extract prepared (Folch et al. 1957). Tricosanoyl glycerol (tri23:0) standard was added to each sample before homogenisation. The amount of tri23:0 standard added varied with the weight of the fish and the lipid content of the respective tissues, and was judged by previous experience such that the correct proportion of 23:0 to deuterated fatty acid and correct loading of 23:0 on the GC-MS was maintained (Bell et al. 2001). Samples were kept on ice under nitrogen during work up and were stored at -20°C under argon.

Quantitation of fatty acids. One mg of total lipid was saponified with 2 ml of 0.1M KOH in 95% (by vol) ethanol under nitrogen for 1 h at 78°C . Non-saponifiable material was removed by extracting with isohexane/diethyl ether (2:1, by vol), the aqueous phase acidified and free fatty acids extracted with diethyl ether. Pentafluorobenzyl esters were then prepared from 100 μg free fatty acid using acetonitrile/diisopropylamine/pentafluorobenzyl bromide (1000:10:1, by vol) at 60°C for 30 min under nitrogen (Pawlosky et al. 1992). Excess reagent and solvent were removed under nitrogen and samples dissolved in isohexane and stored at -20°C under argon until analysis.

Calibration standards of individual fatty acids (18:3n-3, 18:4n-3, 20:4n-6, 21:4n-6, 20:5n-3, 22:5n-3, 22:6n-3 and 24:6n-3) with 23:0 were prepared by varying the amount of each fatty acid while keeping the 23:0 constant and plotting the peak area ratio against the mass ratio of the different fatty acids. Sample volumes for analysis were adjusted such that the amount of 23:0 injected onto the GC-MS was constant. Pentafluorobenzyl esters were chromatographed and quantitated on a Fisons MD 800 GC-MS fitted with an on-column injector and a Chrompack CP wax 52CB column (30 m x 0.32 mm id, 0.25 micron film thickness) (Burke Analytical, Alva, Clackmannanshire, UK), using helium as carrier gas (column head pressure 7 psi) and running in negative chemical ionisation mode with methane as reagent gas (pressure 7 psi). The temperature programme was $80\text{--}190^{\circ}\text{C}$ at $40^{\circ}\text{.min}^{-1}$, $190\text{--}240^{\circ}$ at $1.5^{\circ}\text{.min}^{-1}$, then 240°C for 10 min. Fatty acids were identified by selective ion scanning for the required masses using a dwell time of 80 msec and cycle time of 20 msec, and quantitated by reference to the appropriate fatty acid calibration curve. Arachidonic acid (20:4n-6) was used as the standard for 20:4n-3 and 24:6n-3 as the standard for 24:5n-3.

Analysis of results. Data are presented as means \pm SD. Mean values were compared using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test (Prism statistical package, GraphPad Software Inc., San Diego).

Results

Initial experiments sampling at 3, 7, 14, 24 and 35 days post-dose of $\text{D}_5\text{-}18:3\text{n-}3$ showed that the synthesis and tissue deposition of newly formed $\text{D}_5\text{-}22:6\text{n-}3$ was slow (Fig. 1). Whole body accumulation peaked at day 14 ($4.3 \pm 1.2 \mu\text{g D}_5\text{-}22:6\text{n-}3\text{.g fish}^{-1}\text{.mg D}_5\text{-}18:3\text{n-}3 \text{ eaten}^{-1}$) then decreased slightly thereafter (Fig 1A). This corresponded to a rate of $0.54 \pm 0.12 \mu\text{g D}_5\text{-}22:6\text{n-}3\text{.g fish}^{-1}\text{.mg D}_5\text{-}18:3\text{n-}3 \text{ eaten}^{-1}\text{.day}^{-1}$ over the first 7 days. In liver deposition peaked at day 7 (Fig 1B) at $0.51 \mu\text{g D}_5\text{-}22:6\text{n-}3\text{.g fish}^{-1}\text{.mg D}_5\text{-}18:3\text{n-}3 \text{ eaten}^{-1}$ and fell to $0.07 \mu\text{g D}_5\text{-}22:6\text{n-}3\text{.g fish}^{-1}\text{.mg D}_5\text{-}18:3\text{n-}3$

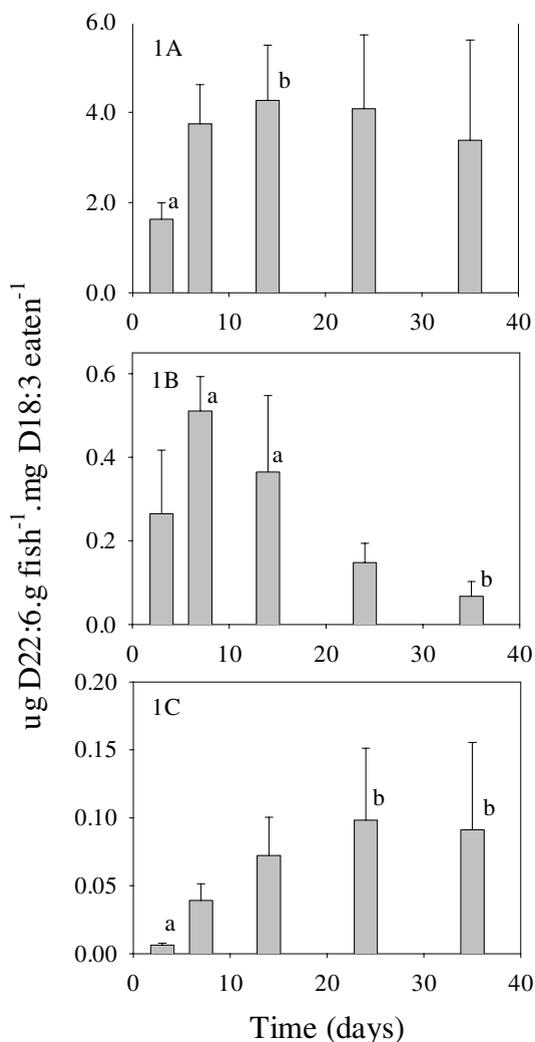


Figure 1. The accumulation of newly synthesised $D_5-22:6n-3$ in whole body (1A), liver (1B) and brain (1C) following an ingested pulse of $D_5-18:3n-3$. $N = 5$ for day 3, 4 for day 7, 5 for day 14, 3 for day 24 and 4 for day 35. One-way analysis of variance (ANOVA) gave differences in mean values of $p = 0.054$ for whole body, $p = 0.0012$ for liver and $p = 0.0114$ for brain. Values with different superscripts are significantly different at $p < 0.05$, those without superscripts are not significantly different from any other value.

eaten⁻¹ by day 35 while brain (Fig 1C) accumulated $D_5-22:6n-3$ to 24 days. Accretion of $D_5-22:6n-3$ in eyes was similar to that in brain peaking at 24 days (data not shown) (Bell et al. 2001).

Newly synthesised $D_5-22:6n-3$ accumulated in the mid gut containing the caeca and in liver to a similar extent at early time points (days 1, 2 & 3 post-dose) (Table 1). There was no significant difference between the values at 1, 2 and 3 days post-dose so the data was pooled. The stomach and gut posterior to the caeca contained little $D_5-22:6n-3$ (Table 1). A second experiment over a longer time course up to 15 days investigated the deposition of newly synthesised DHA in caeca and liver in more detail. Caeca contained 5 times more $D_5-22:6n-3$ than liver on a per mg protein basis two days post-dose of $D_5-18:3n-3$ (Table 2). The amount of newly synthesised $D_5-22:6n-3$ doubled in liver between 2 and 5 days post-dose but fell by almost 30% in caeca over the same time (Table 2). The ratio of $D_5-22:6n-3$ in caeca and liver approached unity by day 9.

All the $n-3$ fatty acid pathway intermediates ($18:4n-3$, $20:4n-3$, $20:5n-3$, $22:5n-3$, $24:5n-3$ and $24:6n-3$) were identified in liver and caeca (Fig 2). In both tissues $D_5-22:6n-3$ was the predominant fatty acid at both times increasing from 34.7 to 73.8% in liver (Fig 2A) and from 40.4 to 66.2% in caeca (Fig 2B) between 2 and 5 days post-dose of $D_5-18:3n-3$. In liver $D_5-20:5n-3$ and $D_5-22:5n-3$ were the next most abundant deuterated fatty acids comprising 16.1% and 13.9% respectively at day 2 (Fig 2A) and by day 5 these values had fallen to 6.6% and 8.0% respectively. In caeca $D_5-20:5n-3$, $D_5-18:3n-3$ and $D_5-18:4n-3$ all comprised between 12.5 and 15.2% at day 2, and by day 5 $D_5-18:4n-3$ totalled 10.8% with all other fatty acids less than 6.1%. $D_5-24:5n-3$ was a trace component in both tissues at both

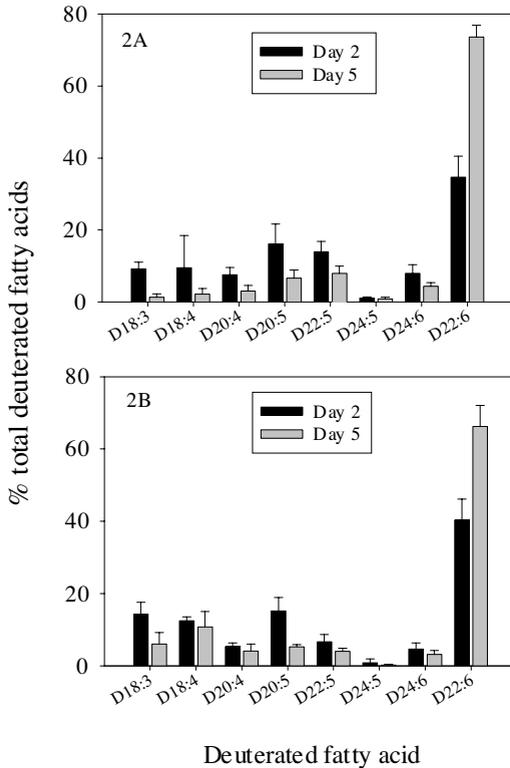


Figure 2. The distribution of deuterated fatty acids in liver (2A) and caeca (2B) two and five days after feeding a pulse of D_5 -18:3n-3. $N = 5$ for day 2 and 6 for day 5.

time points while D_5 -24:6n-3 comprised between 3.2 and 8.0%.

All the above experiments were undertaken with trout fed a diet containing 5% fish meal to aid palatability. A further series of experiments were undertaken using diets with no fish meal to examine whether 22:6n-3 synthesis was fully induced under the earlier conditions. These diets also contained 9% gelatin replacing 9% of the casein (Castell et al. 1972). Groups of fish were reared on diets containing either 11% vegetable oil or 11% fish oil. In addition small trout fed from first-feeding on the 11% vegetable oil diet were also assayed. Trout reared on an 11% vegetable oil diet containing no fish meal gave a rate of 22:6n-3 formation very similar to that found with 11% vegetable

Table 1. The deposition of newly synthesised D_5 -22:6n-3 in liver and gut tissues ($n=9$).

| Tissue | Amount of D_5 -22:6n-3 deposited | | % of body total |
|----------|--|---------------------------------------|-----------------|
| | ng D_5 -22:6n-3. mg tissue ⁻¹ . | mg D_5 -18:3n-3 eaten ⁻¹ | |
| Liver | 22.2 ± 11.5 | | 24.4 ± 10.5 |
| Stomach | 0.37 ± 0.27 | | 0.5 ± 0.2 |
| Caeca | 14.6 ± 10.9 | | 23.2 ± 14.8 |
| Hind gut | 1.97 ± 2.17 | | 1.0 ± 0.8 |

Table 2. Comparison of D_5 -22:6n-3 deposition in liver and caeca at intervals post-dose of D_5 -18:3n-3. The number of fish for each time point is given in parentheses. Values are mean ± SD. One-way ANOVA gave differences in the caeca to liver ratio of $p = 0.0003$. Within columns values with different superscripts are different at $p < 0.01$. Mean weight of fish 15.6 ± 3.8 g.

| Time (days) | ng D_5 -22:6n-3. mg protein ⁻¹ . mg D_5 -18:3n-3 eaten ⁻¹ | | Caeca:liver ratio |
|-------------|---|-------------|--------------------------|
| | Caeca | Liver | |
| 2 (5) | 186.0 ± 164.2 | 47.2 ± 46.3 | 5.18 ± 2.39 ^a |
| 5 (6) | 132.6 ± 62.3 | 93.1 ± 48.7 | 1.40 ± 1.23 ^b |
| 9 (4) | 106.2 ± 51.1 | 99.1 ± 41.8 | 1.06 ± 0.16 ^b |
| 15 (6) | 56.7 ± 21.7 | 59.1 ± 29.8 | 0.93 ± 0.55 ^b |

Table 3. Rates of synthesis of 22:6n-3 in rainbow trout of different sizes and fed different diets.

| Group | Diet | Weight (g) | No. of fish | Rate ($\mu\text{g D}_5\text{-22:6n-3.g fish}^{-1}.\text{mg D}_5\text{-18:3n-3 eaten}^{-1}.\text{7 days}^{-1}$) |
|-------|----------------|-----------------|-------------|--|
| 1 | 11% VO + 5% FM | 7.51 \pm 0.98 | 4 | 3.75 \pm 0.87 |
| 2 | 11% VO | 6.30 \pm 0.88 | 5 | 4.05 \pm 0.81 |
| 3 | 11% FO | 7.97 \pm 1.66 | 5 | 0.38 \pm 0.12 |
| 4 | 11% VO | 0.80 \pm 0.30 | 12 | 33.6 \pm 12.6 |

FM = fish meal, FO = fish oil. One-way ANOVA for 11% VO versus 11% FO (groups 1 and 2 versus 3) gave $p < 0.001$ and for fish size (group 1 and 2 versus 4) gave $p < 0.001$.

oil plus 5% fish meal (4.05 versus 3.75 $\mu\text{g D}_5\text{-22:6n-3.g fish}^{-1}.\text{mg D}_5\text{-18:3n-3 eaten}^{-1}.\text{7 days}^{-1}$ respectively) (Table 3) while fish reared on 11% fish oil gave a rate of approximately one tenth that of fish fed vegetable oil (Table 3). Small trout (weight 0.8 g) gave a rate some 10 fold higher than fish of 6 to 7 g weight reared on the same diet (Table 3).

Discussion

The method described has proved to be versatile and has given the first quantitative information on rates of DHA formation in fish. It is sensitive and in selective ion scanning mode can measure 1 pg of $\text{D}_5\text{-22:6n-3}$ comfortably (signal to noise ratio of circa 10:1). The use of $\text{D}_5\text{-21:4n-6}$ as an ingestion marker, rather than relying on measuring remaining $\text{D}_5\text{-18:3n-3}$, gives a more reliable estimate of the amount of diet eaten by individual fish (see Bell et al. (2001) for more detail). Most of the $\text{D}_5\text{-18:3n-3}$ was catabolised (recovery 1.4-13.5%) and the relative recovery of $\text{D}_5\text{-21:4n-6}$ was 4.3-25.6 more than that of $\text{D}_5\text{-18:3n-3}$.

The finding that caeca accumulate newly synthesised DHA more rapidly than liver and to a higher concentration strongly implies that caeca are an important site of DHA synthesis. This is significant since it was previously thought that liver was the main site of PUFA biosynthesis in fish (Henderson and Tocher 1987). In the absence of precise information on the flux of substrate to the respective tissues it is difficult to quantitate the relative contributions of each tissue but the results here suggest that the caeca are the more important tissue. The identification of all the pathway intermediate fatty acids, including $\text{D}_5\text{-24:5n-3}$ and $\text{D}_5\text{-24:6n-3}$, is consistent with the pathway for DHA synthesis proposed by Voss et al. (1991) where the final stage of the pathway does not involve a direct Δ^4 desaturation of $\text{D}_5\text{-22:5n-3}$ but an elongation to $\text{D}_5\text{-24:5n-3}$, Δ^6 desaturation to $\text{D}_5\text{-24:6n-3}$ and peroxisomal chain-shortening to $\text{D}_5\text{-22:6n-3}$.

The rate of DHA synthesis was slow and in 4-6 g fish reared on a diet containing 11% vegetable oil and 5% fish meal the whole body concentration of DHA fell over the time the fish were on this diet (Bell et al. 2001). DHA synthesis is repressed by dietary EPA and DHA (Buzzi et al. 1996) but the minimal amounts of these fatty acids present in this diet were not thought sufficient to repress synthesis. Fish reared on a diet completely lacking fish meal, and therefore any long-chain PUFA, gave a similar rate of DHA formation suggesting that DHA synthesis was indeed fully active under the earlier dietary conditions. Trout fed a diet containing 11% fish oil, where DHA synthesis

should be fully repressed, showed a rate of DHA formation approximately one tenth that of the fully induced fish fed 11% vegetable oil.

In the original studies on essential fatty acid requirements in rainbow trout by Castell et al. (1972) the whole body concentration of DHA was maintained at a higher level implying a higher rate of synthesis than that found here. Assays of 0.8 g rainbow trout fed the 11% VO diet gave a rate of DHA synthesis almost 10 times higher than in 6 g fish reared on the same diet. It therefore appears that the capacity to synthesise DHA is down-regulated early in development. The effects of fish size and dietary oil content on the rate of DHA synthesis are currently being investigated further.

Modern molecular biological techniques capable of identifying the genes for delta-5 and delta-6 desaturase (Hastings et al. 2001) coupled with this in vivo assay provide powerful tools for furthering our understanding of DHA synthesis and the factors which control it. Apart from the basic interest in elucidating the processes involved, opportunities exist for enhancing DHA production in farmed fish. Two obvious approaches are to identify the molecular switch responsible for down-regulating DHA synthesis early in development and override it, and secondly to inhibit the oxidation of 18:3n-3 so that more is spared for DHA synthesis. Ultimately it may be possible to genetically modify marine fish so they are not dependent on dietary long-chain PUFA.

Acknowledgments

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Accumulation of DHA (docosahexaenoic acid; 22:6n-3) in larval and juvenile fish brain

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Key words: *docosahexaenoic acid, brain, accumulation, metabolism, development, marine fish*

Abstract

As in mammals, a critical functional role for n-3HUFA (highly unsaturated fatty acids), specifically docosahexaenoic acid (DHA; 22:6n-3), in neural tissues has been established in larval and juvenile fish. Accumulation of DHA in brain during development has been demonstrated in several marine fish species. A very low rate of DHA biosynthesis was observed in turbot brain but a rapid accumulation of DHA in brain of turbot and gilthead sea bream was observed during weaning from live to pelleted food. The incorporation of [1-¹⁴C] linolenic acid (LNA; 18:3n-3) and [1-¹⁴C] DHA in juvenile turbot brain cells showed no significant differences between the amounts of LNA and DHA incorporated into brain phospholipids demonstrating no preferential uptake and incorporation of DHA into brain cells. However, during 24h incubation, 1.1% and 8.5% of radioactivity from [1-¹⁴C] LNA and [1-¹⁴C] eicosapentaenoic acid (EPA; 20:5n-3), respectively, were recovered in the DHA fraction of turbot brain lipids. Thus, LNA bioconversion cannot contribute significantly to brain DHA, whereas EPA can to a greater extent. In a further study, the *in vivo* metabolism of intraperitoneally injected [1-¹⁴C] LNA in liver, brain and eyes of juvenile rainbow trout and gilthead sea bream showed that, although the sea bream incorporated more LNA into its lipids, the bioconversion of LNA was greater in the trout. The proportion of radioactivity recovered in desaturated/elongated products was much lower in liver than in brain and eyes in both species, but the recovery of radioactivity in DHA in brain was significantly higher in trout compared to sea bream. Overall, although the results did not eliminate a role for liver in the biosynthesis and provision of DHA for developing neural tissues in fish, they indicated that DHA can be synthesised in fish brain and eye *in vivo*. However, they also suggested that the level of DHA in marine fish brain is largely due to dietary DHA levels than to PUFA bioconversion capabilities. In conclusion, as the DHA in neural tissues is mainly of dietary origin, irrespective of the metabolic capacity of PUFA bioconversion in the different tissues and fish species, the adequate supply of n-3HUFA, particularly DHA, during the early stages of fish is crucial for the normal development of the neural system and to avoid behavioural abnormalities (raptorial or schooling behaviour) due to visual and/or neural impairment. Dietary deprivation of DHA can be particularly deleterious in larvae and juveniles from fast growing large pelagic marine species such as carangids and tunids.

Introduction

The understanding of nutrition during early development in all species of interest is a major prerequisite to counter the major challenge of marine fish larvae culture and, in particular, lipid nutrition in marine fish is considered crucial to ensure successful culture. The importance of lipid biochemistry and metabolism in marine fish has been extensively reviewed, as well as the requirements and possible roles of particular lipid molecules in marine fish larvae during development (Sargent et al. 1993b; Watanabe 1993). The abundance of long chain polyunsaturated fatty acids of the n-3 series (n-3 PUFA), mainly eicosapentaenoic acid (EPA; 20:5(n-3)) and docosahexaenoic acid (DHA; 22:6(n-3)) in fish oils is only surpassed by the greater abundance of these fatty acids in the phosphoglycerides of fish cellular membranes. The very high concentration of DHA in fish neural tissues is also well established (Tocher and Harvie 1988; Bell and Dick 1991).

Marine fish are not capable of bioconverting C18 PUFA to their C20 and C22 homologues, probably due to a deficiency in the $\Delta 5$ fatty acid desaturase and/or the C18-20 elongase (Sargent et al. 1993a), consequently, marine fish invariably obtain EPA and DHA from their natural diet which is rich in these particular fatty acids (Sargent et al. 1995).

DHA has a critical role in neural tissues in mammals, and is accumulated by these tissues during development; nutritional depletion of DHA in the brain and retinal membranes is accompanied by functional defects such as reduced visual acuity and impaired learning abilities (Lauritzen et al. 2001). In fish, neural tissue, especially the eyes, can form a relatively large proportion of the total body mass of the embryo and larvae, so that a large portion of the total DHA required for embryonic and larval growth is directed towards the formation of cell membranes vital for normal development and functioning of the visual and neural systems. Therefore, any deficiency in DHA during embryonic and larval development will have serious consequences for the successful performance of sophisticated predatory larvae, particularly during and immediately following first feeding (Sargent 1995).

The present paper summarises a series of studies which show the specificity of DHA uptake by the brain of larvae and juvenile fish, as well as the metabolism of (n-3) PUFA in the brain, eye and liver clarifying the relative importance of these tissues in the biosynthesis and provision of DHA.

Variations in brain DHA at different stages of development in a wild marine fish species. A study was carried out to determine the changes in composition of brain lipids and fatty acids in wild Atlantic herring (*Clupea harengus* L.) at four different stages of development: larvae at the end of the yolk sac stage, two different juvenile stages and sexually mature adults. In brain total lipids, saturated fatty acids generally decreased and monounsaturated fatty acids and dimethyl acetals (derived from plasmalogens) increased from larvae to adults. However, the proportions of polyunsaturated fatty acids in total lipids and individual brain phosphoglycerides were generally highest in juvenile stages, due mainly to increased DHA, and were lowest in adult fish (Figure 1). In conclusion, a significant accretion of DHA in brain lipids of Atlantic herring was observed during larval and juvenile stages (Mourete and Tocher 1992a).

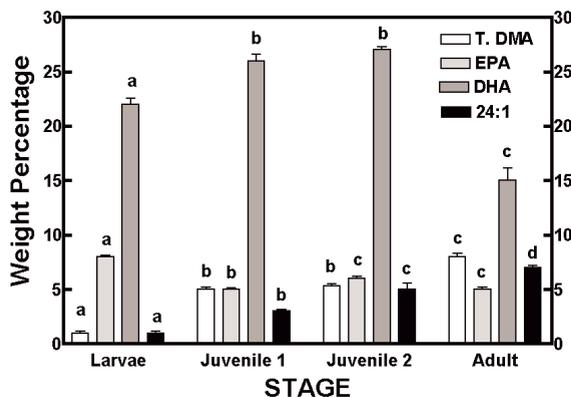


Figure 1. Variations of selected total lipid fatty acids in brain of Atlantic herring (*Clupea harengus* L.), including DHA, at different stages of development. T. DMA, total dimethyl acetals; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; 24:1, nervonic acid. Values not bearing the same superscript are significantly different ($p < 0.05$). Data taken from Mourente and Tocher (1992a).

Specific accumulation of DHA in brain lipids during development of juvenile turbot. Juvenile turbot (*Scophthalmus maximus* L.) were sampled during a 10-week period immediately following weaning from live feed to a pelleted diet and the changes in the lipid class and fatty acid compositions of total lipids and individual glycerophospholipids in brain were investigated during these early developmental stages. The percentage of DHA in brain lipids was low at the beginning of the study period but a specific and significant accumulation of this fatty acid was observed during development. The percentages of DHA increased in total brain lipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) by 3.2-fold, 11.7-fold, 2.7-fold, 2.0-fold and 2.1-fold, respectively, by the end of the 10-week period (Figure 2). The percentages of other n-3 and n-6 PUFAs generally decreased during this period, as did that of monoenes; the percentages of saturated fatty acids remained relatively constant. This result clearly demonstrated that the n-3 PUFA composition of the brains of early developing turbot can be rapidly and markedly influenced by the PUFA composition of the diet (Mourente et al. 1991).

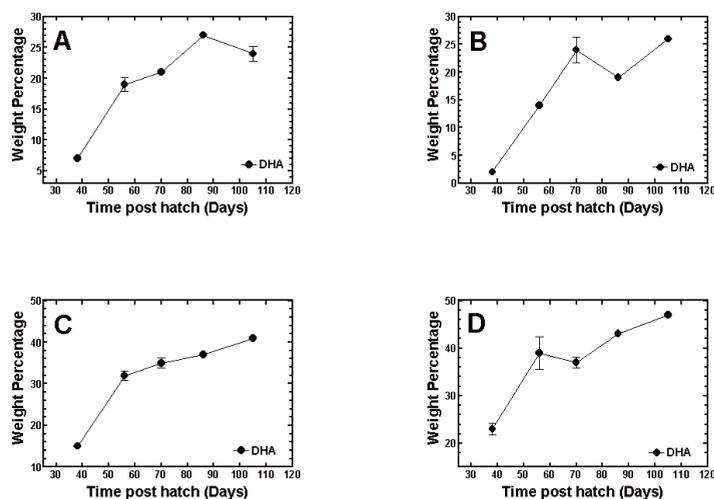


Figure 2. Specific accumulation of DHA in brain lipids during development of juvenile turbot (*Scophthalmus maximus* L.). A, variation of DHA in brain total lipids; B, variation of DHA in brain diradyl glycerophosphocholine; C, variation of DHA in brain diradyl glycerophosphoethanolamine; D, variation of DHA in brain phosphatidylserine. Data taken from Mourente et al. (1991).

Effects of weaning onto a pelleted diet on brain DHA of developing cultured marine fish. The results of the above study prompted a further investigation of the changes that might occur in brain DHA levels during the weaning period. The early larvae of marine fish are generally fed a mixture of live feeds consisting initially of rotifers *Brachionus spp.* and consequently of nauplii of the brine shrimp *Artemia*. Because both live preys are usually naturally deficient in EPA and specifically DHA, they have to be enriched with microalgae and/or supplements rich in these fatty acids (Bell 1998). After growth for a suitable period of time on these live feeds, the small juvenile fish are weaned on to dry pelleted diets consisting mainly of fish meal and fish oil. In this context, we studied the influence of the weaning diet alone on brain lipid and fatty acid composition of two marine fish species farmed in Europe, the turbot *Scophthalmus maximus* L. and the gilthead sea bream *Sparus aurata* L. Each species was maintained on the same dietary regime throughout early development, and fed enriched *Artemia* prior to weaning. At this point, one group of fish was fed a dry pelleted food whereas the other group remained on enriched *Artemia*. Both populations were sampled 7 days later and the brains dissected out and analyzed for lipid

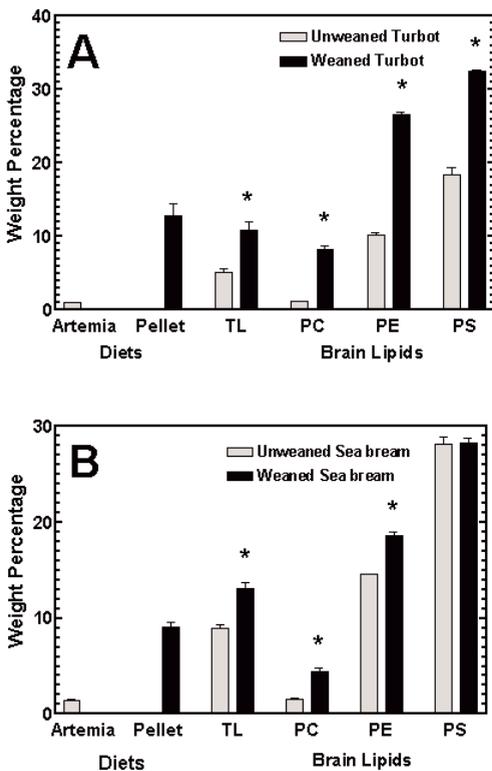


Figure 3. Effects of weaning onto a pelleted diet on brain total lipid DHA level in (A) turbot (*Scophthalmus maximus* L.) and (B) gilthead sea bream (*Sparus aurata* L.). Data taken from Mourente and Tocher (1992b; 1993).

content, lipid class and fatty acid compositions. The brine shrimp metanauplii, although enriched with fish oil, exhibited low levels of DHA. As a result, phospholipids from the brains of fish reared only with enriched *Artemia* were rich in EPA but relatively deficient in DHA. In contrast, in fish that had been weaned on to a fish meal and oil-based diet rich in DHA, the level of this particular fatty acid increased rapidly and significantly in their brain lipids, primarily in PC and PE (Figure 3). The rapid and specific incorporation of DHA into brain phosphoglycerides was accompanied by significant decreases in EPA (Mourente and Tocher 1992b; 1993).

Metabolism of [1-¹⁴C](n-3) PUFA in brain cells from juvenile turbot. The incorporation of [1-¹⁴C] 18:3(n-3) (LNA) and [1-¹⁴C] 22:6(n-3) (DHA), and the metabolism via the desaturase / elongase pathways of [1-¹⁴C] 18:3(n-3) (LNA) and [1-¹⁴C] 20:5(n-3) (EPA) were studied in brain cells from newly-weaned (1-month-old) and 4-month-old turbot. In this study we aimed to determine: i) if there was a differential uptake and incorporation of DHA into brain cells, ii) the extent to which LNA and EPA contribute to brain DHA levels and

iii) if these processes were affected by age and development of the fish. The rank order of the extent of net incorporation of both LNA and DHA into glycerophospholipids was $PC > PE > PS > PI$, and was independent of the PUFA added, the age of the fish and the time of incubation. However, the rate of incorporation of LNA into total lipid, PC, PE and PS was significantly greater than the rate of incorporation of DHA, and there was a significantly greater amount of DHA incorporated into PE than LNA. There was no significant difference between the amounts of LNA and DHA incorporated into total lipids, PC, PS and PI. Therefore, little preferential uptake and incorporation of DHA into brain cells was apparent. During 24 hours incubation, on average 1.1% and 8.5% of radioactivity from $[1-^{14}C]$ LNA and $[1-^{14}C]$ EPA, respectively, were recovered in the DHA fraction. Thus, LNA cannot contribute significantly to brain DHA levels in the turbot whereas EPA can to a greater extent. There were no significant differences between the amounts of radioactivity from either $[1-^{14}C]$ LNA or $[1-^{14}C]$ EPA recovered in the individual products / intermediates of the desaturase / elongase pathways in brain cells from 30-day-old and 120-day-old turbot (Figure 4). These results strongly suggested that the accumulation of DHA in turbot brain during development is not due to specific uptake and incorporation of DHA and that endogenous desaturation of LNA cannot contribute to DHA levels in the brain. This confirms our previous conclusions, from studies on lipid and fatty acid compositions of developing turbot brain, that the level of DHA in the brain was mainly dependent on the level of DHA in the diet (Tocher et al. 1992).

The *in vivo* incorporation and metabolism of $[1-^{14}C]$ linolenate ($18:3n-3$) in liver, brain and eyes of juveniles of rainbow trout *Oncorhynchus mykiss* L and gilthead sea bream *Sparus aurata* L.

Accumulation of docosahexaenoic acid (DHA; $22:6n-3$) in brain and eyes during development had been demonstrated in marine fish and significant biosynthesis of DHA from dietary $18:3n-3$ was not observed in either liver or neural tissues. However, it was not clear whether this was also the case in freshwater fish and whether liver or neural tissues themselves are of greater importance in the biosynthesis of DHA from dietary $18:3n-3$. In this study, we investigated the *in vivo* metabolism of intraperitoneally injected $[1-^{14}C]$ $18:3n-3$ in liver, brain and eyes of young juvenile freshwater and

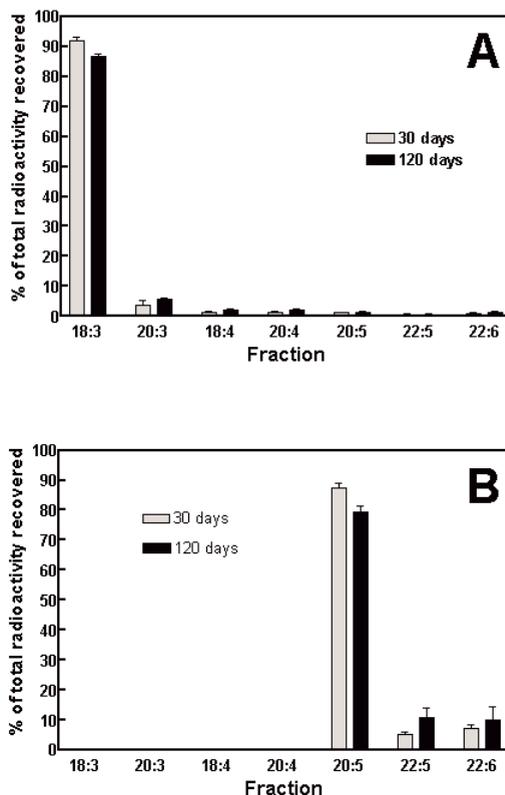


Figure 4. Metabolism of $[1-^{14}C](n-3)$ PUFA in brain cells from juvenile turbot (*Scophthalmus maximus* L.). Metabolism via the desaturase/elongase pathway 30 days and 120 days after hatch of (A) $[1-^{14}C]18:3(n-3)$ and (B) $[1-^{14}C]20:5(n-3)$. Data taken from Tocher et al. (1992).

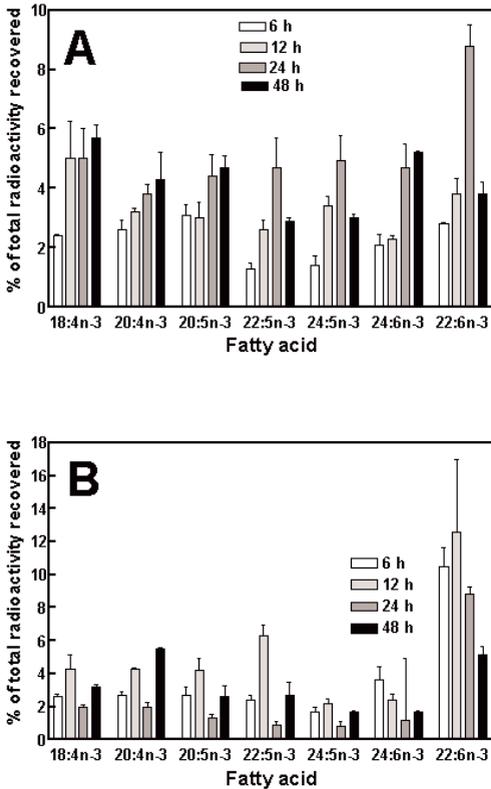


Figure 5. Metabolism via the desaturase elongase pathway of $[1-^{14}\text{C}]18:3n-3$ in brain of (A) gilthead sea bream (*Sparus aurata* L.) and (B) rainbow trout (*Oncorhynchus mykiss* L.). Data taken from Mourente and Tocher (1998).

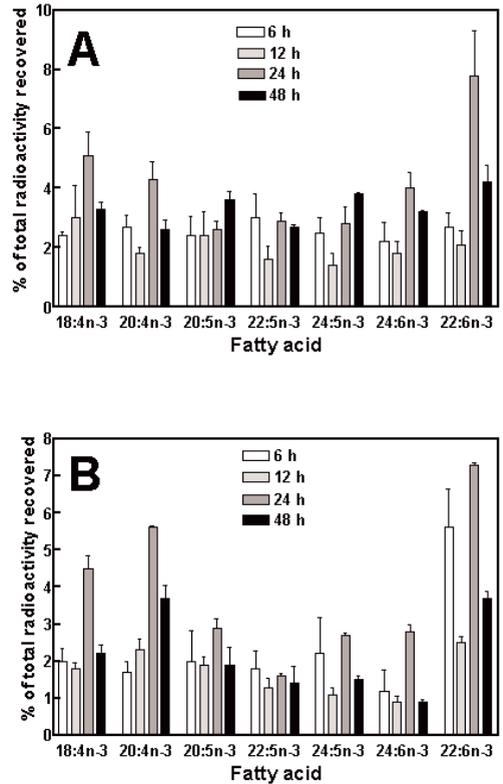


Figure 6. Metabolism via the desaturase elongase pathway of $[1-^{14}\text{C}]18:3n-3$ in eye of (A) gilthead sea bream (*Sparus aurata* L.) and (B) rainbow trout (*Oncorhynchus mykiss* L.). Data taken from Mourente and Tocher (1998).

marine fish. Metabolism was followed over a 48h time course in order to obtain dynamic information that could aid the elucidation of the roles of the different tissues in the biosynthesis and provision of DHA from dietary 18:3n-3. The study was performed in rainbow trout *Oncorhynchus mykiss* L. as an example of a freshwater fish, and gilthead sea bream *Sparus aurata* L., as an example of a marine fish, to determine the effect that low or limiting $\Delta 5$ -desaturase activity may have in this process. As expected, the results showed that although sea bream incorporated more 18:3n-3 into its lipids, metabolism of the incorporated fatty acid by desaturation and elongation was significantly greater in the trout. In liver, the percentages of radioactivity recovered in tetraene and pentaene products were greater in trout than in sea bream although there was no difference in hexaenes. In contrast, the recovery of radioactivity in DHA was significantly greater in brain of trout compared to sea bream. In both species, the percentage of radioactivity recovered in desaturated / elongated products was much

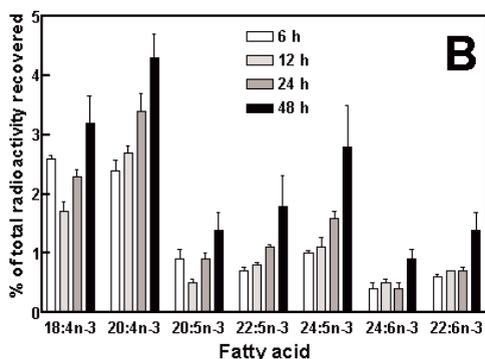
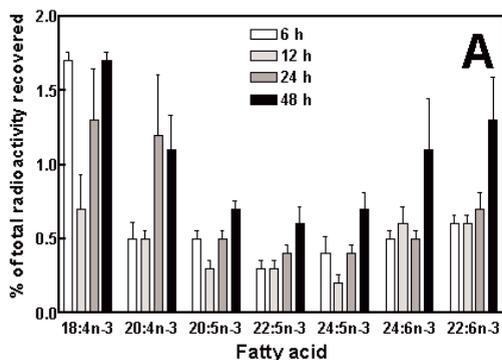


Figure 7. Metabolism via the desaturase elongase pathway of $[1-^{14}\text{C}]18:3n-3$ in liver of (A) gilthead sea bream (*Sparus aurata* L.) and (B) rainbow trout (*Oncorhynchus mykiss* L.). Data taken from Mourente and Tocher (1998).

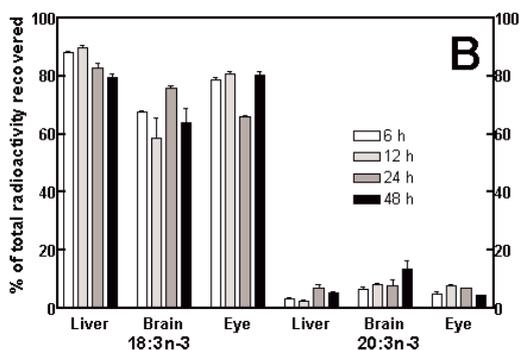
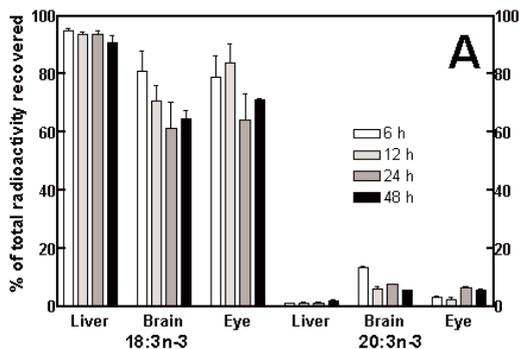


Figure 8. Unmetabolised $[1-^{14}\text{C}]18:3n-3$ recovered in brain, eye and liver of (A) gilthead sea bream (*Sparus aurata* L.) and (B) rainbow trout (*Oncorhynchus mykiss* L.). Percentage of radioactivity from $[1-^{14}\text{C}]18:3n-3$ recovered unmetabolised as $18:3n-3$ or elongated to $20:3n-3$. Data taken from Mourente and Tocher (1998).

lower in liver than in brain and eyes, but the percentages increased over the 48h time course. In trout, the highest percentages of desaturated products in brain and eye were observed after 12 and 24h, respectively. However in sea bream the highest percentages of desaturated products in the neural tissues were observed after 24-48h (Figures 5, 6, 7, and 8). Overall, although the results could eliminate a role for liver in the biosynthesis and provision of DHA for developing neural tissues in fish, they suggest that DHA can be synthesised in fish brain and eye *in vivo* (Mourente and Tocher 1998).

Role(s) of DHA in the formation of neural tissues and its consequences on performance and health of marine fish during development and growth.

Marine fish occupy an environment where the ability to see in low light intensities is of considerable importance. For this reason they have highly developed systems of rods in their retina. The abundance of DHA in the rods of higher vertebrates is well known and the fact that more than

50% of the total fatty acids in PE from rod outer segment membranes can be DHA has revealed the presence of di-DHA molecular species of this phosphoglyceride. The molecular species composition of the phosphoglycerides from the brain and retinas of several fish species were determined (Bell and Tocher 1989; Bell and Dick 1990; 1991) and results indicated that di-DHA species occurred in large proportions in phosphoglycerides of brain and retina. Therefore, a correlation was demonstrated between the appearance of rods in the eyes of herring (*Clupea harengus* L.) and the increasing of di-DHA molecular species of phospholipids (Bell and Dick 1993). Furthermore, dietary deficiency of DHA resulted in impaired vision at low light intensities in juvenile herring (*Clupea harengus* L.) (Bell et al. 1995), deficits of di-DHA phospholipids in eyes of sea bass (*Dicentrarchus labrax* L.) (Bell et al. 1996; Navarro et al. 1997), abnormal pigmentation in flatfish (Estevez and Kanazawa 1996) and behavioural differences in herring larvae (Navarro and Sargent 1992).

It is also relevant and noteworthy that di-DHA molecular species of phosphoglycerides are absent from eggs of marine fish (Bell 1989), so that the di-DHA species abundant in neural tissue must be assembled from pre-existing lipids in the eggs or from dietary lipid ingested by the larvae during embryogenesis and larval development, respectively (Sargent et al. 1993b).

More recent research has elucidated the critical role of dietary DHA during larval stages as essential for the development of schooling behaviour in carangid fish such as yellowtail (*Seriola quinqueradiata*) and the striped jack (*Pseudocaranx dentex*) (Masuda et al. 1998; Masuda and Tsukamoto 1999; Masuda et al. 1999; Ishizaki et al. 2001). Furthermore, mortality of northern bluefin tuna (*Thunnus thynnus*), resulting from trauma caused by collisions with culture tank walls (Miyashita et al. 2000; Masuma et al. 2001), is possibly a result of visual disorientation related to impaired retinomotor responses. Although not as yet demonstrated, it is possible that such visual impairment is related to dietary DHA deficiency.

Conclusions and Perspectives

Marine fish larvae have an absolute requirement for (n-3) HUFA, particularly DHA; dietary deficiencies of these induce a range of pathologies in fish including malpigmentation and visual and behavioural abnormalities. Therefore, carnivorous (piscivorous) marine fish have little or no ability to modify C18 PUFA assimilated from their diets through anabolic reactions in the liver and other tissues, although they can modify assimilated C22 PUFA by catabolic (chain shortening) reactions. This limitation might appear to place a requirement for selectivity of uptake of DHA from blood lipids, enabling the brain and eyes to achieve a PUFA profile from that of circulating blood lipids derived from the intestine and/or liver. Thus, the ease with which changes in brain PUFA compositions of these species can be elicited by changing their dietary PUFA composition, leads to the conclusion that the specificities of the brain fatty acyl transferases incorporating PUFA into major brain phosphoglycerides are very low. Therefore, the neural and visual tissues of these fishes are highly vulnerable to changes in circulating PUFA in the blood lipids resulting from changes in dietary PUFA (Sargent et al. 1993b). These studies emphasize the importance of providing sufficiently high dietary levels of DHA for adequate neural development in species or individuals with limited ability to elongate and desaturate C18 and C20 PUFA to their C22 homologues.

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The critical role of docosahexaenoic acid in marine and terrestrial ecosystems: from bacteria to human behavior

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Abstract

Docosahexaenoic acid (DHA), together with other fatty acids, has been an important research target for both fishery and medical scientists. The aim of this review is to integrate information from both groups of disciplines and, thereby, elucidate the importance of its physiological and ecological functions. DHA is abundant in the brain and retina of vertebrates. Administration of DHA improves the learning capability in mice and rats, and mitigates aggressive behavior in humans. DHA also improves membrane fluidity of cells and reduces activity of cancer cells. Although humans can synthesize DHA from α -linolenic acid and eicosapentaenoic acid, it is by far more efficient to take supplemental DHA to improve development of the central nervous system, visual capability and other health factors. In the case of fishes, DHA-deficiency brings out the disability of feeding at low light intensity, the lack of school formation, and low growth or survival in the long run. Unlike most terrestrial animals, marine fishes cannot synthesize DHA de novo from precursor molecules. For the successful larval fish culture, broodstock should be fed proper amounts of DHA to spawn good quality of eggs, and prey for larval fish have to be enriched with oil containing DHA oil. In the natural marine ecosystem, algae and, to a lesser extent, bacteria, are the major producers of DHA. Copepods and other crustaceans accumulate DHA by feeding on algae, and fish larvae feed on these zooplankton. Since DHA production of algae is reduced by UVB (280-320 nm wavelength) radiation, the increase of UVB on the ocean surface may have a negative impact on fish production through the DHA food chain. Some pelagic fish species have symbiotic bacteria that can produce DHA and, thus, may have an advantage under such conditions. Since DHA-deficiency is easily manipulated in marine fish larvae, the behavior of fish should be a useful experimental model to study the function of DHA. Possible reduction of the amount of DHA in the marine ecosystem should be carefully studied through a multidisciplinary approach applying meteorological, oceanographic, fisheries and biochemical studies.

Introduction

A multi-disciplinary scientific approach often gives deeper insight into certain subjects than digging from each sub-field solely. Reviewing certain topics by covering several different scientific fields is demanding but rewarding. One of such successful efforts was made by Browman (1989), who dealt with research related to the “critical period” in the field of embryology, ethology and ecology, and proposed the hypothesis that the so-called critical period of fish recruitment may be “causally interrelated in a hierarchical manner” with embryological and ethological critical period.

In this paper I would like to make a multidisciplinary review on docosahexaenoic acid (DHA), the nutritious importance of which seems to be well advocated mainly by medical scientists. Indeed the physiological role of DHA has been intensively studied in the medical field for the last couple of decades. Those researchers have advised in their reviews to consume a certain amount of seafood for good health, as fish and mollusks are the major source of DHA (Mostofsky et al. 2001).

The importance of DHA has also been a hot topic in the same period among scientists in another discipline, i.e., aquaculture and fish nutrition. Because most marine fish larvae cannot synthesize DHA from precursor molecules such as eicosapentaenoic acid (EPA) or α -linolenic acid (ALA), feeding DHA to fish larvae is crucial for successful fish culture (Sargent et al. 1989).

Recently, scientists studying ecology are also starting to consider DHA and other fatty acids as a key factor to understanding ecosystems (Arts et al. 2001). There is as yet a limited exchange of information among different disciplines of scientists working on DHA and other fatty acids. It is especially a pity to see that most fisheries scientists and fish biologists are not fully aware of the important medical aspects in their research materials. The goal of this paper was therefore to integrate knowledge related to DHA in medical, fish nutrition, and ecological disciplines from the viewpoint of a fish biologist, and then elucidate the critical importance of DHA in marine and terrestrial ecosystems.

Basics and evolutionary aspects. Fatty acids, in general, have two major functions in animals and plants as an energy storage and membrane components. Among various fatty acids, those with four or more double bonds, i.e., arachidonic acid (ARA), EPA and DHA, are called highly unsaturated fatty acids (HUFAs) and have important physiological functions. Because of the long chain of carbons with six double bonds, DHA has especially high flexibility in shape and thus should have specialized functions in membrane systems and gives high fluidity to cell membranes. In humans and other terrestrial animals, DHA is synthesized from ALA through elongation and desaturation processes. Animals have to take ALA or longer chain n-3 HUFA to synthesize DHA as an essential fatty acid. Yet it is by far more efficient to take DHA itself rather than taking precursor molecules. Unlike animals, many plants can produce ALA *de novo*.

DHA is abundant in neural cells, photoreceptor cells, and spermatozoa of any vertebrate. Crawford (1992) suggested that DHA originated from photoreceptor cells in marine bacteria. This molecule then became utilized as an energy source and for storage in phytoplankton. Then DHA was utilized as a transmitter of information in invertebrates and vertebrates. DHA might have played a critical role in human evolution, according to Crawford’s review. Human ancestors lived near lakes and ocean coastlines in Africa and consumed plenty of fish and shellfish, which should have enabled rapid evolutionary expansion of brain size in a relatively short period (Crawford 1992).

Function of DHA in humans and other mammalian animals. The efficacy of DHA to improve the condition of various diseases such as psychiatric disorders, visual disability, Alzheimer's disease and even cancer has been reported (Mostofsky et al. 2001). DHA occurs in ample quantities in the brain, but it has been difficult to show direct evidence for improving learning capability in humans by providing DHA. Hamazaki et al. (1998) reported the result of providing DHA or DHA-free capsules to college students. Although there was no significant difference in their examination results, the group provided with DHA capsules showed less aggressive behavior compared to the DHA-free control group in double-blinded aggression-estimating tests (Hamazaki et al. 1998; 1999). The effect of mitigating aggressive behavior in DHA was also confirmed in elderly Thai subjects (Hamazaki et al. 2002). Hibbeln (2001) compared the relationship between homicide mortality rates and seafood consumption across 26 countries and found a strong negative correlation between these two factors. He attributed this to the effect of DHA to mitigate aggression, and suggested a threshold seafood consumption of 10-20 kg person⁻¹ year⁻¹ to reduce homicide.

Improvement of learning capability by DHA is clearly shown in rodents. Okuyama and his colleagues conducted intensive work using a Skinner's box on rats (Mostofsky et al. 2001). Individuals fed with a diet rich in ALA (a precursor of DHA) showed significantly better scores in a reverse schedule of learning procedure, compared to a group fed with a diet rich in LA (linoleic acid), from which animals cannot synthesize DHA (Yoshida et al. 1997). The ALA-rich animals tended to have an increased number of synaptic vesicles in the CA1 cells of the hippocampus compared to the LA-rich animals. Interestingly, ALA-rich animals not given the learning task did not show the increase of synaptic vesicles. The HUFA contents of the ALA and LA diets corresponded to the typical meals of Americans and Japanese, respectively (Yoshida et al. 1997). Lim and Suzuki (2000; 2001) conducted experiments to show the effect of DHA on the ability of mice to learn a maze and found that a DHA-fed group required less time to reach the maze exit than a control group. They also found that the DHA level in the brain increased after feeding on a DHA-rich diet for 2 weeks, and improved maze-learning ability was evident 1 month after the start of feeding and was still evident after 3 months. Lim and Suzuki (2001) discussed that it might take time for an improvement in learning ability to become noticeable after the incorporation of DHA.

Implications for fisheries science. Earlier work conducted by Watanabe and his colleagues showed that rotifers enriched by freshwater *Chlorella* lack DHA as the essential fatty acid for marine fish larvae (reviewed by Watanabe 1982). Since then, DHA requirements were quantitatively studied in many species of fish and crustaceans; such as eel *Anguilla japonica*, rainbow trout *Oncorhynchus mykiss*, red sea bream *Pagrus major*, yellowtail *Seriola quinqueradiata*, striped jack *Pseudocaranx dentex*, carp *Cyprinus carpio* and kuruma prawn *Penaeus japonicus* (Takeuchi et al. 1996; reviewed by Sargent et al. 1989), with the typical amount shown to be 1% DHA dry weight or more in the diet. Sargent (1999) suggested that too much DHA and EPA could be harmful to some fish, and emphasized the importance of determining the desired level and ration of DHA, EPA and ARA as the "gold standard".

Fish eggs generally contain high amounts of essential fatty acids. Under hatchery conditions they use yolk-origin fatty acids as they develop and the DHA level especially decreases toward metamorphosis, although it increases again in the early juvenile stage by feeding on a DHA-rich diet. Both wild (Pickova et al. 1997) and hatchery rearing data (Navas et al. 1997; Furuita et al.

2000) support the idea that the hatching rate and the growth of larvae are strongly dependent on the amount of DHA in the eggs. DHA is accumulated from parental fish to yolk just as the human mother intensely accumulates DHA to the fetus through the placenta. The amount of DHA that mother fish can allocate to yolk is dependent on dietary-origin DHA (reviewed by Izquierdo et al. 2001). It is therefore common practice to feed high DHA diets to broodstock fish in the spawning season. Furuita (2000) suggested that the dietary effect of DHA should be reflected on a daily basis. This may also coincide with the diet of a human mother and the development of the fetus.

Although rotifers and *Artemia* nauplii are a commonly used diet for larval fish culture, efforts to search for alternative diets have been made. Both wild and cultured copepods are known to be superb as a diet (ex. Nanton and Castell 1998).

The importance of DHA in larval fish culture has been studied as a remedy for problems in hatchery production, such as low survival, low growth rate and deformities, with strong similarity to the research related to n-3 HUFA deficiency in humans. A typical symptom of DHA deficiency in larval and juvenile fish is spinning at the surface, as well as malpigmentation of juveniles in flatfishes. Estévez et al. (2001) showed that malpigmentation of flounder increases with high enrichment of ARA in *Artemia* and suggested that the malpigmentation can be attributed to deficiency in the nervous system, because ARA enzymatically competes with DHA and high-dose ARA enrichment induces a decrease of DHA in the brain. It is however notable that the effect of ARA is more directly reflected in the development of the retina in turbot and halibut (McEvoy et al. 1998), probably due to the high metabolic rate of DHA in the retina (Mostofsky et al. 2001). Since flatfish change their body color by their visual information, it is possible that impaired capability of vision and/or visual processing may prevent normal development of coloration and thus result in malpigmentation. This could be clarified by measuring visual capabilities of DHA-provided and DHA-deficient flatfish through behavioral approaches, such as testing visual acuity and feeding capability under a low light intensity.

Deficiency of certain nutrients should be reflected in behavior before resulting in low growth or survival rate. This should be especially true in the case of DHA, which is the major component of the central nervous system. Bell et al. (1995) found that herring larvae could not capture their prey at a low light intensity when they were deficient in DHA. Masuda et al. (1998) found that yellowtail did not form schools when they were raised on a DHA-deficient diet. We measured the schooling-related capability as a visual mutual attraction index and an optokinetic response. The DHA-deficient fish did not show mutual attraction; although they showed normal optokinetic responses. Considering that dietary DHA is incorporated into the brain in the yellowtail, especially the optic tectum (Masuda et al. 1999; Ishizaki et al. 2001), DHA deficiency is suggested to result in the lack of visual processing capability in juvenile fish.

The wild diet of fish larvae generally contains high amounts of DHA. However, this may not always be true. Davis and Olla (1992) demonstrated that the DHA content of wild copepods (*Acartia*) fluctuated dramatically within as short a period as 12 days in the same open-ocean area.

DHA deficiency in larval fish can be summarized as follows (Fig. 1). Low growth and survival rates of DHA-deficient fish may result from lack of DHA in the brain, which induces ineffective feeding behavior in addition to the direct shortage of DHA in membrane systems. In the wild, such a deficiency may have a more serious outcome, since behaviorally deficient fish would be an easy target for predators.

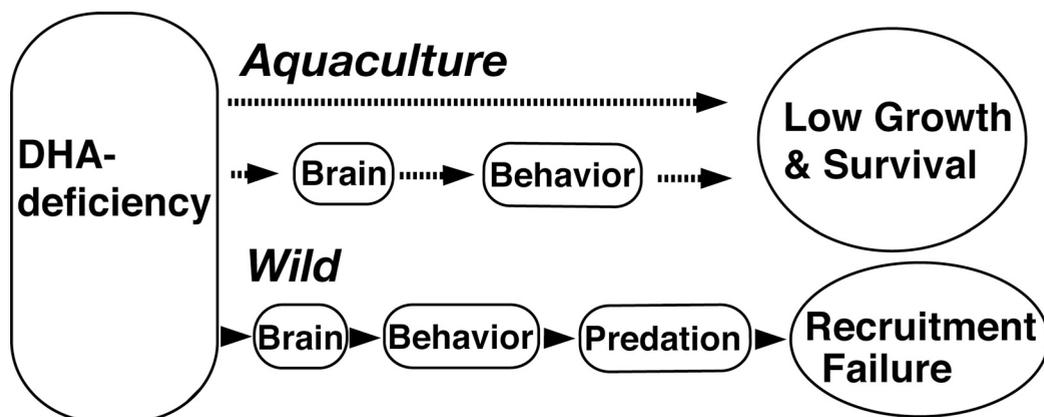


Figure 1. Schematic drawing of the effect of DHA deficiency under aquaculture and wild condition.

Environmental factors controlling DHA in the marine ecosystem. DHA production in the marine ecosystem is mostly attributable to phytoplankton and, to a much lesser extent, bacteria. DHA production in phytoplankton is influenced by water temperature and light conditions, with notable negative impact of UVB (280-320 nm wavelength) light; Wang and Chai (1994) demonstrated that the production of EPA and DHA in algae such as *Isochrysis galbana* and *Prorocentrum micans* decreased to 50 % and 20 %, respectively, after 5-6 days of UV exposure under laboratory conditions.

Most marine copepods and other zooplankton rely on phytoplankton for their DHA source. The increase of UV radiation on the ocean surface may therefore have profound effects on the marine ecosystem through the food chain (Fig. 2) in addition to direct negative effects that have previously been reported in copepods (Rodriguez et al. 2000) and fishes (reviewed by Zagarese and Williamson 2001). Browman et al. (2000) studied the impact of UV on the survival of copepods and cod eggs and found that UVB with negative impact on the survival of those animals would penetrate at least 3-4 m depth in the Gulf of St. Lawrence, Canada. He also pointed out that indirect effects of UV through the food-chain can be far more profound than the direct effects. The relatively short life span of phytoplankton and copepods may result in a rapid collapse of DHA content in copepods. If fish larvae encounter a nutritionally inferior diet when they critically require high-DHA, presumably during the metamorphosis stage, such larvae may face catastrophic mortality (Fig. 2).

Some marine bacteria that produce DHA and other fatty acids live symbiotically in the intestine of pelagic fishes, such as jack mackerel *Trachurus japonicus* and chub mackerel *Scomber japonicus*, and thus help the physiological DHA requirement of the host (Yazawa et al. 1987). Since DHA requirement can be a limiting factor of long-term growth and survival for any marine fishes, such symbiosis may give great advantage to the host. Furthermore, even when the DHA production of marine algae decreases for some reason, such as a temporal increase of UV radiation, those pelagic fishes may be relatively secure from the DHA shortage. These inter-specific differences in DHA acquisition routes could also contribute to population changes among pelagic fish

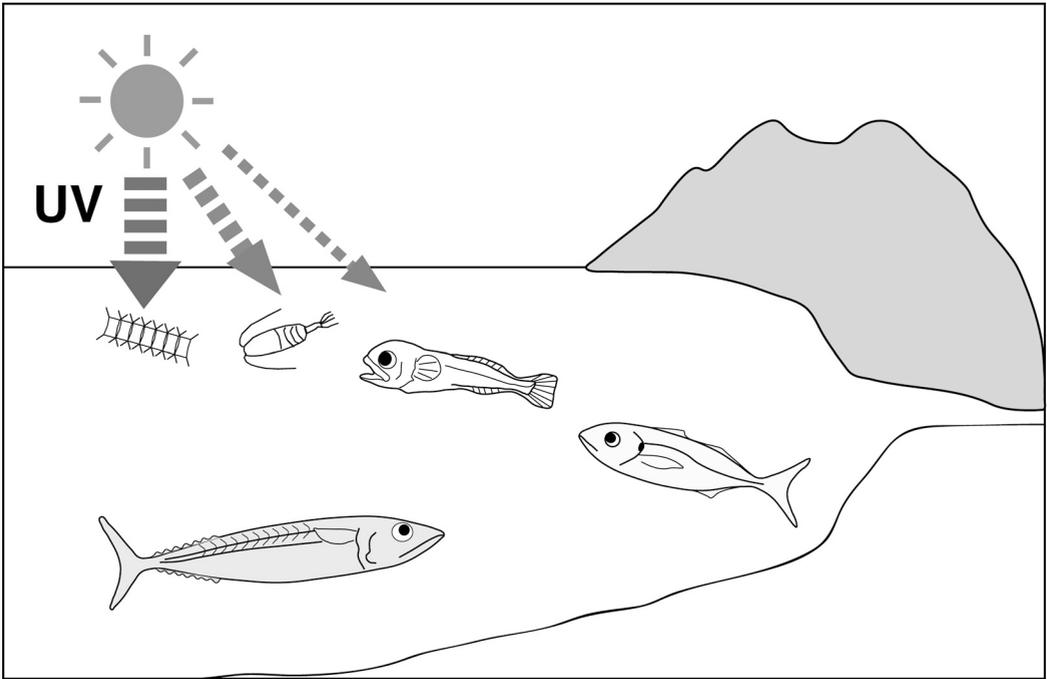


Figure 2. Hypothetical impact of UV radiation on the DHA production in marine ecosystem through the food chain.

species. In the west Pacific Ocean, the abundance of jack mackerel, chub mackerel and sardine *Sardinops melanostictus* is replaced in a cycle of a few decades (Matsuda et al. 1991), and the interaction between UV and DHA or EPA producing symbiotic bacteria may play some role in the replacement of the dominant fish in the pelagic ocean.

Future directions of research related to DHA. In rodents and mammals, the DHA-deficient condition requires careful experimental manipulation, since they can produce DHA from precursor n-3 HUFA and also retain a substantial amount of maternal DHA. Medical scientists therefore raise mice for two to three generations to acquire DHA-depleted animals. In fish, however, DHA deficiency can be easily manipulated because they cannot produce it de novo and egg-origin DHA is used up during the larval stage. Therefore, the direct effects of DHA can be more easily and clearly studied by using marine fishes compared to terrestrial animals.

The effect of DHA on learning capability is a promising field for the use of fish as experimental animals. Learning capability of fish can be measured by applying Pavlovian conditioning (Masuda and Ziemann 2000).

To elucidate the potentially fluctuating DHA production in the marine ecosystem, an interdisciplinary approach will be required among scholars in biochemistry, meteorology, oceanography and fishery science. Such research is of high priority in view of the critical importance of DHA in both marine and terrestrial ecosystems.

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An assessment of the development and survival of wild rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) exposed to elevated selenium in an area of active coal mining

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Abstract

Elevated levels of selenium (Se) have recently been detected downstream from uranium and coal mining activity in Canada. While Se is an essential dietary element, elevated concentrations can lead to decreased growth, reproductive impairment and increased mortality. A hallmark of Se toxicity is the appearance of teratogenic deformities in the progeny of exposed females that result from the deposition of Se to their eggs. Teratogenesis is an effective indicator of Se toxicity in fish communities. This study compares three methods of evaluating deformities in the larvae of rainbow trout and brook trout inhabiting seleniferous waters downstream from active coal mining activity in the Northern East Slopes Region of Alberta, Canada. Gametes were stripped from adult fish and transported to the laboratory where they were fertilized and reared to the swim-up stage. Fry were assessed for deformities using frequency analysis, a graduated severity index (GS), and morphometric analysis. Adults show no signs of Se toxicity but accumulated higher concentration of Se in muscle and eggs compared to fish from the reference sites. There were increased incidences of edema and spinal deformities in rainbow trout fry and increased frequency of craniofacial deformities in brook trout fry from the seleniferous site compared to those from the reference sites. Of the three methods used to assess deformities in larval fish, frequency analysis was found to be the most practical, rapid, and produced the most meaningful results.

Introduction

Selenium is a naturally occurring metalloid that is typically present in uncontaminated waters at concentrations of 0.1-0.4 µg/L (Lemly 1985a; Dobbs et al. 1996). It is both an essential and toxic trace element, with a very narrow range between levels required in the diet and toxic threshold concentrations. Fish require dietary Se levels of 0.1-0.5 µg/g (all concentrations reported as dry weight) (Hodson and Hilton 1983; Gatlin and Wilson 1984). However, concentrations of only 7-30 times greater than those required (i.e. >3 µg/g) can result in bioaccumulation and toxicity (Lemly 1997a). Field and laboratory studies have demonstrated that excessive Se can result in a variety of toxic effects including reduced growth, tissue damage, reproductive effects and increased mortality (e.g. Hodson et al. 1980; Hodson and Hilton 1983; Lemly 1993b).

The potential for widespread Se contamination of aquatic ecosystems due to anthropogenic activities has been recognized for nearly two decades (Lemly 1993a). One major source of Se contamination is subsurface drainage from the irrigation of seleniferous agricultural soils, notably in the semiarid and arid regions of the United States (Lemly 1993a). Toxic effects and impaired reproduction in birds and fish at Kesterson Reservoir, California, were attributed to elevated Se levels in agricultural drain water that bioaccumulated in the food chain (Saiki and Lowe 1987; Ohlendorf et al. 1988a, 1988b). Another major human-related source of Se is the procurement, processing and combustion of fossil fuels (Lemly 1993a). Tissue damage, reproductive failure and the elimination of fish communities was well documented in such locations as Belews Lake, North Carolina, which served as a cooling reservoir for a coal-fired electric power plant (Cumbie and Van Horn 1978; Sorenson et al. 1984; Lemly 1985b, 1993b) and contained high concentrations of Se.

Most of the reported cases of Se poisoning in natural fish populations have been from standing, warm-water habitats. Consequently, the applicability of guidelines and indexes developed from these systems to cold, flowing water habitats is presently a matter of contention in the literature (Lemly 1997a; Brix et al. 2000; Kennedy et al. 2000). Recently, elevated Se levels have been detected in cold, flowing water habitats downstream from uranium (TAEM, 1996) and coal (McDonald and Strosher 1998; Casey and Siwik 2000; Kennedy et al. 2000) mining activities in Canada. Cold-water species inhabiting these systems have elevated concentrations of Se in tissues that exceed published toxic effects thresholds for warm water fishes (Lemly 1993a). However, to date no toxic responses have been reported in these fish. One study, examined mortality and the incidence of teratogenic deformities in the larvae of cutthroat trout (*Oncorhynchus clarki lewisi*) inhabiting Se-rich cold water systems and found no significant relationships between these parameters and the concentration of Se in eggs (Kennedy et al. 2000).

Se poisoning resulting from the transfer of Se from females to their eggs during vitellogenesis can result in the appearance of terata as permanent pathological markers. Teratogenesis is restricted to the egg-larval stage of development when the larvae utilize yolks contaminated with Se (Lemly 1997a). The most common types of terata include spinal curvatures (lordosis, scoliosis, or kyphosis), missing or deformed fins, gills, opercula and eyes, as well as abnormally shaped heads and mouths (Lemly 1993b, 1997a). Other symptoms of Se poisoning include pericardial and abdominal edema, exophthalmia (bulging or protrusion of eyes), and cataracts. Because these latter symptoms appear to be reversible and are not formed strictly in the embryo-larval stage, they are

not considered true terata; rather, they are considered acute or chronic toxic responses to high concentrations of Se (Lemly 1993b). Nevertheless, these conditions may progress to, or be associated with, true terata (Lemly 1997a).

There are several advantages to using teratogenic defects as bioindicators of Se toxicity in fish communities. Teratogenesis is a direct expression of Se toxicosis and represents the sum total of parental exposure, regardless of temporal, spatial or chemical variations in Se exposures. Thus, terata represent a measure of existing, rather than potential hazard (Lemly 1997a). Moreover, teratogenic deformities can be subtle, but important causes of recruitment failure in fish communities. That is, a significant loss of the early life stages of a fish population can occur at the same time that adult fish appear healthy (Lemly 1997a).

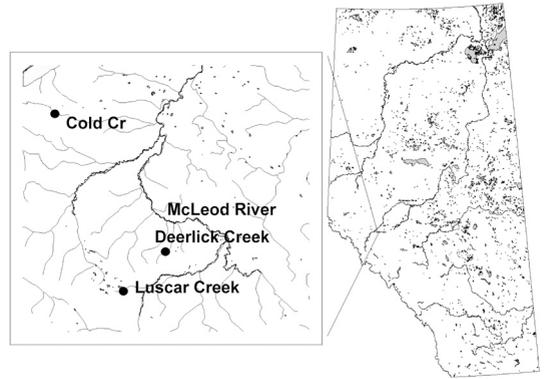
The prevalence of teratogenic deformities is regularly used to evaluate the toxicity of various environmental contaminants. A number of field and laboratory studies have already used Se induced teratogenesis to assess the impacts of Se on the reproductive fitness of fish (e.g. Bryson et al. 1985; Gillespie and Baumann 1986; Woock et al. 1987; Pyron and Beitinger 1989; Schultz and Hermanutz 1990; Hermanutz et al. 1992; Hermanutz 1992; Lemly 1993b, 1997b; Kennedy et al. 2000). However, in each of these studies no attempt was made to quantify the degree of severity of observed malformations. Consequently, these types of evaluations are dependent on the experience and subjectivity of the investigator in diagnosing the deformities. This paper examines the use of morphometrics to quantitatively characterize teratogenic malformations in the offspring of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) collected from sites with high concentrations of selenium downstream from coal mining activity in the northeastern slopes region of Alberta, Canada. This method is compared to two other approaches that have previously been used to assess the effects of Se on fish reproduction, including the frequency of gross morphological malformations and the use of graduated severity index scores.

Materials and Methods

Sites and Fish Collection. Spawning rainbow trout were collected in June 2000 from Luscar Creek (high Se site) and Deerlick Creek (reference site) and brook trout were collected from Luscar Creek and Cold Creek (reference site) in October 2000 in the Northeastern slopes region of Alberta, Canada (Figure 1). Precise locations and water chemistry parameters for these three sites at sampling times are given in Table 1. Concentrations of Se for Deerlick and Cold creeks were not available for 2000. However, concentrations at other reference locations in the area ranges from <0.5 to 2.2 µg/L (Casey and Siwik 2000). Spawning times were monitored by visual identification of redd formation as well as by recording daily water temperatures. For rainbow trout, collection of spawning fish began when daily maximum temperatures reached 6°C, while for brook trout collections began at 3°C.

Ripe and running males and females were collected using a Smith-Root type VII backpack electroshocker. Fish were netted, separated by sex, and held in cages at the appropriate location for <24 hours before sampling. At sampling, fish were individually anesthetized with 0.4 g/L MS-222 (tricaine methane sulfonate) (SIGMA Chemical Company, St. Louis, MO) until fin movement ceased

Figure 1. Collection sites for rainbow and brook trout in McLeod River drainage of Alberta, Canada. Luscar Creek is a selenium exposed site downstream from coal mining activity and Deerlick and Cold Creeks are reference sites for rainbow and brook trout, respectively.



(~ 3 min.). Each fish was then carefully blotted dry, weighed and measured, and then the area surrounding the urogenital opening was dried thoroughly with paper towels. Eggs or milt were expressed by light pressure on the abdomen. Gametes from each fish were stored on ice in sealed plastic bags filled with oxygen and protected from light.

Egg and Larvae Rearing. After transportation from the Alberta sites to the Freshwater Institute laboratories in Winnipeg (< 24 hours), eggs from each female were fertilized with a consistent volume (10 L/50 mL) of composite milt derived from 3-5 males captured at the same site. Eggs were combined with the milt in a stainless steel bowl by gentle mixing with the tip of a sterilized goose feather. After standing for 60 seconds, the eggs were covered with dechlorinated Winnipeg City tap water (approximately 100mL, see water chemistry in Table 1) and gently swirled 3 minutes. An additional 500mL of water was then added and the eggs were allowed to water harden for 5 minutes. The eggs were then placed in vertical Heath tray type incubators. The eggs from each female were assigned randomly to one of four trays, each with twelve compartments. Weekly monitoring of the water in trays showed no significant differences in temperatures (8 ± 0.8 (C)), flow rate (6 L water/min), dissolved oxygen saturation (>98%), or Se concentrations (1.6 ± 0.1). Dead eggs and fry were removed daily and preserved in Davidson's solution with 10% glycerin added (Humason 1962). Time, in degree/days (DD = mean temperature X days at that temperature), to the eyed stage and hatch were recorded. At swim-up, fry were sacrificed with an overdose of MS-222

Table 1. Typical study site water characteristics

| Site | Temperature (°C) | % DO | pH | Conductivity (mS/cm) | [Se] (µg/L) |
|--------------------|------------------|------|-----|----------------------|---------------|
| Field Sites | | | | | |
| Luscar | 2–6 | 93 | 8.3 | 0.538 | 6–32 |
| Deerlick | 2–7 | 91 | 7.5 | 0.116 | N/A |
| Cold | 2–8 | 97 | 8.0 | 0.393 | N/A |
| Laboratory | | | | | |
| FWI | 8.0 ± 0.8 | >98 | 7.8 | 0.18 | 1.6 ± 0.1 |

N/A = not available

(0.8 g/L) and preserved in Davidson's Solution. Percent fertilization was calculated based on the number of dead eggs removed where visual examination did not reveal a developing embryo compared to the total number of eggs. Mortality was calculated based on the number of dead eggs removed compared to the total number of fertilized eggs until the swim-up stage. Fertilization, hatch and survival data, as well as adult female endpoints were compared between sites by Mann-Whitney U test (SPSS Inc., 1999).

Tissue Se. Concentrations of Se were determined in eggs and tissues of adult rainbow trout and brook trout by ICP-MS at the Alberta Research Council Laboratory in Vegreville, AB, as previously described (Feng et al. 1999). Because several other elements are known to affect the availability and toxicity of Se (Hamilton and Palace 2001) concentrations of Al, Sb, Ba, Be, Bi, B, Cd, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Hg, Mo, Ni, Ag, Sr, Tl, Th, Sn, Ti, U, V, Zn were also determined. However, with the exception of Se, there were no significant differences in the concentrations of metals or metalloids in fish tissues or eggs collected from Luscar Creek and the reference sites (data not shown). Note that Se concentrations in tissues from this study are reported on a wet weight basis. To reduce the introduction of error, values from the literature are reported as either wet or dry weight according to how they were originally reported. Wet weight Se concentrations can be approximated from dry weight concentrations for most tissues by assuming 75% moisture content.

Assessment of Morphological Deformities

i) Frequency Analysis

All preserved offspring were examined using a dissection microscope for gross external malformations. Abnormalities were recorded in four categories: spinal, craniofacial, finfold and edema. Skeletal deformities included kyphosis (convex curvature of the thoracic region of the spine), lordosis (concave curvature of the lumbar region of the spine) and scoliosis (lateral curvature of the spine), as well as stunting in the spinal plane. Craniofacial defects included reduction or absence of the jaws and ocular deformities such as reduced eye diameter (microphthalmia) or pigment irregularities as well as asymmetry. Finfold defects included a reduction in thickness or absence of a fin. Edema included accumulation of body fluid in the region of the yolk sac or head. For the frequency assessment, only the number of deformed fry were determined for each category. In order to account for differences in clutch sizes between individual females, counts for each category were converted into percentages based on the total number of preserved fry obtained from each female. Frequency data were analyzed using a Mann-Whitney U test (SPSS Inc., 1999). Regression analysis was also used to examine relationships between concentrations of Se in eggs and the percentage of fertilization, survival, and deformities (SPSS Inc., 1999).

ii) Graduated Severity Index

The severity of the four deformity categories was scored using a graduated severity index (GS) (Middaugh et al. 1988; Hose et al. 1996), where 0 = normal; 1 = slight defect of size or structure; 2 = moderate defect of size or structure; 3 = severe defect of size or structure. A total GS score was

calculated for each fry by summing the individual scores in each category. Comparisons between reference and high Se sites for the GS data were made using approximate randomization statistical analysis, based on 1000 shuffles (Noreen 1989).

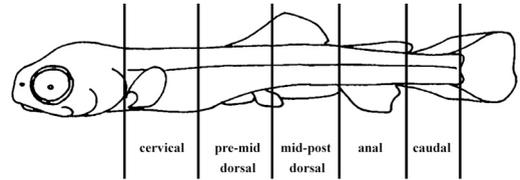


Figure 2. Regions for spinal angle measurements in rainbow and brook trout fry.

iii) Morphometric Analysis

Morphometric analysis consisted of eight length measurements made on a subsample of 100 fry per female under low power magnification using a SummaSketch III digitizing pad connected to a personal computer using a custom designed software application. Measurements included total body length, head height, head width, snout length, right and left eye diameter, and right and left opercular length. All length measurements were corrected for size variance by dividing by total body length. Spinal angles were measured in Corel Draw version 5 (Corel Corporation, 1995) after larvae were digitized along the lateral line (for dorso-ventral curvatures) and the fin fold (for lateral curvatures). The angles were recorded in 5 regions: cervical; pre-mid dorsal fin; mid-post dorsal fin; anal fin; and caudal, as shown in Figure 2. The total lateral and dorso-ventral spinal curvatures were calculated by adding the deviation from 180° at each of these regions. Measurement errors were calculated using three nonconsecutive repeated measurements of 25 randomly selected larvae (Bailey and Byrnes 1990). When both left and right side measurements were made, only left side measurements were used in measurement error analysis. Morphometric data was analyzed using a mixed model ANOVA (SAS Institute, 1999). Before ANOVA, total dorso-ventral and lateral spinal curvature data were log transformed. Quality control measurements were analyzed following the methods of Bailey and Byrnes (1990) by model II nested ANOVA (SAS Institute, 1999).

Table 2. Mean somatic measures and concentrations of Se in adult rainbow trout and brook trout (mean \pm S.E.).

| Site | Rainbow trout | | Brook trout | |
|--------------------------|-------------------|-----------------|------------------|------------------|
| | Luscar | Deerlick | Luscar | Cold |
| n | 3 | 4 | 11 | 7 |
| Weight (g) | 177.0 \pm 112.0 | 70.0 \pm 7.5 | 167.8 \pm 45.3 | 117.4 \pm 22.8 |
| Length (cm) | 22.7 \pm 5.2 | 19.2 \pm 0.6 | 23.4 \pm 1.8 | 22.0 \pm 1.6 |
| CF ^a | 1.12 \pm 0.02 | 0.98 \pm 0.04 | 1.10 \pm 0.04 | 1.04 \pm 0.04 |
| [Se] muscle ^b | 1.50 \pm 0.28 | 0.48 \pm 0.15 | 3.79 \pm 0.51 | 0.55 \pm 0.10 |
| [Se] eggs ^b | 8.37 \pm 1.62 | 2.05 \pm 1.06 | 6.37 \pm 0.78 | 1.35 \pm 0.24 |
| [Se] liver ^b | NT | NT | 13.14 \pm 0.24 | 2.13 \pm 0.33 |

^a = weight (g) / length (g)³ x 100

^b (μ g/g, wet weight)

NT = not taken for Se analysis

Results

Adult fish. Weights, lengths, condition factors (CF), and concentrations of Se in tissues of adult female brook trout and rainbow trout are shown in Table 2. Weight, length and CF were not significantly different between Se exposure sites and the reference sites.

No observable deformities were identified in adult rainbow trout from either site. However, 25 % of brook trout collected from Luscar Creek were observed to have opercular deformities, including shortened opercula, most often with exposed gills. Less than 1% of brook trout in Cold Creek had shortened opercula. Routine histopathological evaluation of gill, liver, and kidney tissues showed no evidence of any lesions in either the Se-exposed fish or fish collected from the reference sites.

Significantly higher concentrations of Se (mean \pm S.E., on a wet weight basis) were found in the muscle, liver and egg tissues of both species from Luscar Creek compared to the reference sites (Table 2). Luscar Creek rainbow trout had significantly higher concentrations of Se in axial muscle ($1.50 \pm 0.28 \mu\text{g/g}$) and eggs ($8.37 \pm 1.62 \mu\text{g/g}$) compared with muscle ($0.48 \pm 0.15 \mu\text{g/g}$) and eggs ($2.05 \pm 1.06 \mu\text{g/g}$) of fish from the reference site. Similarly, Luscar Creek brook trout had significantly higher concentrations of Se in axial muscle ($3.79 \pm 0.51 \mu\text{g/g}$), liver ($13.14 \pm 1.41 \mu\text{g/g}$), and eggs ($6.37 \pm 0.78 \mu\text{g/g}$) versus muscle ($0.55 \pm 0.10 \mu\text{g/g}$), liver ($2.13 \pm 0.33 \mu\text{g/g}$), and eggs ($1.35 \pm 0.24 \mu\text{g/g}$) in fish from the reference site. Selenium in eggs was positively correlated to selenium in muscle tissue for both rainbow trout ($r = 0.864$; Figure 3a) and brook trout ($r = 0.954$; Figure 3b).

Embryos

i) Frequency Analysis

There were no significant differences in fertilization (rainbow, $63.3 \pm 8.0 \%$; brook, $96.9 \pm 0.7 \%$), mortality (rainbow, $1.9 \pm 0.8 \%$; brook, $11.3 \pm 2.4 \%$) or temperature units to the eye stage (rainbow, $297.7^\circ/\text{days}$; brook, $233.6^\circ/\text{days}$) or to hatch (rainbow, $433.4^\circ/\text{days}$; brook, $468.8^\circ/\text{days}$) for either species from the Se exposed site and the reference sites. Fish from the Se-exposed site had a significantly higher percentage of fry with abnormalities based on frequency analysis. Specifically, rainbow trout from Luscar Creek had 38.9% of fry with deformities compared to 0.7% in Deerlick Creek. Brook trout from Luscar Creek had 14.4% of fry with deformities compared to 4.0% of fry from Cold Creek. Luscar Creek rainbow trout fry had a significantly higher incidence of fin (3.2%) and craniofacial (7.7%) deformities versus fry from the Deerlick Creek ($<0.2\%$). The prevalence of spinal curvatures was also significantly higher in rainbow trout fry from the exposed site (13.8%) compared to the reference site (0.7%). The most frequent type of spinal abnormality was concave curvature of the spine relative to normal fry, as shown in Figure 4 a+b.

The most marked difference between rainbow trout fry from Luscar and Deerlick Creeks was the incidence of edema. While less than 0.2% of fry from Deerlick had visible signs of edema, 30.8% of fry from Luscar exhibited fluid accumulation. In most cases, edema appeared as an accumulation of fluid surrounding the yolk sac (Figure 4c). Yolk sac edema was often associated with spinal curvatures. Edematous fry were most often shorter and had utilized less yolk than had fry that had not developed edema at the time of sampling. In addition to yolk sac edema, fluid also accumulated in the head, resulting in a spreading of the cranial features as illustrated in Figure 4d.

In contrast to the rainbow trout fry, the incidence of edema, spinal or finfold deformities in

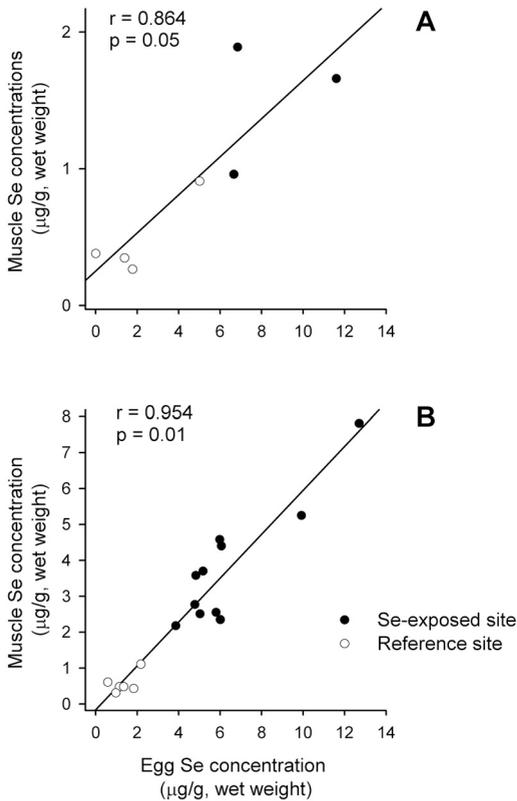


Figure 3. Correlation between muscle and egg Se concentrations in (A) rainbow trout and (B) brook trout. Each symbol represents data from tissues obtained from one female.

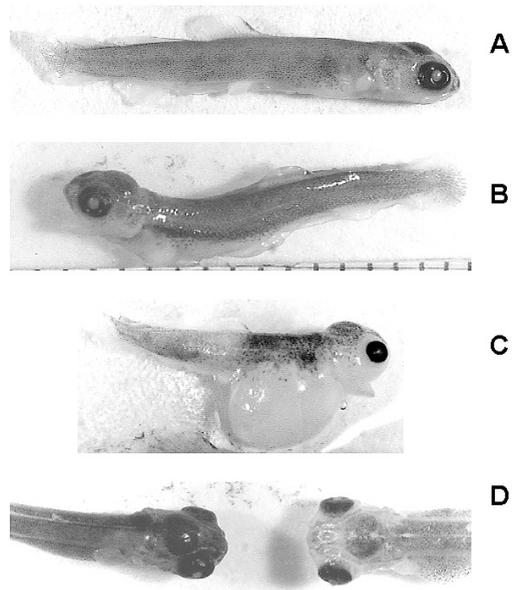


Figure 4. Rainbow trout fry from the selenium-exposed site, Luscar Creek showing the most commonly observed deformities. Craniofacial (CF), skeletal (SK), finfold (FF) and edema (ED) deformities were noted and scored in these fry using a severity index, from no deformity = 0 to multiple or severe deformity = 3. Panel A is a normal fry, scored as CF 0, SK 0, FF 0, ED 0, panel B is a fry with spinal curvature, scored as CF 0, SK 1, FF 0, ED 0, panel C is an edematous fry, scored as CF 3, SK 2, FF 0, ED 2, and panel D shows a fry on the right with spreading of the cranial features, scored as CF 0, SK 0, FF 0, ED 1.

brook trout were not significantly different between sites (<0.7%, <2%, <2%, respectively). Luscar Creek brook trout were observed to have significantly higher incidence of craniofacial abnormalities (13.6%) versus those from Cold Creek (3.0%). Approximately 70% of the craniofacial deformities recorded were shortened or misshapen jaws, with another 20% attributable to eye defects (Figure 5).

There was a significant relationship between concentrations of Se in rainbow trout eggs from Luscar and Deerlick Creeks and percentage of: deformed fry ($r^2 = 0.988$, $p = 0.002$), craniofacial deformities ($r^2 = 0.898$, $p = 0.01$), spinal deformities ($r^2 = 0.884$, $p = 0.013$), finfold deformities ($r^2 = 0.980$, $p = 0.005$), and edema ($r^2 = 0.999$, $p = 0.000$) (Figure 6). The point of rapid rise in each of the Se regressions occurs near 6 $\mu\text{g/g}$. No such relationships were identified between the concentrations of Se in the eggs of brook trout and any of the parameters examined (Figure 7).

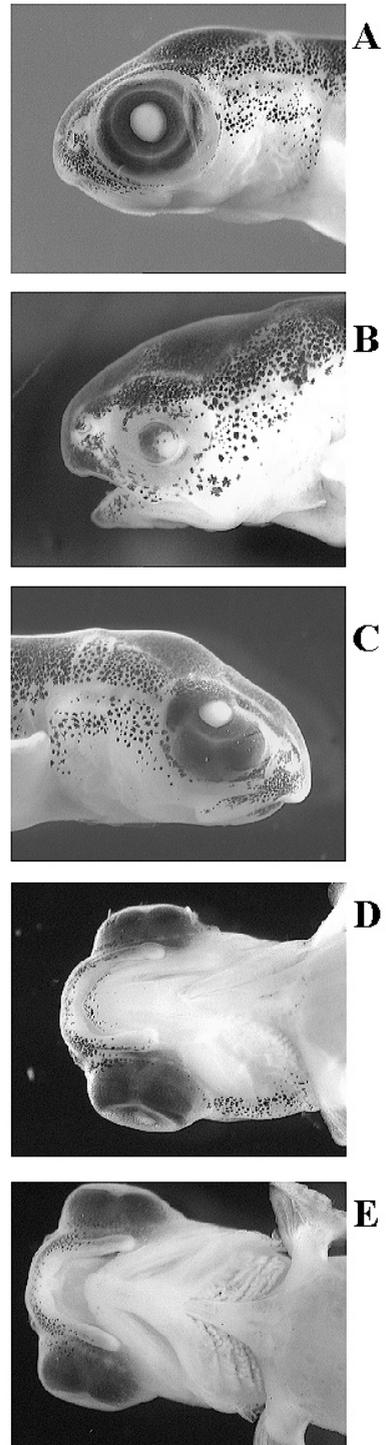
Figure 5. Brook trout fry from the selenium-exposed site, Luscar Creek showing the most commonly observed deformities. Craniofacial (CF), skeletal (SK), finfold (FF) and edema (ED) deformities were noted and scored in these fry using a severity index, from no deformity = 0 to multiple or severe deformity = 3. Panels A and D are normal fry, scored as CF 0, panel B is a fry with microphthalmia, scored as CF 3, panel C is a fry with an ocular malformation, scored as CF 1, and panel E is a fry with a shortened, malformed jaw, scored as CF 1.

ii) GS Analysis

Total GS scores were significantly higher in rainbow trout fry at the Se-exposed site than in those from the reference sites (Figure 8a). The edema index contributed the most to the total index score and accounted for nearly 50% of its value. For brook trout, the difference in mean total GS scores between Luscar and the reference site was not as great that observed for rainbow trout (Figure 8b). Of the individual indices, only craniofacial scores were significantly higher in brook trout fry from Luscar compared to those from the reference site, contributing approximately 60% to the total GS score. Conversely, finfold GS scores were significantly higher in Cold Creek brook trout compared to those from the Se-exposed site. Neither edema nor spinal index scores differed significantly between sites.

iii) Morphometric Analysis

Morphometric analysis revealed substantial variation in sizes among offspring of females within a single site, limiting statistical power for comparisons of results from females captured at the Se-exposed sites versus the reference sites (Table 3). Still, with this limitation, rainbow trout fry were found to have significantly greater lateral and dorso-ventral spinal curvatures at the Se-exposed compared to the reference sites. Similar results were obtained for brook trout. Luscar Creek brook trout also had significantly shorter left and right opercular lengths compared to those from Cold Creek. Among all eight parameters measured, percent measurement error (%ME) of the morphometric variables ranged over two orders of magnitude (Table 4), from 0.2% for brook trout body length to 95.0% for brook trout snout length.



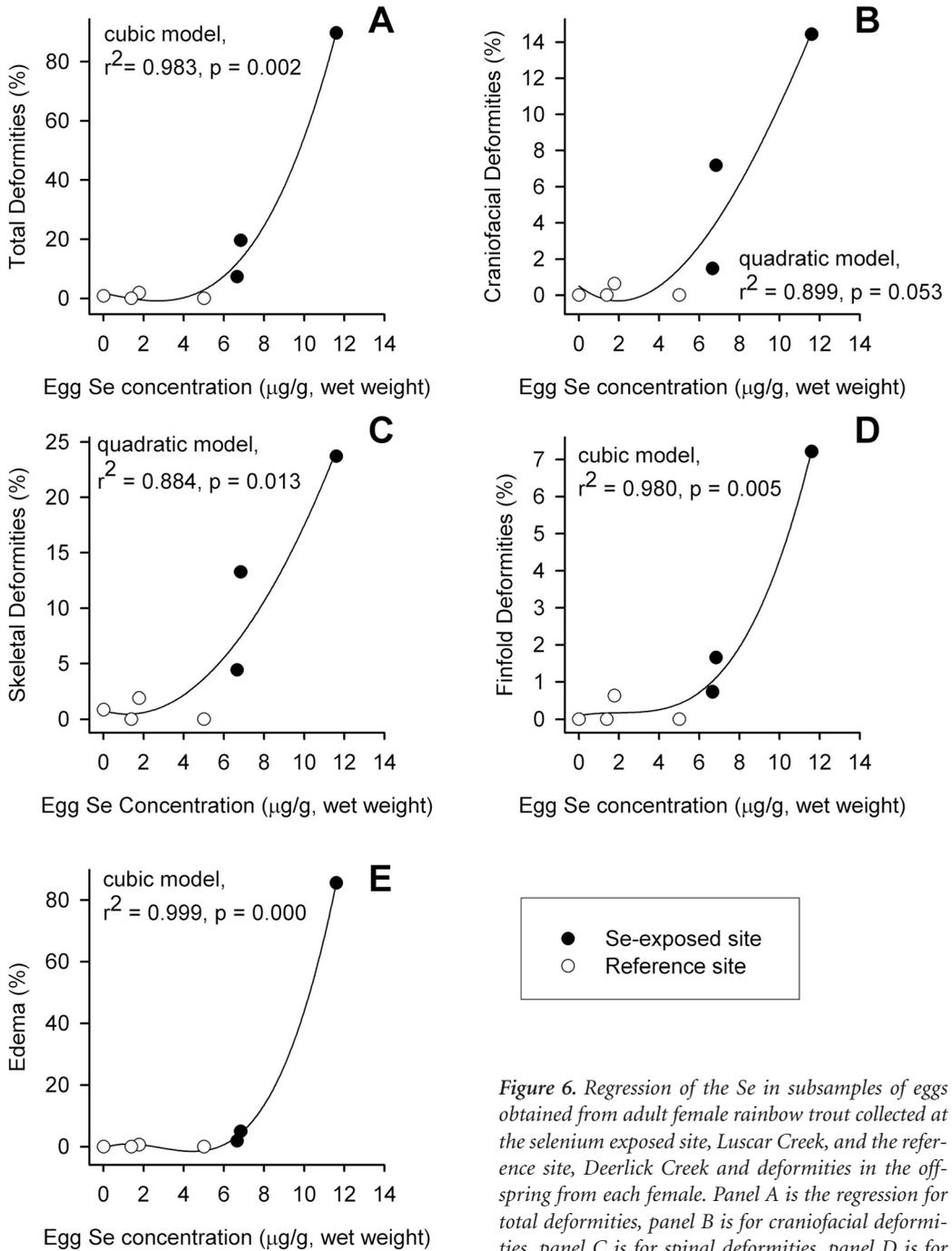


Figure 6. Regression of the Se in subsamples of eggs obtained from adult female rainbow trout collected at the selenium exposed site, Luscar Creek, and the reference site, Deerlick Creek and deformities in the offspring from each female. Panel A is the regression for total deformities, panel B is for craniofacial deformities, panel C is for spinal deformities, panel D is for finfold deformities and panel E is for edema. Each symbol represents data from one female's eggs.

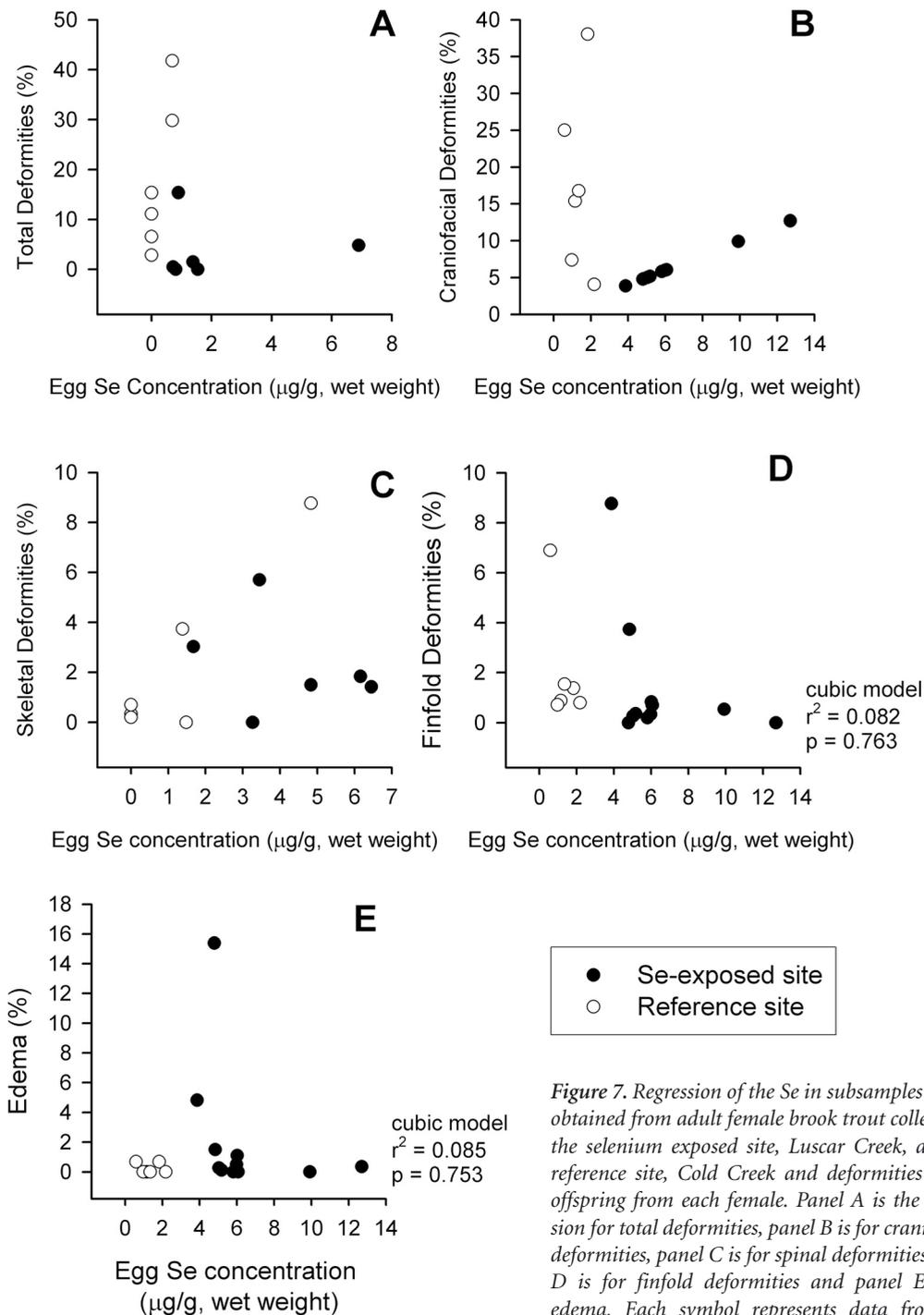


Figure 7. Regression of the Se in subsamples of eggs obtained from adult female brook trout collected at the selenium exposed site, Luscar Creek, and the reference site, Cold Creek and deformities in the offspring from each female. Panel A is the regression for total deformities, panel B is for craniofacial deformities, panel C is for spinal deformities, panel D is for finfold deformities and panel E is for edema. Each symbol represents data from one female's eggs.

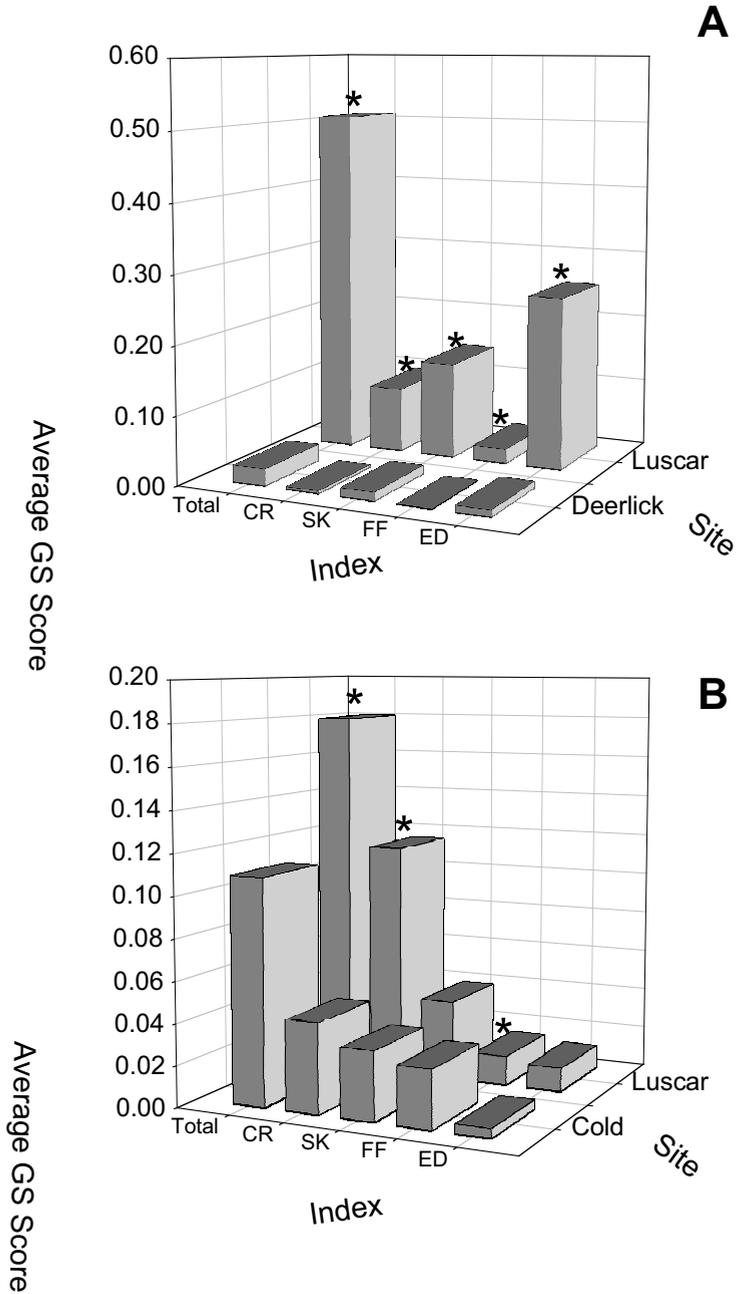


Figure 8. Mean graduated severity index (GS) scores for Total, craniofacial (CF), skeletal (SK), finfold (FF) and edema (ED) defects in rainbow trout (panel A) and brook trout (panel B) from the selenium exposed site, Luscar Creek, and the reference sites, Deerlick and Cold Creeks. Scores significantly different between sites are marked with an asterisk.

Discussion

Elevated levels of Se were found in the tissues of two salmonid species captured downstream from coal mining activity in the northeastern slopes region of Alberta, Canada. These levels were found to exceed toxic effect thresholds of 8 $\mu\text{g/g}$ in muscle, 12 $\mu\text{g/g}$ in liver, and 10 $\mu\text{g/g}$ in eggs (dry weight; Lemly 1993a). In spite of high Se body burdens, adult rainbow and brook trout show no evidence of Se toxicosis. Histopathological effects including hematological changes, as well as gill, liver, kidney and ovary damage have been reported in redear sunfish (Sorenson 1988) and green sunfish (Sorenson et al. 1984) collected from Se contaminated power plant cooling reservoirs. The Se concentrations in redear livers at 7.5 $\mu\text{g/g}$, after conversion to wet weight, are comparable to the concentrations of Se in livers of brook trout from this study (13.14 $\mu\text{g/g}$) and Se concentrations in green sunfish muscle 13 $\mu\text{g/g}$, after conversion to wet weight, exceed those in the muscle of both rainbow and brook trout at (1.5 $\mu\text{g/g}$ and 15.2 $\mu\text{g/g}$). Differences in species susceptibility to Se-induced tissue damage in adults warrants further consideration.

Rainbow trout and brook trout consistently had higher concentrations of Se in their eggs than in muscle tissue, which is similar to the pattern of accumulation observed in cutthroat trout (Kennedy et al. 2000), largemouth bass and bluegill sunfish (Baumann and Gillespie 1986). Moreover, Se concentrations in the eggs of both species showed a strong relationship with Se concentrations in muscle, suggesting that maternal transfer of Se is efficient in both brook trout and rainbow trout. A similar relationship between Se concentrations in muscle and eggs have been reported for razorback suckers (Hamilton and Waddell 1994) and cutthroat trout (Kennedy *et al.* 2000).

Se levels in the tissue of females from these studies were highly variable. This was true even for females collected from the same site. This variability is likely a consequence of fish mobility within the study area. For example, fish are free to move into or out of Luscar Creek via the McLeod River. This means that exposure to Se laden waters or prey items may be intermittent. In fact, fish captured in Luscar Creek may feed at sites other than Luscar Creek that differ widely in their Se concentrations. Similarly, trout captured at the reference sites may have spent a significant portion of their feeding times at sites with elevated Se concentrations. This is one of the most important reasons that actual tissue Se concentrations must be considered in examining potential teratogenesis rather than comparisons based only on capture site.

Fertilization, percent mortality and development time was not different between the Se-exposure site and the reference sites for either species. These findings are consistent with the results of a number of other studies that have reported no significant reduction in either percent fertilization or percent hatch in response to elevated Se burdens in bluegills (Coyle et al. 1993; Gillespie and Baumann 1986), fathead minnows (Ogle and Knight 1989), perch (Crane et al. 1992) or cutthroat trout (Kennedy et al. 2000).

The most sensitive indicators of Se toxicity are reproductive based endpoints (Lemly 1993a). As such, the assessment of the incidence of deformities in larvae is an important component of evaluating the impact of Se on fish populations. The primary goal of this study was to compare three methods of evaluating deformities in larval fish: frequency assessment, GS method, and morphometrics.

Although rainbow trout and brook trout were not found to exhibit comparable types of defor-

mities, the results of the frequency and GS analyses were generally consistent for each species. Frequency assessment showed that Luscar rainbow trout had a significantly higher number of fry with skeletal deformities and edema compared to the reference site, while GS analysis indicated a significant increase in the severity of skeletal deformities and edema. At first it may appear that the finfold and craniofacial malformation results of these two methods are contradictory. However, the differences indicate that although there were not significantly more craniofacial or finfold deformities in Luscar Creek rainbow trout, the overall severity of their deformities was greater than those from the reference site. Brook trout had a significantly higher incidence of craniofacial deformities in Luscar Creek compared to the reference site. GS scores also indicated that the severity of craniofacial deformities was higher in these fish.

Morphometric analysis generally did not return similar results as the other two methods. Significant differences between sites were seen only for dorso-ventral and lateral spinal curvatures in both species. For rainbow trout then, morphometric analysis only confirmed that spinal curvatures are the most consistent teratogenic deformity observed at the seleniferous site. Results from morphometric analysis of brook trout were similar, even though this type of deformity was not identified by the two other analyses. None of the measured craniofacial parameters (HW, HH, SL, LED, RED) were found to differ by morphometric analysis even though these were identified in the prior two analyses. Finally, morphometrics did reveal shorter opercula in brook trout fry from Luscar Creek compared to those from Cold Creek. Interestingly, opercular anomalies were observed in the adult brook trout that were collected from Luscar Creek.

Several advantages and disadvantages are associated with each of the methods used to evaluate deformities. Frequency assessment is a cost-effective method of evaluation, requiring few specialized instruments and lending itself well to rapid analysis. Up to 5,000 fry can be assessed in approximately 40 hours. Statistical analysis of data using nonparametric t-test equivalent is appropriate as the data consist of percentages. Finally, regression analysis to evaluate the relationships between Se content of the eggs and the incidence of the endpoints examined are most appropriate and can be readily performed with this type of data.

The GS method has comparable equipment and analysis requirements. However, the data generated is on a non-continuous scale (0-16), requiring analysis using computer-intensive statistics, for which software application packages are not readily available. Furthermore, because GS scores were scaled values, they were not particularly suitable for regression analysis, which is likely the most appropriate tool to examine toxicological effects in populations.

Morphometric analysis requires specialized equipment to magnify as well as digitize the images of fry. Analysis requires considerably more time, with an analysis of 1,500 fry taking about 700 hours. Statistical analysis of the morphometric data also required complex, nested designs to account for variation between the offspring of different females in addition to variation among sites. Morphometric evaluation also proved inadequate to detect some deformities while being oversensitive to others. The lower power of this method to detect craniofacial deformities in brook trout likely resulted from the craniofacial categories used in the other two tests being, in effect subdivided into several different parameters (HH, HW, SL, RED, LED) for this analysis. Some deformities identified by frequency analysis, including edema, fin alterations, differences in jaw length, and ocular pigment, could not be detected with morphometric analysis. The possibility of adding

more morphometric parameters to a particular study in order to ensure more thorough inspections, is time prohibitive. As evidenced by its detection of differences in opercular lengths, morphometric analysis may be more suited to detecting differences in the sizes of structures than GS or frequency evaluations.

Teratogenic effects observed in fish from cold flowing water habitats in this study were similar to those reported in other studies where fish from warm standing waters were evaluated. For example, Gillespie and Baumann (1986) reported that artificial crosses involving female bluegills (ovary Se, 6.96 µg/g) from a Se-contaminated reservoir produced up to 100% edematous larvae. Similarly, bluegill sunfish (Hermanutz et al. 1992) and fathead minnows (Schultz and Hermanutz 1990) exposed to elevated levels of Se in outdoor experimental streams had a high incidence of edema, lordosis and internal hemorrhaging at ovary Se concentrations of 5.96 µg/g. In contrast, Kennedy et al. (2000) reported no increase in the occurrence or severity of teratogenic deformities in cutthroat trout (egg Se, 21.2 µg/g, dry weight) inhabiting a Se-contaminated river impacted by coal mining.

Of the three methods used to assess developmental abnormalities, frequency assessment was the most time and cost effective method. As well, the data generated was the most useful to derive site specific toxicity thresholds. With regard to the relationships between Se eggs concentrations and the frequency of larval deformities in rainbow trout, results from this study closely resembled that reported by Lemly (1993b) for centrarchid species inhabiting a Se-laden reservoir. Both data sets exhibit cubic model regression curves, with the point of rapid rise in deformities occurring in rainbow trout at egg burdens of ~ 6 µg/g, wet weight.

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The effect of lead and cadmium on LDH and G-6-PDH isoenzyme patterns exhibited during the early embryonic development of the teleost fish, *Ctenopharyngodon idellus* with emphasis on the corresponding morphological variations

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Key words: Grass carp, *Ctenopharyngodon idellus*, embryonic development, lead, cadmium, LDH, G-6-PDH, antioxidants

Abstract

Using electrophoresis, the patterns of LDH and G-6-PDH isoenzymes were identified in the early embryonic and larval stages of *Ctenopharyngodon idellus* until the 14th day post-hatching. Such control patterns were compared with those elucidated after treatment with lead and cadmium sublethal doses of 100, 200 and 300 ppm. The behaviour of the maternal and zygotic genes were found to be influenced by such doses of heavy metals. Significant differences were recorded and interpreted in genetic terms since the heavy metals led to activation of some alleles and inactivation of others. These variations were found to be correlated with certain morphological and histological variations in some body organs including the skeletal and spinal elements and gills. The role of these metabolic enzymes as antioxidants was considered.

Introduction

In natural aquatic ecosystems metals occur in low concentrations, normally at the nanogram to microgram per liter level. In recent times the occurrence of nondegradable nonnutritive pollutants such as lead, cadmium and mercury in excess of natural loads has become a problem of increasing concern. Different studies on the impacts of heavy metals on Egyptian freshwater and marine adult

fish were carried out by Abdel-Moneim et al. (1994), Shakweer and Abbas (1996), and Hussein and Mekkawy (2001). However, few studies were executed on the embryonic and larval stages including those of Lashein (1996, 1999a, b, 2000) and Hamdy et al. (2000). These studies were restricted to the morphological and histopathological effects of some heavy metals on the developmental stages. None of these studies explains the nature of the relationship between the heavy metal-induced morphological and histopathological variations and the antioxidant defense system which utilizes enzymatic and non-enzymatic mechanisms. Due to the aforementioned facts and the record of lead in sediments of Lake Nasser and the Nile (Abdel-Moneim et al. 1994), the present work aims at studying the effect of lead and cadmium on LDH and G-6-PDH isoenzyme patterns exhibited during the early embryonic development of the teleost fish, *Ctenopharyngodon idellus*; with emphasis on the corresponding morphological and histological variations. Do such metabolic enzymes act as antioxidants? A question to be considered in this respect. This work was also a continuation of the work of Lashein (1996).

Materials and Methods

Gametes of the grass carp *Ctenopharyngodon idellus* were obtained by artificial spawning of adult fish in the fish culture station at EL-Ahaiwa, Sohag Governorate, Upper Egypt according to Oyen et al. (1991). Carps of 3 kg were intramuscularly injected with adult carp pituitary powder suspended in a 0.9 % NaCl solution. Females were first injected with 1 mg kg^{-1} fish, while males received 4 mg kg^{-1} fish. Twelve hours later, the females took an additional dose of 1 mg kg^{-1} fish. Twenty-four hours after the first injection, gametes of males and females were collected and mixed; fertilization was induced by addition of water. Immediately thereafter, fertilized eggs were placed in the egg incubators ($25\pm 1^\circ\text{C}$).

Prior to the stage of swelling of the fertilization membrane (6 hours after fertilization), eggs were transported to the laboratory. In a dechlorinated drinking water of pH around 7.5 and 26°C , the fertilized eggs were divided into a control and groups treated with 100, 200 and 300 ppm of lead and cadmium. Such doses were found to be sublethal by Lashein (1996). Control and treated groups were sampled for electrophoresis at intervals of 8, 12, 16, 20, 24 and 36 hours (after fertilization; 36 hours is the hatching time) and 1, 3, 4, 6, 10 and 14 day post-hatching and frozen at -83°C . Each sample consists of 10 fertilized eggs or larvae. Larvae were fed daily on commercial fish food. Daily observations of larval mortality and developmental abnormalities were made. Such observations confirm those recorded by Lashein (1996).

Pure cadmium chloride ($\text{CdCl}_2 \cdot 2.5\text{ H}_2\text{O}$) and lead chloride (PbCl_2) were used. Stock solution of each metal salt was prepared using acidified distilled water as dissolving media (duplicate 5 liter aquaria per group were used, 500 eggs in each). Test solutions were statically renewed daily (100%) and the dissolved oxygen, pH and temperature were recorded and were nearly constant.

For electrophoresis, adopting the method used by Partington and Mills (1988), 10-specimen samples of the control and treated groups were homogenized by hand using a glass rod. The homogenates were centrifuged under cooling (-4°C) at 12000 rpm for 15 minutes. The extractions were electrophoresed according to the procedures mentioned in Helena Laboratories Publications

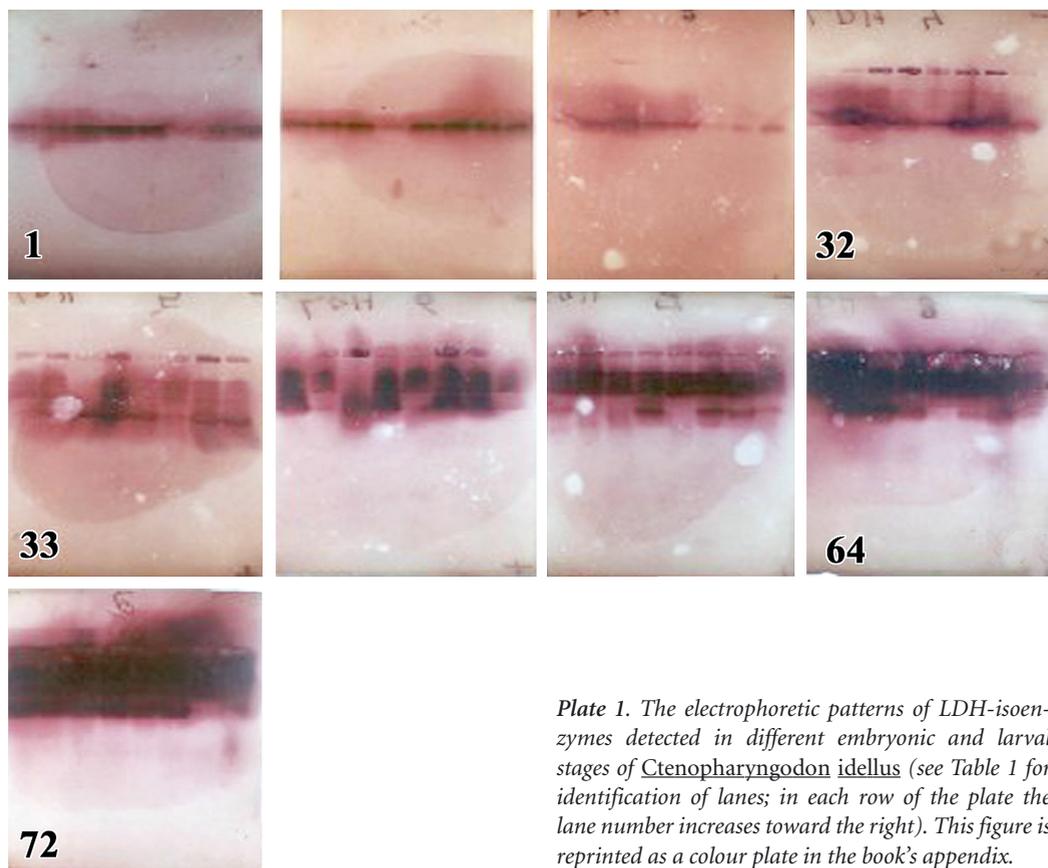


Plate 1. The electrophoretic patterns of LDH-isoenzymes detected in different embryonic and larval stages of Ctenopharyngodon idellus (see Table 1 for identification of lanes; in each row of the plate the lane number increases toward the right). This figure is reprinted as a colour plate in the book's appendix.

(1984 and 1985) for detection of LDH and G-6-PDH using cellulose acetate plates. The electrophoretic isoenzyme patterns (Plates 1 & 2) were analyzed and graphed by Gel-Pro Analyzer Software V3.1 for Windows95/NT (Media Cybernetica, 1993-97).

For the histopathological study fresh embryos of the control and treated groups were fixed in Davison's fluid (20% formalin, 10% glycerol, 30% ethanol (95%), 10% glacial acetic acid and 30% distilled water) (Gabe 1976). After 24 hours of fixation, the specimens were stored in Davison's fluid without acetic acid. It was found that this fixative prevents the yolk from becoming brittle (Gabe 1976). Larval specimens of control and treated groups were fixed in Bouin's and Carnoy's fluids (Drury and Wallington 1967).

Alternative sections of the prepared serials were stained with Haematoxylin and Eosin, and Alcian blue stain (Drury and Wallington 1967). Periodic Acid Schiff's (PAS) reaction was used for mucoid and carbohydrate detection (Gabe 1976). Appropriate sections of the control and treated embryonic and larval stages were selected to investigate the lead and cadmium effects on skeletal elements, notochord, gills, chloride cells, liver and kidney.

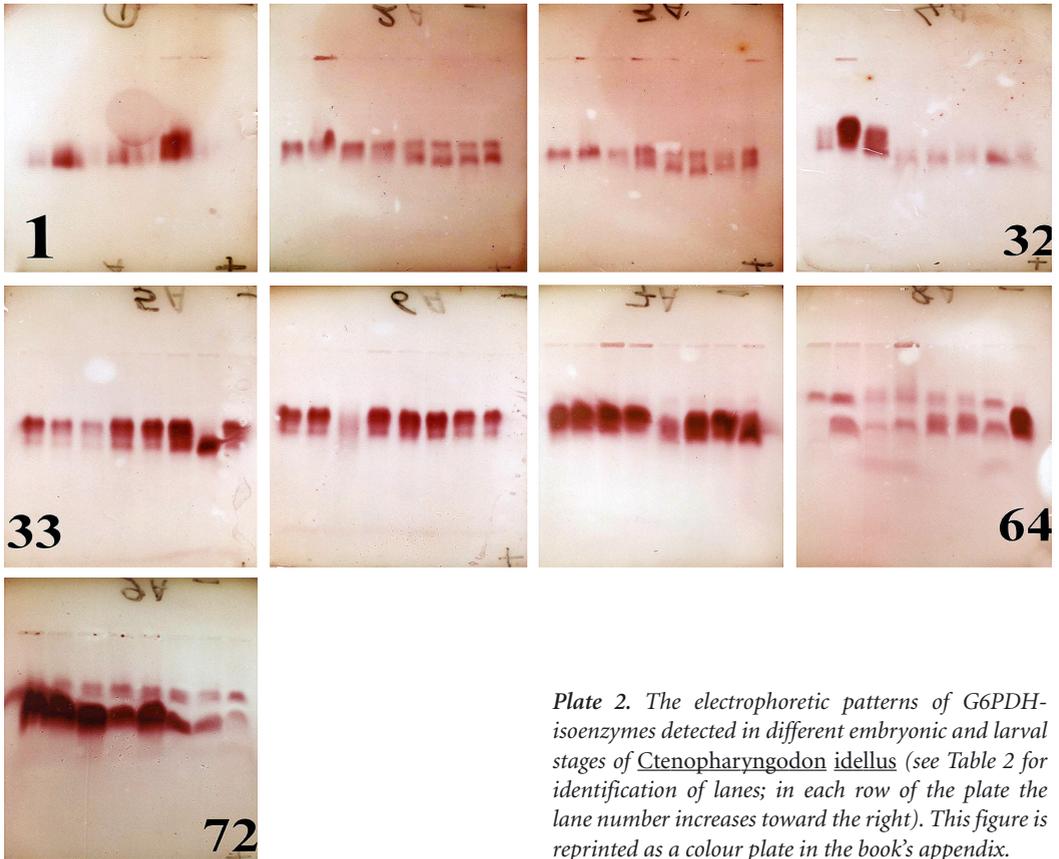


Plate 2. The electrophoretic patterns of G6PDH-isoenzymes detected in different embryonic and larval stages of *Ctenopharyngodon idellus* (see Table 2 for identification of lanes; in each row of the plate the lane number increases toward the right). This figure is reprinted as a colour plate in the book's appendix.

Results and Discussion

LDH isoenzymes. Table 1, Plate 1 and Figure 1a show the patterns of LDH isoenzymes detected by cellulose acetate electrophoresis in the embryonic and larval stages of *Ctenopharyngodon idellus* treated with lead and cadmium in comparison with the control. LDH isoenzymes (A4, A3B, A2B2, AB3 and B4) detected in such stages were found to be controlled by two loci, LDH-A and LDH-B. Such isoenzymes in control individuals exhibited variable activities during development (Table 1).

From 8-hour-post-fertilization (8h-stage) to 24h-stages, only the maternal B4-isoenzyme was detected. Such enzyme in addition to other maternal metabolic ones is necessary to initiate the embryonic development. The amount of maternal B4-isoenzyme fluctuates until the 24h-stage, in which it decreases to 11%. LDH-B locus and other enzymes involved in glycolysis and in carbohydrate metabolism are important as housekeepers (Neyfakh and Abramova 1979) in developing embryos and almost remain constant or slowly decrease (Shaklee and Whitt 1977). Generally, such situations refer to the utilization of maternal enzymes stores and their subsequent degradation before zygotic translation of mRNA. 16h-stage up to 36h-stage is the time of gastrulation and

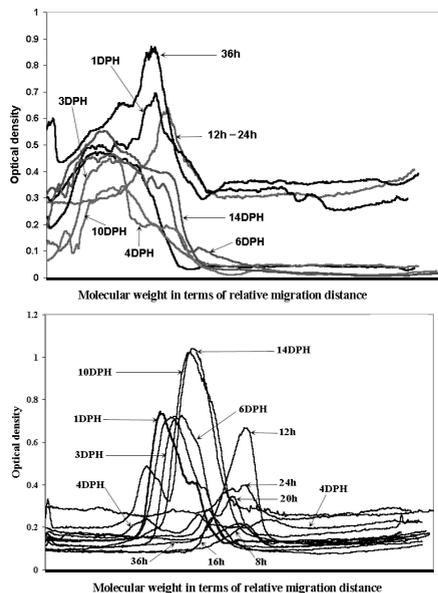


Figure 1. The electrophoretic LDH- (a) and G-6-PDH isozymogram (b) of different developmental stages of grass carp, *Ctenopharyngodon idellus* representing the control.

heart muscles. In the subsequent stages, the contribution of A-subunit equalizes or exceeds that of B-subunit. Such a situation is in accordance with the development of the skeletal muscles and the liver. There is some evidence that synthesis of the subunit-A is regulated by oxygen tension (Rider and Taylor 1980). The variability in patterns of LDH isoenzymes reflects their particular metabolic characteristics including reactivity with various analogues of the coenzyme NAD during developmental stages. Also, such asymmetry of the electrophoretic pattern of zygotic LDH in each stage reflect that their rates of synthesis, rates of breakdown of their polypeptide units and the specific activity of LDH subunits are not equal. These differences may depend on the action of regulatory genes linked to LDH-A and LDH-B loci. Rider and Taylor (1980) postulated that the transient isoenzyme patterns discernable at particular times in embryonic and neonatal life may be essential to the solution of specific metabolic problems at those stages of development.

The maternal B4-isoenzymes fluctuate in their activities in 12h-stage to 20h-stage under the effect of lead and cadmium doses. Does this situation reflect differential sensitivity and at the same time reflect that isoenzyme expression tends to be more tightly coordinated between individuals? In the later stage, decline in B4 activity is evident especially with lead doses. So, one can conclude that early embryonic development of *Ctenopharyngodon idellus* up to the first steps of organogenesis is less sensitive to lead and cadmium in spite of the process of water hardening. It is probable that the highest percentages of lead and cadmium are accumulated in the chorion not in the

epiboly during which the body axis formation and organogenesis are initiated (Plate 3). This developmental period corresponds to the initial differentiation of tissues of liver kidney, gonads and nervous system etc. Accordingly, the zygotic genes of *Ctenopharyngodon idellus* embryo appear to be inactive up to the process of organogenesis. Numerous observations of various fish species refer to the fact that maternal proteins are synthesized up to the blastula stage or even up to gastrulation (Whitt 1970; Shaklee and Whitt 1977; Neyfakh and Abramova 1979).

Only, in 36h-stage (the hatching stage), a major switching process from maternally determined LDH translation to embryonic (zygotic) LDH translation occurred. The zygotic LDH-isoenzymes varied in their individual activities and their number from stage to stage (Table 1). Moreover, total activity varied from stage to stage without a definite trend in the control individuals. The contribution of B-subunit is larger than that of A-subunit in 36h-stage and 1DPH-stage (1-day-post-hatching stage) that have well developed

Table 1. The percentages of LDH isoenzymes detected in lead- and cadmium-induced embryonic and larval stages of Ctenopharyngodon idellus in comparison with the control stages and the total amount of LDH enzyme (%) relative to each electrophoretic lane (5 µl-extract) (Lane No. corresponds to that of Plate 1).

| Stage | Lane | LDH isoenzymes (%) | | | | | % |
|-------|------|--------------------|------|------|------|------|----|
| | | A4 | A3B | A2B2 | AB3 | B4 | |
| C8h | 1 | | | | | 100 | 64 |
| C12h | 2 | | | | | 100 | 70 |
| Cd100 | 3 | | | | | 100 | 70 |
| Cd200 | 4 | | | | | 100 | 58 |
| Cd300 | 5 | | | | | 100 | 53 |
| Pb100 | 6 | | | | | 100 | 39 |
| Pb200 | 7 | | | | | 100 | 48 |
| Pb300 | 8 | | | | | 100 | 57 |
| C16h | 9 | | | | | 100 | 69 |
| Cd100 | 10 | | | | | 100 | 66 |
| Cd200 | 11 | | | | | 100 | 57 |
| Cd300 | 12 | | | | | 100 | 43 |
| Pb100 | 13 | | | | | 100 | 72 |
| Pb200 | 14 | | | | | 100 | 77 |
| Pb300 | 15 | | | | | 100 | 61 |
| C20h | 16 | | | | | 100 | 70 |
| Cd100 | 17 | | | | | 100 | 29 |
| Cd200 | 18 | | | | | 100 | 40 |
| Cd300 | 19 | | | | | 100 | 34 |
| Pb100 | 20 | | | | | 100 | 28 |
| Pb200 | 21 | | | | | 100 | 24 |
| Pb300 | 22 | | | | | 100 | 5 |
| C24h | 23 | | | | | 100 | 11 |
| Cd100 | 24 | | | | | 100 | 24 |
| Cd200 | 25 | | 48.2 | | 35.4 | 16.4 | 50 |
| Cd300 | 26 | | 33.5 | | 34 | 32.5 | 61 |
| Pb100 | 27 | 12.9 | 13.1 | 18.9 | 15.3 | 39.8 | 71 |
| Pb200 | 28 | 17.5 | 6.2 | 26 | | 50.3 | 52 |
| Pb300 | 29 | 20.4 | | 17.7 | | 61.9 | 74 |
| C36h | 30 | 21.6 | | 26.5 | | 51.9 | 68 |
| Cd100 | 31 | 14.9 | | 10 | | 75.1 | 65 |
| Cd200 | 32 | | | | | 100 | 45 |
| Cd300 | 33 | 13.9 | 10.2 | 21.4 | 11.2 | 43.3 | 69 |
| Pb100 | 34 | 28 | 15.9 | 15.9 | 14 | 26.2 | 53 |
| Pb200 | 35 | | | | 27.3 | 72.7 | 77 |
| Pb300 | 36 | 18.5 | 9.6 | 19.2 | 12.2 | 40.5 | 75 |

Table 1 (cont.). The percentages of LDH isoenzymes detected in lead- and cadmium-induced embryonic and larval stages of Ctenopharyngodon idellus in comparison with the control stages and the total amount of LDH enzyme (%) relative to each electrophoretic lane (5 μ l-extract) (Lane No. corresponds to that of Plate 1).

| Stage | LDH isoenzymes (%) | | | | | | % |
|--------|--------------------|------|------|------|------|------|-----|
| | Lane | A4 | A3B | A2B2 | AB3 | B4 | |
| C1DPH | 37 | 18.2 | 11.4 | 8.4 | 8.6 | 53.4 | 73 |
| Cd100 | 38 | 28 | 13.3 | 14 | 5.7 | 39 | 75 |
| Cd200 | 39 | 18.2 | 11.6 | 17.2 | 25.4 | 27.6 | 58 |
| Cd300 | 40 | 13.1 | 10.8 | 11.9 | 11.5 | 52.7 | 81 |
| Pb100 | 41 | 37.2 | 25.6 | 8.5 | 13.1 | 15.6 | 80 |
| Pb200 | 42 | 39.4 | 18.6 | 16.7 | 8.6 | 16.7 | 84 |
| Pb300 | 43 | | | | 66.8 | 33.2 | 84 |
| C3DPH | 44 | 22.7 | 29.0 | 8.1 | 16.8 | 23.4 | 88 |
| Cd100 | 45 | 44.4 | 10.1 | 14.5 | 7.3 | 23.7 | 89 |
| Cd200 | 46 | 24.7 | 18.1 | 17.5 | 17.8 | 21.9 | 87 |
| Cd300 | 47 | 26.8 | 21.4 | 14.6 | 12.3 | 24.9 | 85 |
| Pb100 | 48 | 30.8 | 20.4 | 26.5 | | 22.3 | 85 |
| Pb200 | 49 | 20 | 33.8 | 27.6 | | 18.7 | 64 |
| Pb300 | 50 | 45.9 | 19.3 | 29.2 | | 5.6 | 75 |
| C4DPH | 51 | 28.7 | 23.3 | 21.3 | 9.2 | 18.9 | 84 |
| Cd100 | 52 | 27.6 | 13.1 | 27.9 | 7.3 | 24.2 | 83. |
| Cd200 | 53 | 31.8 | 35.4 | 32.8 | | | 68 |
| Cd300 | 54 | 30.2 | 10.9 | 34.6 | | 24.4 | 79 |
| Pb100 | 55 | 28.6 | 26.1 | 26.5 | | 18.8 | 81 |
| Pb200 | 56 | 26.4 | 27.6 | 29.7 | | 16.3 | 74 |
| Pb300 | 57 | 33.3 | 21.6 | 25.9 | | 19.1 | 78 |
| C6DPH | 58 | 20.5 | 17.3 | 25.9 | | 36.2 | 77 |
| Cd100 | 59 | | 27.1 | 30.4 | | 42.6 | 64 |
| Pb100 | 60 | 17.7 | 28.7 | 24.5 | | 8.1 | 76 |
| Pb200 | 61 | 25.6 | 52.5 | 21.9 | | | 95 |
| Pb300 | 62 | 24.6 | 47.9 | 20.5 | | 6.9 | 93 |
| C10DPH | 63 | 24.4 | 38.5 | 12 | | 25.1 | 65 |
| Cd100 | 64 | 14.4 | 19 | 43.7 | 14.2 | 8.7 | 99 |
| Pb100 | 65 | 4.8 | 12.7 | 30.8 | 17.3 | 34.6 | 79 |
| Pb200 | 66 | 6.8 | 14.4 | 35.5 | 16.5 | 26.7 | 85 |
| Pb300 | 67 | 8.0 | 12.6 | 36.5 | 16.3 | 26.6 | 85 |
| C14DPH | 68 | 5.8 | 13.2 | 38.6 | 28.2 | 14.2 | 88 |
| Cd100 | 69 | 9.3 | 11.9 | 43.9 | 13.5 | 21.3 | 86 |
| Pb100 | 70 | 6.6 | 14.5 | 42.9 | 14.5 | 21.6 | 90 |
| Pb200 | 71 | 8.7 | 13.9 | 43.7 | 24.5 | 9.2 | 87 |
| Pb300 | 72 | 8.3 | 10.2 | 49.1 | 18.1 | 14.4 | 82 |

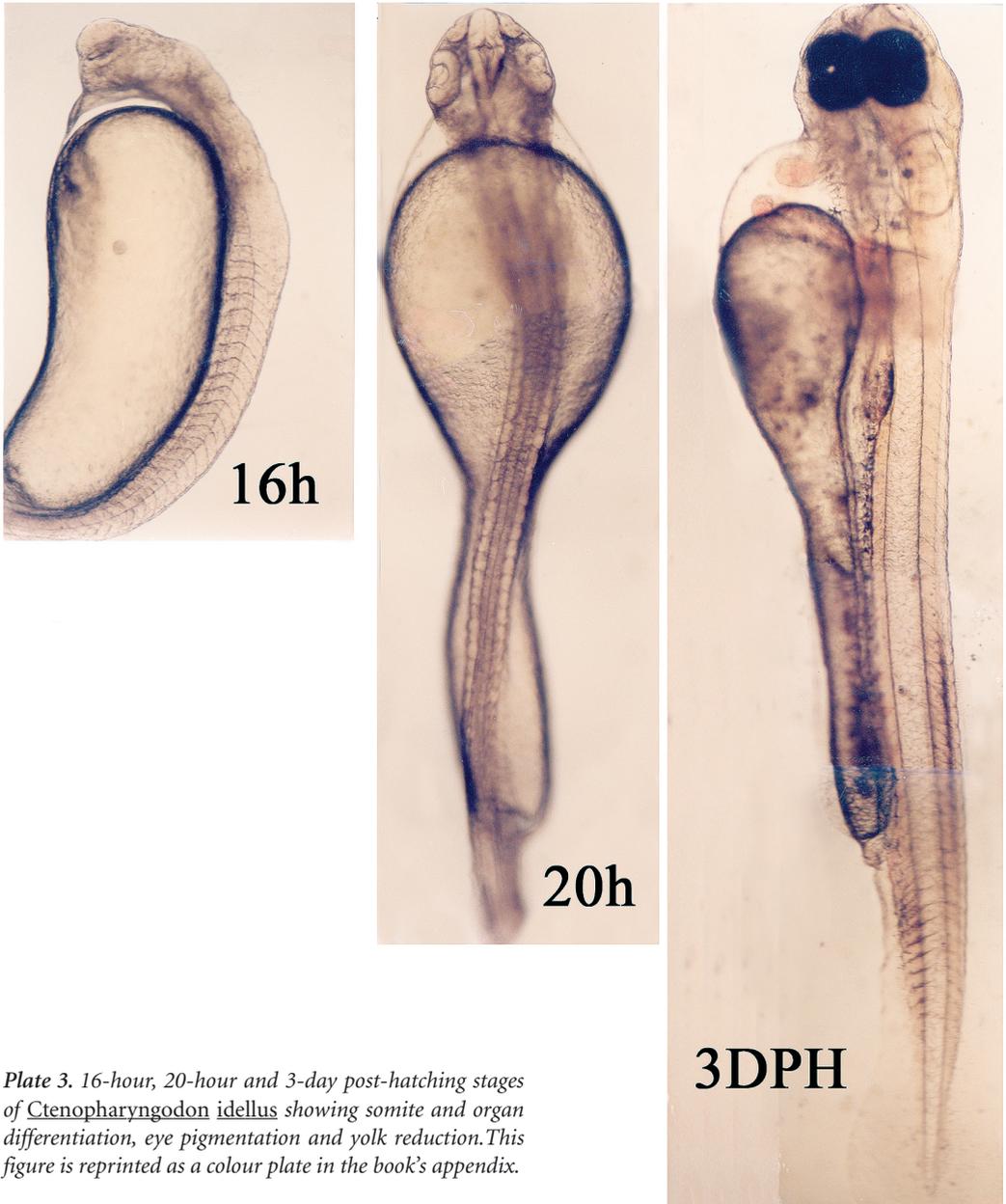


Plate 3. 16-hour, 20-hour and 3-day post-hatching stages of Ctenopharyngodon idellus showing somite and organ differentiation, eye pigmentation and yolk reduction. This figure is reprinted as a colour plate in the book's appendix.

embryo. This conclusion is emphasized by the low decreases in the percentages of egg swelling (62-61% for cadmium; 63-62% for lead), and hatching (71-65% for cadmium; 79-74% for lead) and increase in mortality percentages (18-22% for cadmium; 9-17% for lead) in comparison with the control (63, 82 and 6% respectively). Up to the 24h-stage, the external morphology of the embryo was not severely affected due to cadmium and lead doses considered (100-300ppm). However,

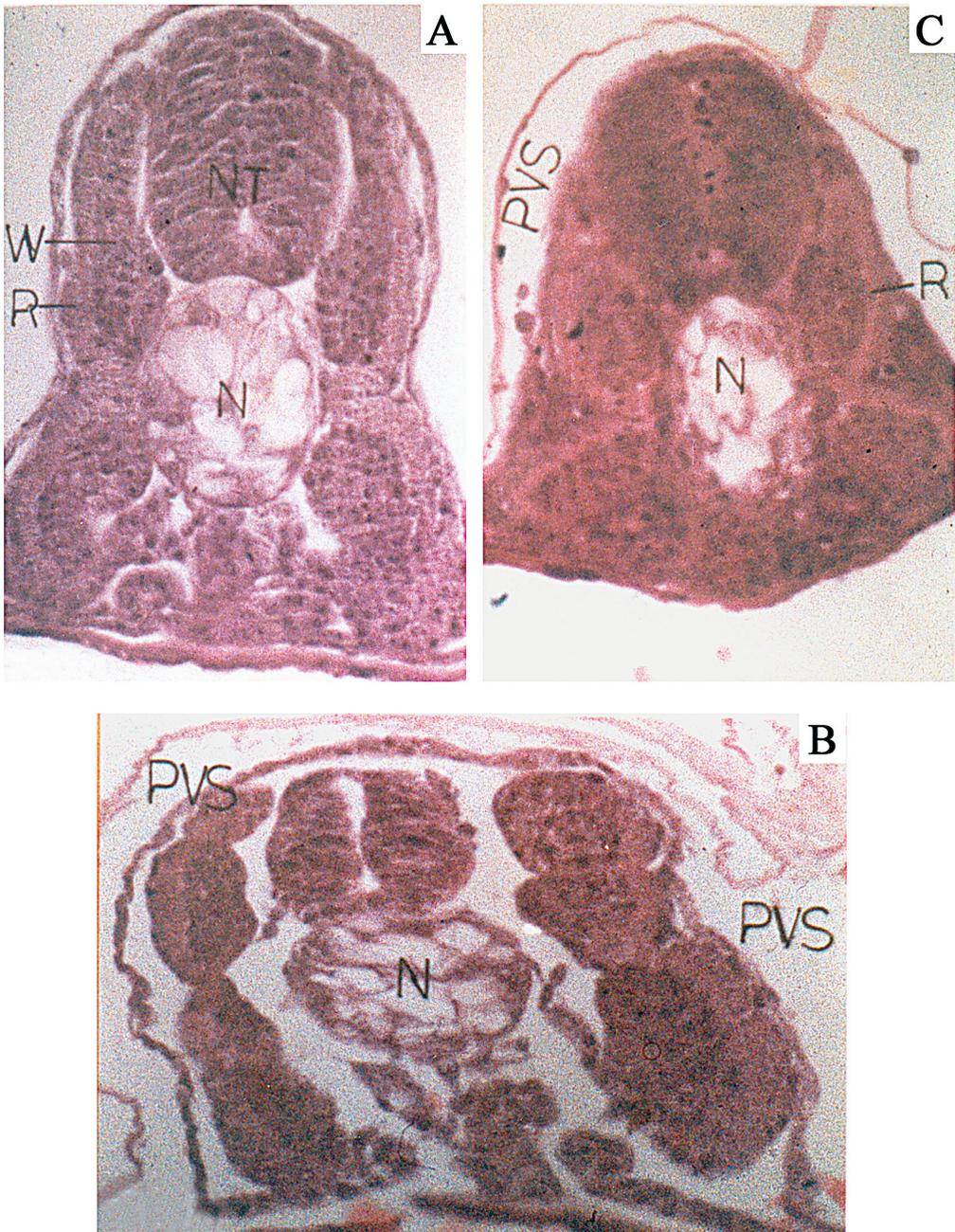


Plate 4. Transverse sections at the trunk level of 18-hour embryo of Ctenopharyngodon idellus treated with cadmium (B) and lead (C) in comparison with the control (A). See the reduced perivitelline space, PVS; disorganized peripheral red myogenic cells, R and distorted notochord, N (Pb less toxic than Cd). W: white muscles; NT: neural tube. This figure is reprinted as a colour plate in the book's appendix.

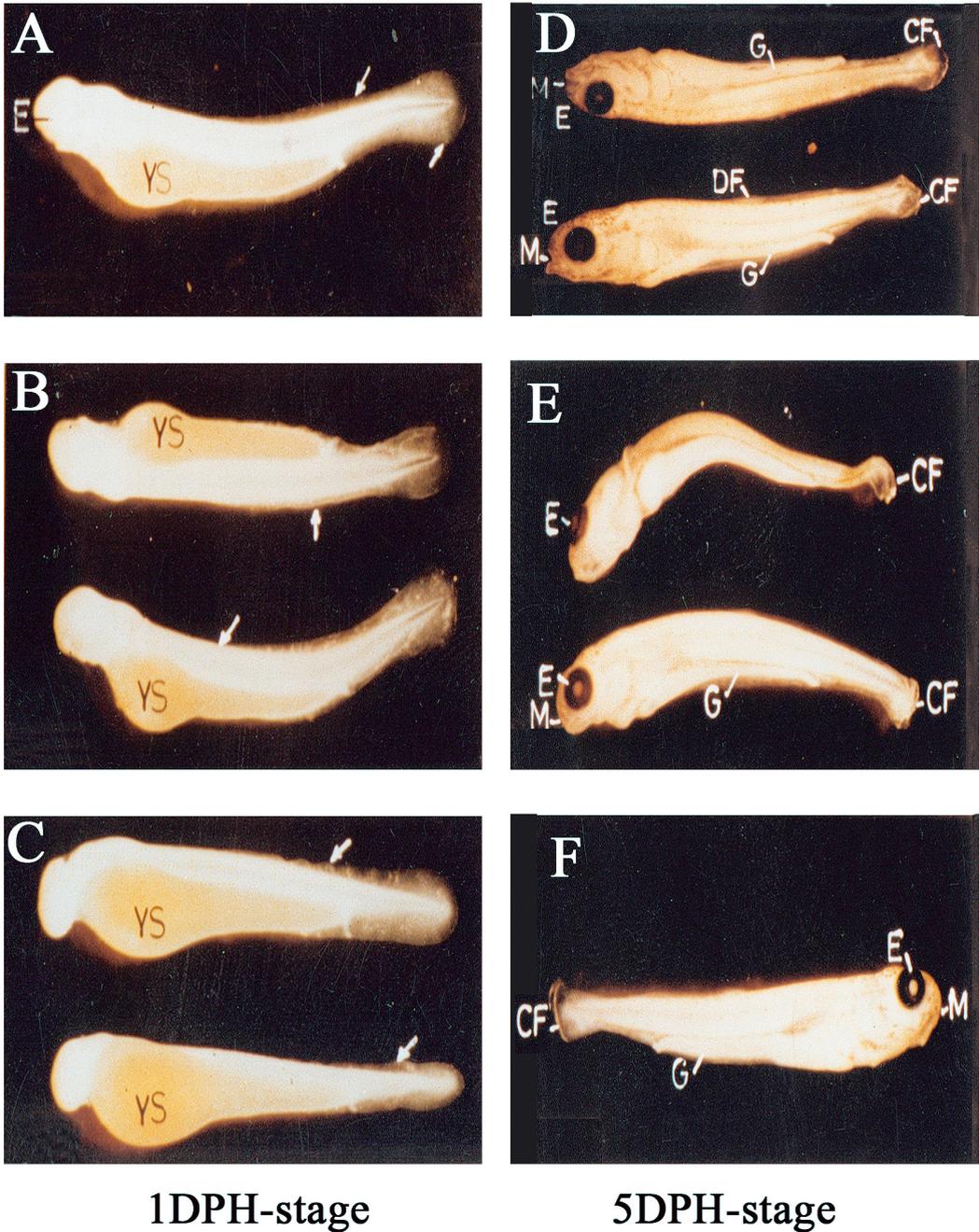
there were some abnormalities appearing during the process of organogenesis that began early in the 16h-stage. In spite of the low amount of lead and cadmium reaching the embryo itself, in the cadmium- and lead-treated 18h-stages, a reduction of the perivitelline space, disorganized myogenic cells and notochordal distortion were observed as compared to the control embryos (Plate 4). Beatie and Pascoe (1978) found that 98% of the cadmium was associated with the chorion of rainbow trout rather than with the embryo itself or the yolk. Similar findings were recorded for cadmium-induced medaka embryos (Michibata 1981). Weis and Weis (1989) reported that salmon embryos were more resistance to cadmium with very low metal uptake even when the chorion was removed. Stouthart et al. (1994) stated that lead was mainly located in the chorion, probably bound to its mucopolysaccharides.

In 24h-stage, in comparison with the control of that stage, lead and cadmium higher doses led to early activation of the zygotic LDH-A and LDH-B loci which were differentially expressed according to different lead and cadmium doses (Table1); LDH-enzyme amount increased under heavy metal stress. Such early activation of zygotic loci may be an adaptive mechanism that protects the embryos against the increased heavy metal exposure especially in the period of organogenesis. Contribution of the B-subunit exceeded that of the A-subunit especially with high doses of lead. It is concluded that the efficiency at which zygotic translation and hence the maternal-zygotic switching process occurs is related to lead- and cadmium-induced degradation of maternal mRNA .

The current doses of cadmium and lead had no effect on hatching time (36 hours after fertilization) of *Ctenopharyngodon idellus*. It is delayed with doses above 400 ppm of such metals (Lashein 1996). In the 36h-stage, the contribution of B-subunit generally increased in the treated embryos. Lead and cadmium led to synthesis of new isoenzymes or increase of synthesis rate of others except in the case of 200ppm-Cd and 200ppm-Pb doses where some isoenzymes disappeared. Vallee and Ulmer (1972) and Prasada-Rao et al. (1983) reported that cadmium inactivates some enzymes while activating others. This metal also alters ribonucleic acid synthesis (Bryan and Hidalgo 1976) and probably destabilizes deoxyribonucleic acid (Eichhorn 1962; Stoll et al. 1976).

No obvious external morphological variations were detected in the newly hatched larvae. In cadmium-treated larvae, 200 ppm-doses slightly affected the peripheral red myogenic muscle cells; cadmium doses equal to or above 400 ppm caused undifferentiation of such muscles. Cadmium-induced notochord was more or less collapsed. Similar results were recorded with lead doses. Using PAS staining, myogenic cell stainability decreased with lead- and cadmium-dose increase. No significant impact of lead and cadmium on the brain and sensory organs of the newly hatched larvae was detected.

In subsequent stages, the five isoenzymes, A4, A3B, A2B2, AB3 and B4 were represented with few exceptions in 3DPH-, 4DPH- and 6DPH-stages. However, the amount of the LDH enzyme increased in these larval stages. Lead and cadmium doses affects the rate of synthesis of such isoenzymes and the contribution of each LDH-A and LDH-B subunits in comparison with the control (Table1). In 10 DPH- and 14 DPH-stages, AB3 which was not detected in the previous larval stages, was synthesized. Moreover, the percentage of A2B4 increased under the stress of lead and cadmium doses (Table1). Such lead- and cadmium-induced isoenzyme variability was associated with necrosis of caudal fin margins, spinal and vertebral curvature and pale body pigmentation (Plate5). Cadmium early affected spinal and vertebral curvature in comparison with lead impact that firstly appeared in 10DPH-stage. The vertebral curvature may be associated with the severe



1DPH-stage

5DPH-stage

Plate 5. Control (A, D), Cd-100 ppm (B, E) and Pb-100 ppm (C, F) treated 1-day and 5-day post-hatching larvae of Ctenopharyngodon idellus showing spinal-vertebral curvature, paleness of body and necrosis of caudal fin (CF). E: eye, M: mouth, G: gut, YS: yolk sac, DF: dorsal fin. This figure is reprinted as a colour plate in the book's appendix.

Table 2. The percentages of G-6-PDH isoenzymes detected in lead- and cadmium-induced embryonic and larval stages of Ctenopharyngodon idellus in comparison with the control stages and the total amount of G-6-PDH enzyme (%) relative to each electrophoretic lane (5µl-extract) (Lane No. corresponds to that of Plate 2).

| Stage | Lane | G-6-PDH isoenzymes (%) | | | | | % | |
|-------|------|------------------------|------|------|------|------|---|------|
| | | A1A1 | A2A2 | A3A3 | A4A4 | B1A1 | | B1B1 |
| C8h | 1 | | | 100 | | | | 37 |
| C12h | 2 | | | 100 | | | | 64 |
| Cd100 | 3 | | | 100 | | | | 29 |
| Cd200 | 4 | | 19.7 | 80.3 | | | | 55 |
| Cd300 | 5 | | 48.8 | 51.2 | | | | 52 |
| Pb100 | 6 | | 41.0 | 59 | | | | 46 |
| Pb200 | 7 | | 39.8 | 60.2 | | | | 82 |
| Pb300 | 8 | | | 100 | | | | 18 |
| C16h | 9 | | 100 | | | | | 30 |
| Cd100 | 10 | | 100 | | | | | 9 |
| Cd200 | 11 | | 100 | | | | | 19 |
| Cd300 | 12 | | 100 | | | | | 10 |
| Pb100 | 13 | | 55.1 | 44.9 | | | | 29 |
| Pb200 | 14 | | 35.3 | 64.7 | | | | 38 |
| Pb300 | 15 | | 32.8 | 67.2 | | | | 23 |
| C20h | 16 | | 31.1 | 68.9 | | | | 38 |
| Cd100 | 17 | | | 100 | | | | 36 |
| Cd200 | 18 | | | 100 | | | | 37 |
| Cd300 | 19 | | | 100 | | | | 13 |
| Pb100 | 20 | | | 48.3 | 51.7 | | | 36 |
| Pb200 | 21 | | | 28.3 | 71.7 | | | 29 |
| Pb300 | 22 | | | 38.1 | 61.9 | | | 37 |
| C24h | 23 | | | 44.1 | 55.9 | | | 33 |
| Cd100 | 24 | | | 43.2 | 56.8 | | | 44 |
| Cd200 | 25 | | | 47.3 | 53.0 | | | 32 |
| Cd300 | 26 | | 58.1 | 41.9 | | | | 60 |
| Pb100 | 27 | | | 78.5 | 21.5 | | | 59 |
| Pb200 | 28 | | | | 100 | | | 19 |
| Pb300 | 29 | | | | 100 | | | 26 |
| C36h | 30 | | | | 100 | | | 18 |
| Cd100 | 31 | | | | 100 | | | 46 |
| Cd200 | 32 | | | | 100 | | | 25 |
| Cd300 | 33 | | 85.2 | 14.8 | | | | 71 |
| Pb100 | 34 | | 91.6 | 8.4 | | | | 60 |
| Pb200 | 35 | | 84.7 | 15.3 | | | | 35 |
| Pb300 | 36 | | 85.8 | 14.2 | | | | 79 |

Table 2 (cont.). The percentages of G-6-PDH isoenzymes detected in lead- and cadmium-induced embryonic and larval stages of Ctenopharyngodon idellus in comparison with the control stages and the total amount of G-6-PDH enzyme (%) relative to each electrophoretic lane (5 μ l-extract) (Lane No. corresponds to that of Plate 2).

| Stage | Lane | G-6-PDH isoenzymes (%) | | | | | % | |
|--------|------|------------------------|------|------|------|------|------|------|
| | | A1A1 | A2A2 | A3A3 | A4A4 | B1A1 | | B1B1 |
| C1DPH | 37 | | 83.8 | 16.2 | | | | 82 |
| Cd100 | 38 | | 52.0 | 48 | | | | 54 |
| Cd200 | 39 | | | 100 | | | | 73 |
| Cd300 | 40 | | 67.9 | 32.1 | | | | 73 |
| Pb100 | 41 | | 85.7 | 14.3 | | | | 70 |
| Pb200 | 42 | | 83.1 | 16.9 | | | | 72 |
| Pb300 | 43 | | 100 | | | | | 46 |
| C3DPH | 44 | | 69.4 | 30.6 | | | | 46 |
| Cd100 | 45 | | 100 | | | | | 89 |
| Cd200 | 46 | | 84.7 | 15.3 | | | | 82 |
| Cd300 | 47 | | 82.2 | 17.8 | | | | 74 |
| Pb100 | 48 | | 78.5 | 21.5 | | | | 77 |
| Pb200 | 49 | | | 100 | | | | 88 |
| Pb300 | 50 | | | 100 | | | | 83 |
| C4DPH | 51 | | | 100 | | | | 78 |
| Cd100 | 52 | | | 100 | | | | 78 |
| Cd200 | 53 | | | 100 | | | | 71 |
| Cd300 | 54 | | | 100 | | | | 66 |
| Pb100 | 55 | | | 100 | | | | 73 |
| Pb200 | 56 | | | 100 | | | | 86 |
| Pb300 | 57 | 100 | | | | | | 27 |
| C6DPH | 58 | 43.0 | 57.0 | | | | | 58 |
| Cd100 | 59 | 31.3 | | | | 29.7 | 39 | 38 |
| Pb100 | 60 | 29.8 | | | | 39.3 | 30.9 | 36 |
| Pb200 | 61 | 26.1 | 73.9 | | | | | 47 |
| Pb300 | 62 | 24.8 | 75.2 | | | | | 42 |
| C10DPH | 63 | 29.9 | | | | 42.5 | 27.6 | 49 |
| Cd100 | 64 | | | 100 | | | | 76 |
| Pb100 | 65 | 17.3 | 82.7 | | | | | 20 |
| Pb200 | 66 | 13.9 | 86.1 | | | | | 24 |
| Pb300 | 67 | 16.9 | 83.1 | | | | | 48 |
| C14DP | 68 | 14.9 | 85.1 | | | | | 64 |
| Cd100 | 69 | 11.8 | 88.2 | | | | | 74 |
| Pb100 | 70 | 22.9 | 77.1 | | | | | 58 |
| Pb200 | 71 | 23.7 | 76.3 | | | | | 40 |
| Pb300 | 72 | 57.1 | 42.9 | | | | | 28 |

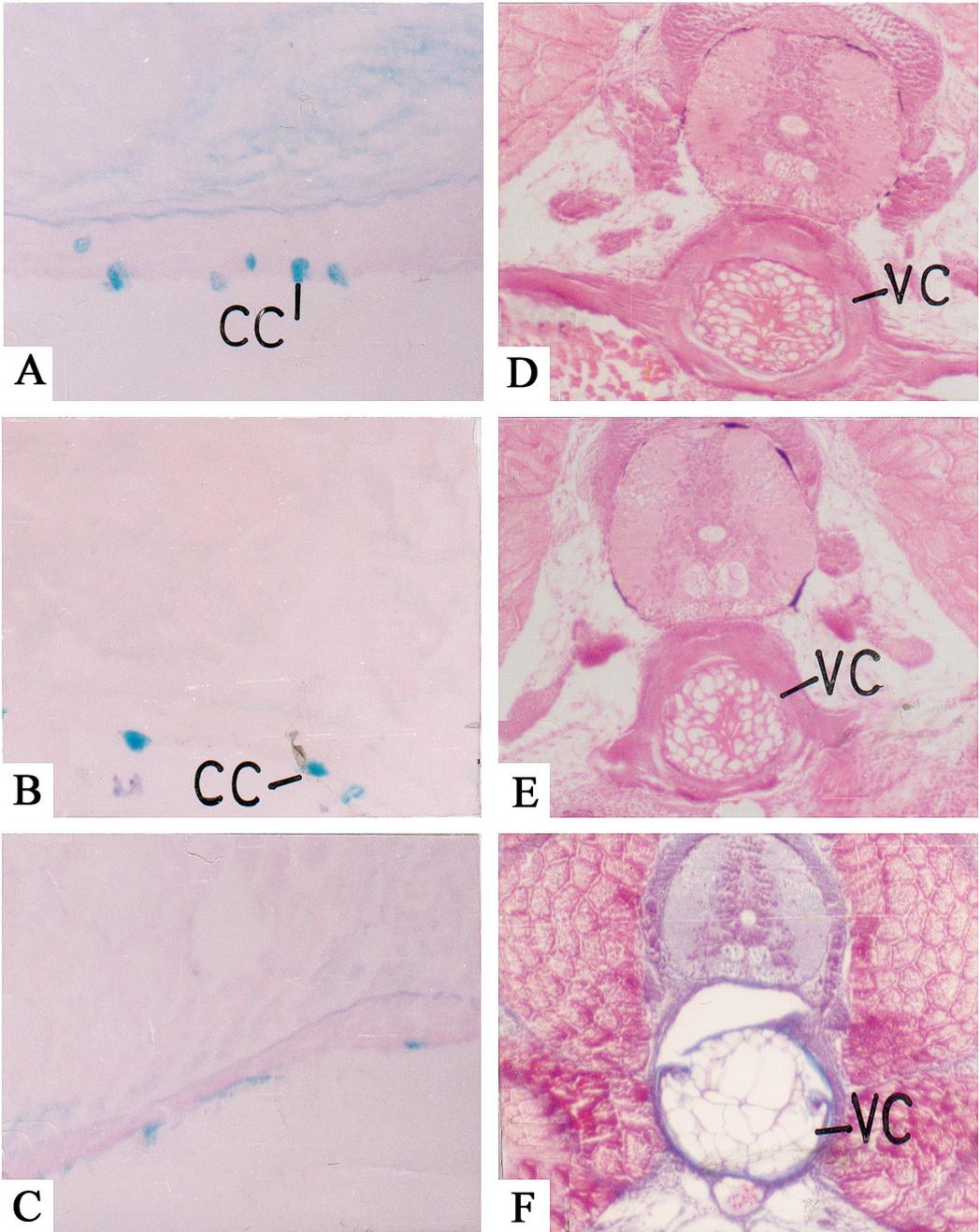


Plate 6. Transverse sections of the control (A), Cd-treated (B) and Pb-treated (C) 14-day post-hatching larvae of Ctenopharyngodon idellus showing chloride cells (CC) in the skin and the corresponding vertebral centrum (VC) deposition (D, E, F respectively). This figure is reprinted as a colour plate in the book's appendix.

degenerative effect of lead and cadmium on the abundance of chloride cells that appear in the skin of *Ctenopharyngodon idellus* 3DPH-larvae at the ventral side of the yolk sac (Lashein 1999a). Plate 6 shows the frequency of the chloride cells and the corresponding vertebral depositions in the control and Cd- and Pb-treated larvae. Since these cells are the active site of calcium uptake, deposition of skeletal elements is influenced by the degree of lead and cadmium effects on these cells. These two metals have been found to interfere with calcium uptake and metabolism in fish embryos and early larval stages (Shephard and Simkiss 1978; Stouthart et al. 1994; Lashein 1999a). The latter author stated that lead has more destructive effect on the chloride cells of *Ctenopharyngodon idellus* than cadmium. Rosenthal and Alderdice (1976) reviewed similar malformation observable after hatching and originating from tissue injury during early stages. Longwell et al. (1992) stated that some of these problems of development are probably the results of abnormal chromosome division of the fish embryo.

G-6-PDH isoenzymes. Table 2, Plate 2 and Figure 1b show the patterns of G-6-PDH isoenzymes detected by cellulose acetate electrophoresis in the embryonic and larval stages of *Ctenopharyngodon idellus* treated with lead and cadmium in comparison with the control. G-6-PDH isoenzymes detected in such stages were found to be controlled by two loci, G-6-PDH-A (a multiple locus having four alleles, A1 to A4) and G-6-PDH-B. The expression of the latter locus was only detected in the control 10d-post-hatching stage (Table 2). The G-6-PDH-A locus seemed to be sex linked on Lyon Hypothesis basis (Rider and Taylor 1980) since no hybrid isoenzymes are formed. Such allelic isoenzymes in control individuals exhibited variable activities during development (Table 2). Such variability must be considered a normal developmental requirement that represents a state of balanced polymorphism during the programmable translation of the genetic system in terms of cell and organ differentiation in variable environments.

From the 8 h- to 16 h-stages, only the maternal G-6-PDH-A isoenzymes were detected. The amount of G-6-PDH in these stages fluctuated and was influenced by allele A2 or A3. The contribution of different alleles in the subsequent stages varies. However, that of A2 was higher in the majority of larval stages. Lead doses (<300 ppm) and cadmium higher doses (> 100 ppm) led to the early activation of the zygotic alleles of G-6-PDH in 12h-stage in comparison with the control (Table 2). In the 16 h-stage, cadmium doses only affect differentially the rate of synthesis of the maternal G-6-PDH. Lead doses activated early the zygotic alleles with a higher contribution of allele A3 in comparison with the control stage. In the 20 h-stage, cadmium prefers only the product of allele A3 and a new allele, A4 was activated in high percentage by lead doses. In the subsequent stages, except for 4-day- and 6-day-post-hatching stages, the pattern of isoenzymes exhibited no definite trend for generalization. In 4-day-post-hatching stage, only the A3 allele expressed itself in the control and treated larvae, except for larvae treated with 300 ppm lead where low A1-allele contribution was detected. The expression of alleles of G-6-PDH-B locus was early detected under the stress of low doses of cadmium and lead in 6-day-post-hatching larvae in comparison with the control stage (10-day-post-hatching larvae) in which such locus was detected. Similar to the situation in LDH, the aforementioned protective variations in G-6-PDH isoenzyme patterns can be considered in association with the morphological ones.

Most of the egg carbohydrates are associated with the egg membrane and therefore are proba-

bly unavailable for use by the developing embryo at least until hatching; the time of their release from that membrane. However, intensive catabolism of carbohydrates commences at fertilization indicating that carbohydrates play an important nutritive role during initial cleavage (Diwan and Dhakad 1995). Such very early metabolism of carbohydrates is associated with LDH and G-6-PDH isoenzymes. So, can one consider the interference of lead and cadmium with synthesis of metabolic enzymes through gene activation and in turn with carbohydrate metabolism as adaptive or antioxidant mechanism for the embryo to tolerate the heavy metal effects? Cadmium does not appear to generate free radicals (Ochi et al. 1987), but does elevate lipid peroxidation in tissues soon after exposure (Muller 1986). Lipid peroxidation occurs in hepatocyte cultures with leakage of LDH and transaminase enzymes (Morel et al. 1990; Carini et al. 1992). Studies by Fariss (1991) have shown that free radical scavengers and antioxidants are useful in protecting against cadmium toxicity. Naughton et al. (1996) reported that exposure of lactate to sources of $\cdot\text{OH}$ radical generated pyruvate and subsequently acetate and CO_2 providing evidence for its powerful $\cdot\text{OH}$ radical scavenging ability. This scavenger may play an important role in neutralizing the toxic effect of $\cdot\text{OH}$ radical arising from any residual H_2O_2 . The production of lactate and pyruvate is associated with the production of NADH which may help in reducing the oxidative stress. Similarly, the increased activity of G-6-PDH results in an increased availability of NADPH which is responsible for protecting liver GSH and preventing lipid peroxidation. G-6-PDH has also been shown to confer resistance to oxidizing agents in *Escherichia coli* and thus may act directly as an antioxidant (Beutler and Yoshida 1988). G-6-PDH is one of 40 proteins that is induced during oxidative stress.

Heath (1995) reported that exposure of embryos to metal at sublethal level can (but not necessarily always) induce a greater tolerance to that metal in the subsequent larvae. This situation was found for cadmium in rainbow trout (Beatie and Pascoe 1978) and may be attributed to metallothionein synthesis in adult fish which serves to sequester the metal (Heath 1995). A similar mechanism was recorded for mercury-induced *Fundulus* embryos (Weis 1984) and generally can be detected during the oxidative stress of other metals. The increased lead- and cadmium-morphological and histological variations of *Ctenopharyngodon idellus* with developmental progress did not emphasize such metal tolerance. In conclusion, the antioxidant system of embryonic and larval stages of *C. idellus* needs to be characterized in order to better understand the enzymatic and non-enzymatic mechanisms that control this system. Moreover, further studies are required to discover the nature of heavy metal impacts on the structural genes and on the regulatory genes that control their expression on the light of homo- and heterozygosity. Finally, one must answer the question: what is the role of the pituitary and thyroid hormones in connection with the oxidative mechanism and activation and inactivation of genes of the embryonic and larval development of *Ctenopharyngodon idellus* under stress.

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Complexity and constraints combined in simple models of recruitment

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Abstract

A simple model is described that uses a combination of stochastically varying mortality during early life and deterministically-driven mortality in the juvenile phase to simulate recruitment. The model is applied to data on walleye pollock *Theragra chalcogramma* in the Gulf of Alaska and Pacific hake *Merluccius productus* in the California Current region to simulate a recruitment time series for each stock. When compared with observed recruitment time series, the model simulations accurately capture the trends and characteristics of recruitment for these stocks, demonstrating the interplay of high frequency activating factors and low frequency constraining factors in the complex process of recruitment.

Introduction

Recruitment is a complex biophysical process, but macro-ecological principles and deterministic factors put boundaries and constraints on the array of possible outcomes. The interaction of biological and environmental factors influencing larval survival is complex and noisy. For example, larval feeding success is influenced by turbulence, light levels, temperature and prey availability and their interactions. Larval feeding increases with moderate turbulence due to higher contact rates with potential prey, but reaches a maximum and declines with higher turbulence due to interference with larval feeding behavior; thus the relationship of larval feeding success with turbulence is nonlinear (MacKenzie et al. 1994). In the case of walleye pollock (*Theragra chalcogramma*), their larvae also avoid turbulence by diving, which removes them from the turbulent regime (Davis 2001), but also takes them out of more illuminated depths with higher prey densities (Kendall et al. 1994). There are other relatively high frequency environmental events that influence larval survival besides feeding conditions, such as outbursts of planktonic predators (like jellyfish),

advection, and environmentally driven shifts in spatial and temporal interactions with biota (e.g., blooms, predator-prey overlaps) and physical features (e.g., seasonal warming, fronts).

On the other hand, boundary conditions and deterministic processes constrain and dampen the oscillations generated by variability in larval survival. For example, the amount of suitable habitat area places a strong constraint on population size. A long-term buildup of large long-lived predators in a community may also constrain recruitment levels. In the Gulf of Alaska, such an increase in groundfish predators, possibly due to an environmental regime shift (Anderson and Piatt 1999), is responsible for increased mortality of juvenile pollock and decreased recruitment (Bailey 2000). Density-dependent processes also may dampen recruitment variations (van der Veer 1986).

Recruitment may be described, therefore, as the interplay of high frequency activating factors (Levin and Pacala 1997), that is, noisy environmental conditions occurring during early life, and constraining factors acting on juveniles. Changes in these constraining (and sometimes boundary or even emergent) conditions occur at relatively low frequency. In this paper, we describe some early stage "hybrid" models by which larval survival is described stochastically (but under the influence of environmental conditions), and juvenile survival is influenced deterministically by constraining factors such as predation and density-dependence. In the cases presented here for walleye pollock (*Theragra chalcogramma*) in the western Gulf of Alaska and Pacific hake (*Merluccius productus*) in the California Current system, these models fairly accurately describe the pattern and magnitude of recruitment, illustrating the concept of bio-complexity, importance of initial and boundary conditions, and interaction of high and low frequency events.

Materials and Methods

A simple type of recruitment model was formulated that describes early life events as stochastically varying, but under the influence of environmental factors and the deterministic control of low frequency and boundary conditions. Similar models have been formulated by Stenseth et al. (1999) and Bjornstad and Grenfell (2001). The form of the survival model for walleye pollock was the standard population dynamics equation:

$$R = E * e^{-(m_1 * t_1 + m_2 * t_2 + m_3 * t_3)}$$

where R is the number of age-2 recruits, E is the initial number of eggs spawned and m_1 is randomly generated mortality over the egg and larval period t_1 , with a low or high range of mortality that depends on environmental conditions. Walleye pollock spawn mainly in Shelikof Strait between Kodiak Island and the Alaska Peninsula in the Alaska Coastal Current (Fig. 1A). Wind speed and sea surface temperature (SST) are two factors associated with survival of larval pollock in this region (Bailey and Macklin 1994; Bailey et al. 1996). Therefore, a low range of mortality ($m_1 t_1 = 5$ to 6.5) was generated if SST (April-June) was above average and wind speed (May) was below average, otherwise a high range of mortality ($m_1 t_1 = 7.5$ to 9.5) was generated. The overall range of larval mortality was determined from a life table (Bailey et al. 1996); non-overlapping high and low ranges under differing environmental conditions were estimated to reflect the "boom

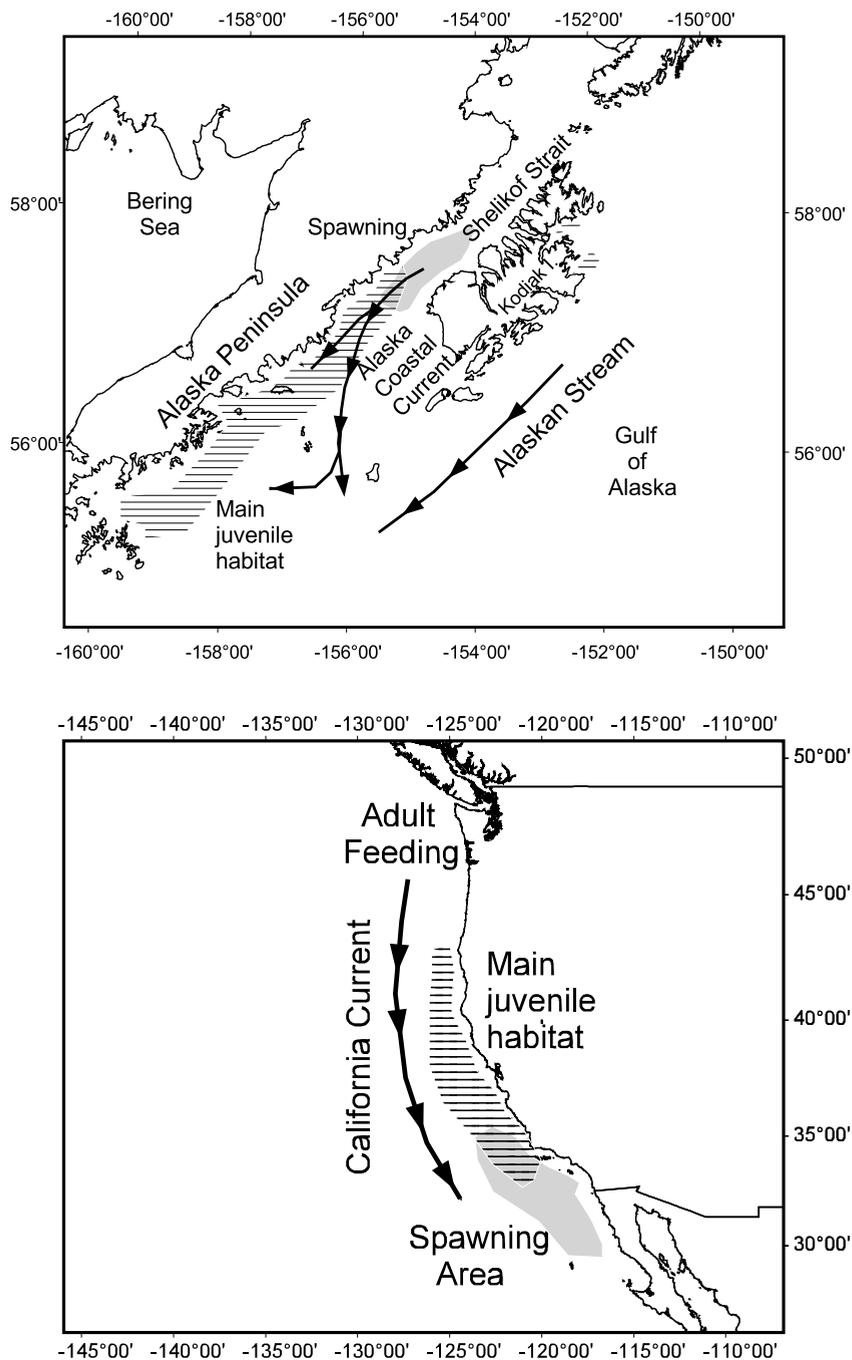


Figure 1. The area of spawning and transport scheme of eggs and larvae of a) walleye pollock *Theragra chalcogramma* and b) Pacific hake *Merluccius productus*.

or bust" nature of larval survival. Late larval mortality rate, m_2 , is density dependent (Schumacher and Kendall 1991). The range of late larval mortality over the period t_2 was determined from life tables (Bailey et al. 1996) and mortality was thus scaled between 1 and 5 depending on cohort density. Juvenile mortality, m_3 , was assumed to depend on potential consumption by large predators over period t_3 ; thus $m_3 t_3$ was scaled between 1 and 3 against the predation potential of large piscivorous groundfishes as presented by Bailey (2000). There was no attempt to fit or optimize the model. The model was run 200 times, each with a fresh set of random numbers, to simulate variation in the early larval mortality.

For comparison of simulated and observed recruitment, a recruitment time series of age-2 pollock in the Gulf of Alaska from catch-at-age analysis was obtained from stock assessment documents (NPFMC 1997). Natality was calculated for the spawning biomass in the western Gulf of Alaska accounting for eggs kg^{-1} and sex ratios and was provided by B. Megrey (Alaska Fisheries Science Center, Seattle WA, pers. comm.). Predation pressure on juvenile pollock was taken from Bailey (2000), calculated from a model accounting for predator biomass, daily ration, overlapping of predator and prey, and percent pollock in the diet. SST was the average value over April-June and was obtained from the NCEP Reanalysis data set provided by N.A. Bond (Pacific Marine Environmental Laboratory, pers. comm.) and was centered on 56°N 156°W . The average May wind mixing parameter ($\text{m}^3 \text{s}^{-3}$) was derived from the surface stress from gradient winds and was provided by S.A. Macklin (Pacific Marine Environmental Laboratory, pers. comm.).

In the case of Pacific hake a similar model form was used. Pacific hake spawn in offshore waters of central to Baja California within the California Current system (Fig. 1B). As with the pollock model, R and E are the number of age-2 recruits and the number of eggs spawned. The ranges of mortality of larvae and juveniles was determined from a life table in Hollowed (1992). SST is correlated with larval survival of Pacific hake (Bailey 1981). Therefore early larval mortality, m_1 , was randomly generated over the larval period t_1 , with a low range ($m_1 t_1 = 7$ to 8.5) if temperature was above average and a high range ($m_1 t_1 = 9.5$ to 11.5) if temperature was below average. Density-dependent mortality due to cannibalism on age-0 and age-1 juvenile hake by older fishes is believed to occur (Buckley and Livingston 1997); density dependent mortality was described here by an empirical fitted relationship between the abundance of the immediately preceding three older year classes (age-1 to age-2 mortality was linearly scaled between 0.15 and 0.4 depending on the three year $\{t, t-1$ and $t-2\}$ running average of observed age-2 recruits). For comparison with the simulated recruitment time series, values for the observed number of recruits were from catch-at-age analysis of commercial harvests (Dorn et al. 1994; 1999). SST was the average value over January-March and was obtained from the NCEP Reanalysis data set provided by N.A. Bond (Pacific Marine Environmental Laboratory, pers. comm.) and was centered on 33°N 120°W .

Results

The time series of data on recruitment of walleye pollock in the Gulf of Alaska extends back to the late 1960s although the early years are probably not reliable. There is evidence of eight strong or moderately strong year classes in the 30 year series, occurring in 1972, 1976-79, 1984, 1988 and 1993 (Fig. 2). Since the late 1970s there has been a trend for decreasing recruitment (slope of time

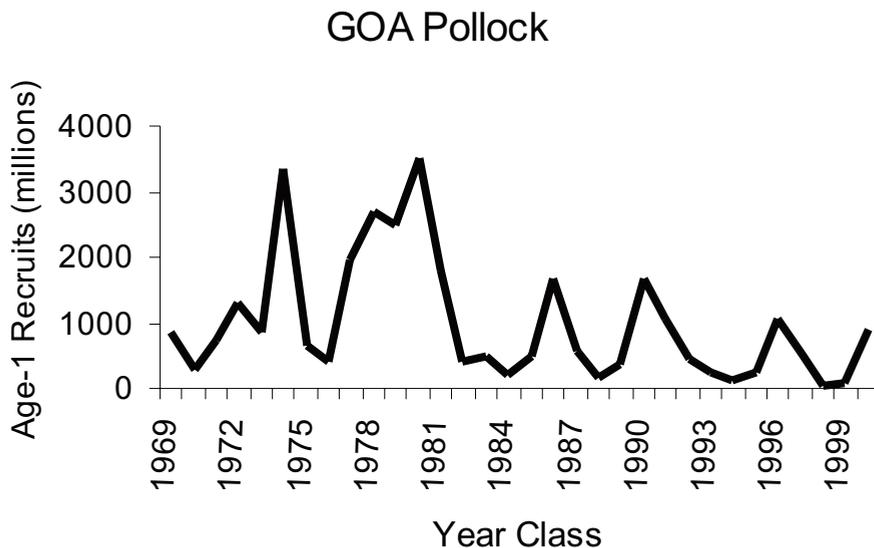


Figure 2. The time series of recruitment of walleye pollock *Theragra chalcogramma* in the western Gulf of Alaska.

trend on the log of observed recruits is -0.11 , $p < 0.001$). There is a significant auto correlation coefficient (AC) at a 1 year time lag ($r=0.48$), indicating that adjacent year classes tend to be either weak or strong, perhaps suggesting runs of strong or weak year classes or possibly some blending of age classes due to aging errors. ACs at other time lags were not significant.

A recruitment time series for walleye pollock was simulated with environment-scaled stochastic larval mortality, intra-cohort density-dependent late larval mortality, and predator-dependent juvenile mortality (Fig. 3). The modeled time series of recruitment is similar to the observed time series ($R^2=0.65$, $p < 0.001$). Comparisons of the simulated and observed means (1.01×10^9 ; 1.63×10^9), standard deviations (9.73×10^8 ; 1.38×10^9) and ranges (3.48×10^9 ; 5.28×10^9) were similar. The modeled series began in 1975 because data on predator abundance were not available for earlier years. In the model, a series of strong year classes in the late 1970s was described, as well as the 1984 and 1988 year classes. The model had several moderately strong year classes that did not appear in the observed data. The declining trend in recruitment noted above in the observed series was captured by the simulation (slope of time trend on the log of modeled recruits is -0.09 , $p < 0.001$).

The observed time series for Pacific hake recruitment extends back to 1960. There have been peaks in recruitment in the hake population occurring every 3-4 years (Fig. 4). There is no trend in the recruitment data (slope of time trend on the log of observed recruits is 0.01 , $p = 0.84$). The ACs for the hake time series were not significant, although at lags of 1 and 2 years they were negative, and at lags of 3 and 4 years they were positive.

For Pacific hake, the recruitment time series was simulated with environment-scaled stochastic larval mortality and inter-cohort density-dependent cannibalistic juvenile mortality resulting from the abundance of the three immediately preceding year classes. The modeled series describes

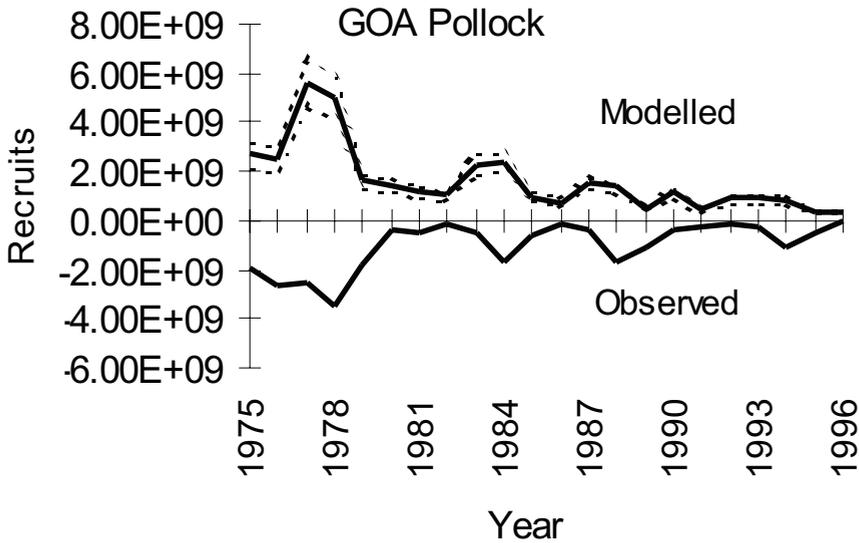


Figure 3. Comparison of observed recruitment (below axis line) to simulated recruitment of walleye pollock *Theragra chalcogramma*. The dotted lines represent ± 1 sd from 200 simulations.

the major peaks in recruitment of hake and the 3-4 year periodicity observed. Fournier analysis showed the major spectral peaks at 3.2 years for both the observed and simulated time series. The magnitude of actual recruitment varies quite a bit from the model results (Fig. 5). The simulated mean was higher than the observed (3.89×10^9 ; 1.81×10^9) as was the standard deviation (3.38×10^9 ; 2.55×10^9). These differences were due to the tendency of the model to identify consecutive strong year classes. There was no trend in recruitment in the model results (slope of time trend on the log of modeled recruits is 0.003, $p < 0.90$), similar to the lack of trend in the observed series.

Discussion

Our objective in working with these simple models was not to obtain “best fits”, but to capture patterns and trends in recruitment with simple models by describing recruitment as a noisy process that is constrained by boundary effects. Thus we described the high frequency recruitment processes in early life stochastically, although they are influenced by environmental proxies such as SST. Deterministic factors such as density-dependence and predation act to constrain recruitment in the models presented here.

Recruitment of walleye pollock in the Gulf of Alaska is dynamic and perhaps periodic, with an apparent downward trend in recruitment since the 1980s. The pollock model incorporated random larval mortality scaled by SST and wind speed, and was constrained by density dependence and predation pressure. The model simulated peaks in recruitment at the same frequency and usually the same years as the observed time series, and captured the long-term trend.

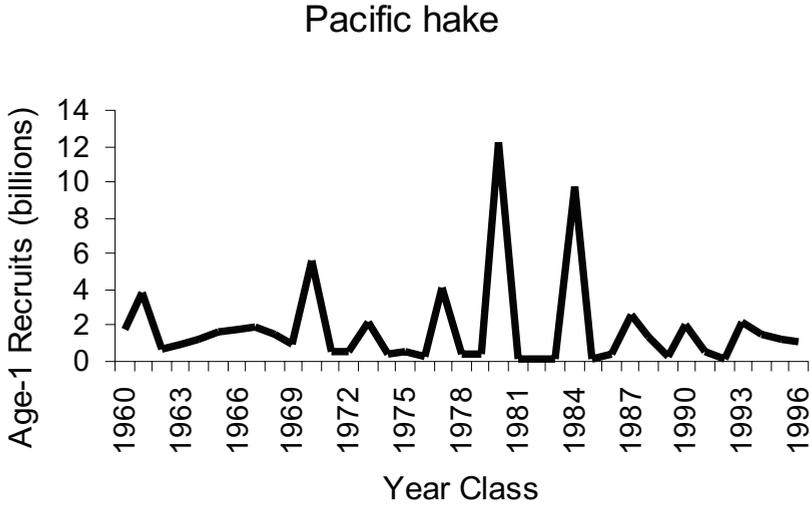


Figure 4. The time series of recruitment of Pacific hake *Merluccius productus* in the California Current region.

In the case of Pacific hake, recruitment is apparently dynamic and periodic, but without a trend. The model incorporated random larval mortality scaled by SST and constrained by cannibalism. Within the frame of the time series used in the analysis, the hake model simulated the 3-4 year periodicity of strong year classes in the population. Strong and weak year classes were accurately captured by the models, but the magnitude of strong year classes often differed from observed strengths. The model generally constructed coupled strong year classes, which did not occur in the observed time series, resulting in a poor statistical fit of modeled results to the observed time series. Although the hake model is promising, it would benefit from refinements, in particular the formulation of density dependence and cannibalism.

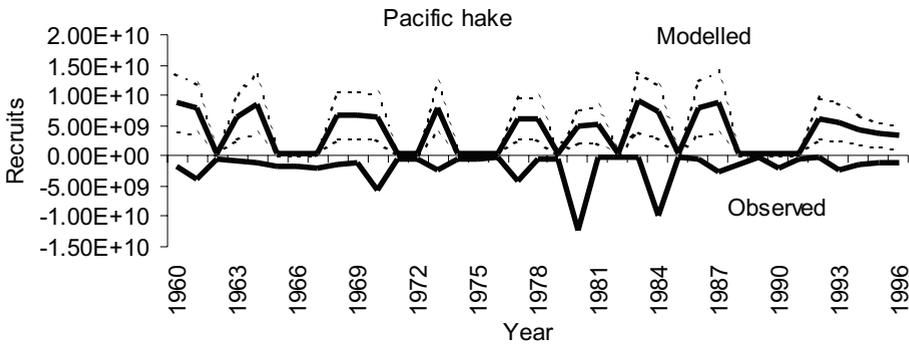


Figure 5. Comparison of observed recruitment (below axis line) to simulated recruitment of Pacific hake *Merluccius productus*. The dotted lines represent ± 1 sd from 200 simulations.

There are many sources of error and variability in the data utilized in the models, including measurement of environmental conditions, spawning biomass, egg production and predator biomass and consumption (Bailey 2000). There is probably a lot of error in the estimates of the observed time series of recruitment as well. Therefore, we stress that capturing relatively strong patterns and trends has been our main objective rather than obtaining an exact fit of simulated and observed time series; we believe that these simple models are of interest because they are successful at demonstrating the interplay of complexity and constraints underlying the patterns and trends in the recruitment time series for these species. Future work on these models might involve refinement of the biological processes represented in the models, better representation of the error structure in the models, and including the interaction of density-dependence and predation with environmental conditions.

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The role of ichthyoplankton surveys in recruitment research and management of South African anchovy and sardine

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Abstract

Anchovy *Engraulis encrasicolus* and sardine *Sardinops sagax* are ecologically and economically important components of the southern Benguela upwelling ecosystem situated off the west coast of South Africa. The importance of these species has resulted in substantial research effort directed towards understanding their population dynamics, with much of this effort going into ichthyoplankton surveys. This review describes the methodologies used and results obtained from ichthyoplankton surveys conducted off South Africa during the past 50 years. This research has allowed the development of a detailed knowledge of the life history cycles of small pelagic species in the southern Benguela, particularly that of anchovy. Spatially distinct spawning and nursery areas have been identified, highlighting the critical influence that transport of eggs and early larvae from the spawning to nursery grounds has on subsequent recruitment strength. Temporal changes in the location of spawning habitat of both anchovy and sardine have been tracked, and environmental parameters characterizing spawning habitat differentiated. Frequent monitoring of early life history stages in the transport region shows promise as a predictor of recruitment success for anchovy but not necessarily sardine, although this predictor is not yet formally incorporated into management procedures. Insights gained from ichthyoplankton surveys have been used to develop individual-based models (IBMs) coupled with a 3D hydrodynamic model of the region; these have allowed the testing of various hypotheses concerning transport success and various physical and biological parameters through experimental simulation. Ichthyoplankton survey data have been used for stock management by application of the DEPM to estimate anchovy spawner biomass from 1986-1993; these absolute estimates were then used to calibrate relative estimates of anchovy biomass made using hydroacoustic methods. These examples demonstrate that ichthyoplankton surveys of anchovy and sardine are widely used off South Africa and have made a substantial contribution toward identifying key mechanisms impacting on recruitment success and hence management of these small pelagic species.

Introduction

The oceanography off South Africa is dominated by two major current systems, the poleward flowing, warm Agulhas current off the east and south coasts, and the equatorward flowing, cool Benguela current off the west coast (Fig. 1). These current systems clearly influence the species diversity of the marine ecosystems in this region; the east and south coasts range from subtropical to warm temperate marine systems with a high diversity of fish species and a low productivity, whereas the west coast is characterized by a cool temperate upwelling system with relatively few fish species and a high productivity. This upwelling system is known as the southern Benguela, and is separated from the northern Benguela off Namibia by a perennial cell of intense upwelling off Lüderitz (26-27.5°S). The Benguela is one of the world's major eastern boundary upwelling

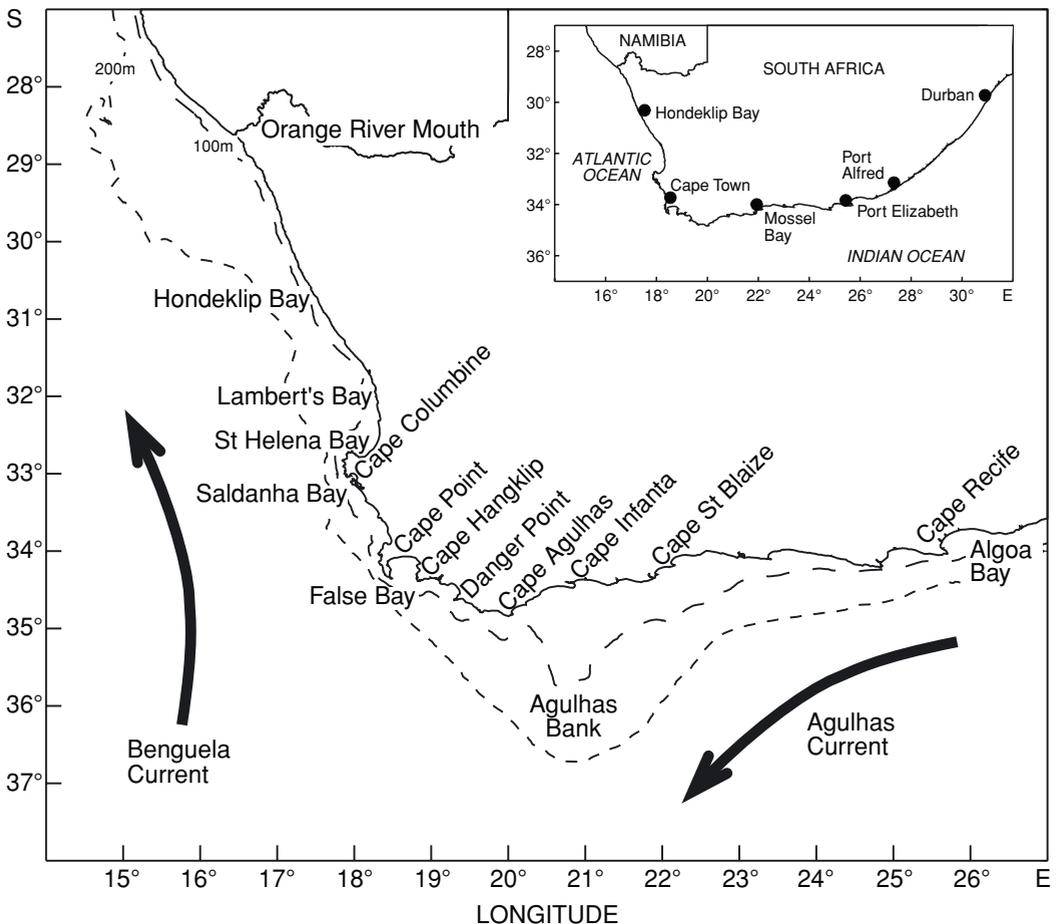


Figure 1: Map of southern Africa showing the continental shelf (the 100 m and 200 m isobaths are shown), the Agulhas and Benguela current systems, and places mentioned in the text.

systems; in the southern Benguela intense coastal upwelling occurs from late spring (September to November) to early autumn (March to May). Detailed reviews of various aspects of the Benguela upwelling system may be found in Shannon (1985), Chapman and Shannon (1985), Shannon and Pillar (1986), and Crawford et al. (1987).

Like other upwelling systems (Schwartzlose et al. 1999), the southern Benguela supports large populations of small pelagic fish, principally anchovy *Engraulis encrasicolus* (formerly known as *E. capensis*; Grant and Bowen 1998) and sardine *Sardinops sagax*. These fish are small schooling species that occur in the upper water layers and are found primarily over the continental shelf. Both species are important prey items for fish, birds and marine mammals (Cury et al. 2000).

These large populations of small pelagic fish support a substantial fishery, South Africa's largest in terms of annual landings and the second-most valuable. Purse-seine fishing for sardine off South Africa commenced in the 1930s (Beckley and van der Lingen 1999), but only became commercial in 1943 following the war-time demand for canned fish (Crawford 1981a). The fishing industry expanded rapidly over the next few years and was landing 50-100 000 tons of fish annually during the late 1940s. Over the past 50 years the pelagic fishery has made large catches averaging around 300 000t annually (Fig. 2a). Sardine dominated the catches initially, but overfishing of this population in the early 1960s led to a stock collapse and the fishery changed to smaller-meshed nets in 1964 to target the smaller anchovy. Anchovy have dominated catches since, but catches of sardine have increased steadily since 1990. The fishery is located off the west and southwest coasts, with anchovy caught inshore, principally in St Helena Bay, whereas sardine are caught farther offshore and over a greater geographical range than anchovy (Fig. 2b). Most of the anchovy caught are less than one year old, and are reduced to fish meal and oil in industrial scale factories. Adult sardine are canned or frozen for human consumption in addition to being packed for bait; juvenile sardine that shoal together with anchovy are taken as bycatch in the anchovy fishery.

Hydroacoustic surveys to estimate the population size of anchovy and sardine have been conducted off South Africa since 1984 (Hampton 1987, 1992; Barange et al. 1999). Anchovy recruitment has been moderately variable over most of the time series, with consequent inter-annual fluctuations in stock size, but has been exceptionally strong since 2000 (Fig. 3). Sardine recruitment has also been variable, but has shown an increasing trend that has resulted in a steady growth of the sardine stock. The commercial importance of anchovy and sardine off South Africa, and the difficulties in managing stocks prone to such interannual variability in population size, has led to substantial research effort being directed towards these species, including a variety of ichthyoplankton surveys.

This review describes the methodologies used and results obtained from ichthyoplankton surveys conducted off South Africa over the past 50 years (see Table 1). The consequent increased knowledge of life history strategies of pelagic fish off South Africa, how this has improved understanding of recruitment variability, and examples of how survey data have been incorporated into methodologies attempting to predict pelagic fish recruitment strength or used directly for management, are discussed. Finally, the incorporation of ichthyoplankton survey data into individual-based models (IBMs) that encapsulate the understanding of the physical and biological processes impacting on recruitment variability, is described.

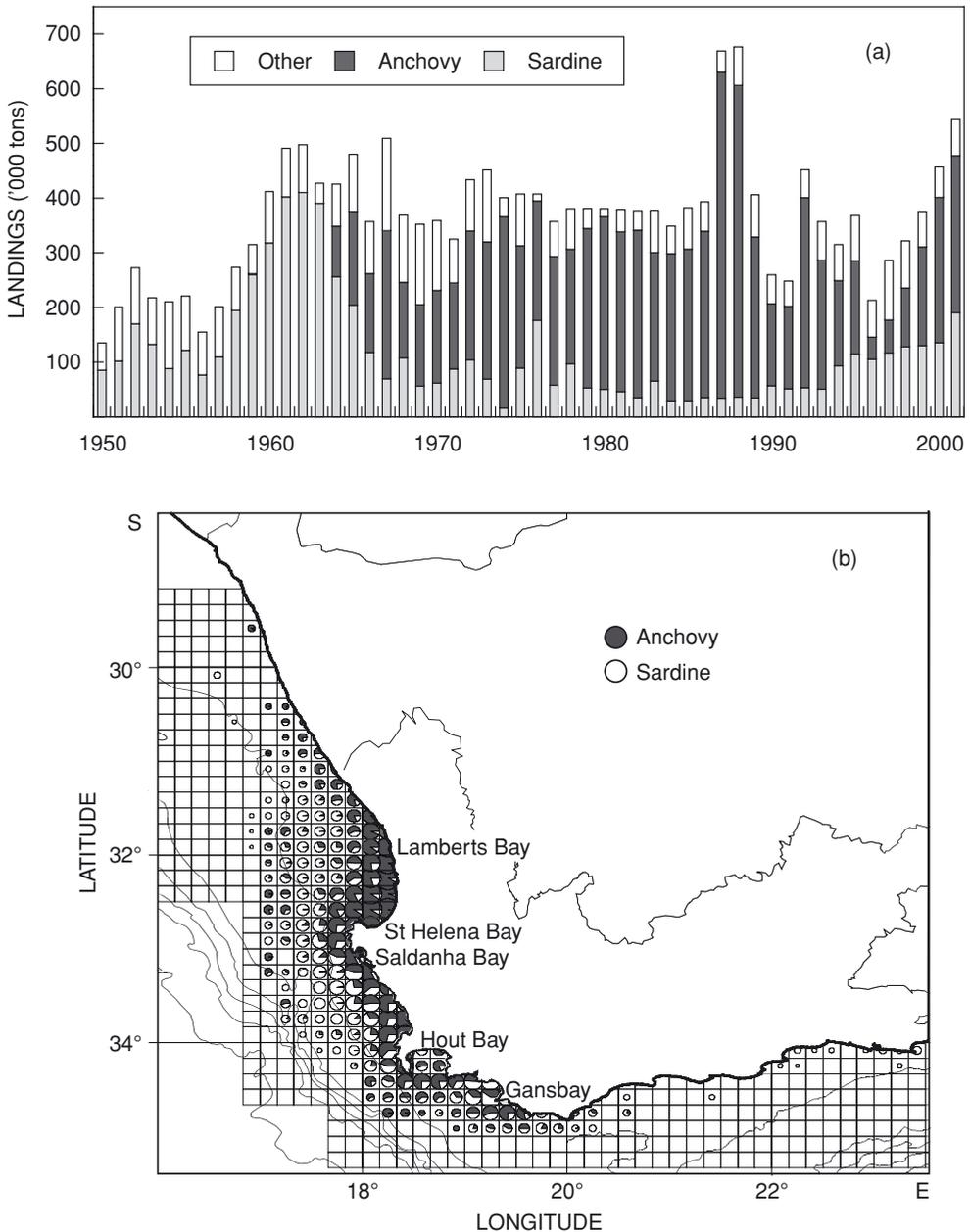


Figure 2: (a) Time-series of annual landings of anchovy (*Engraulis encrasicolus*), sardine (*Sardinops sagax*) and other species (primarily round herring *Etrumeus whiteheadi* and Cape horse mackerel *Trachurus trachurus capensis*) made by South Africa's pelagic fishery, 1950-2001 (from Marine and Coastal Management, unpublished data). (b) Output from ArcView GIS showing anchovy and sardine catches by area (10' by 10' block) over the period 1987-1998. Circle size is proportional to average catch size, and landing sites are indicated (courtesy of L. Drapeau, IDYLE, IRD, Cape Town).

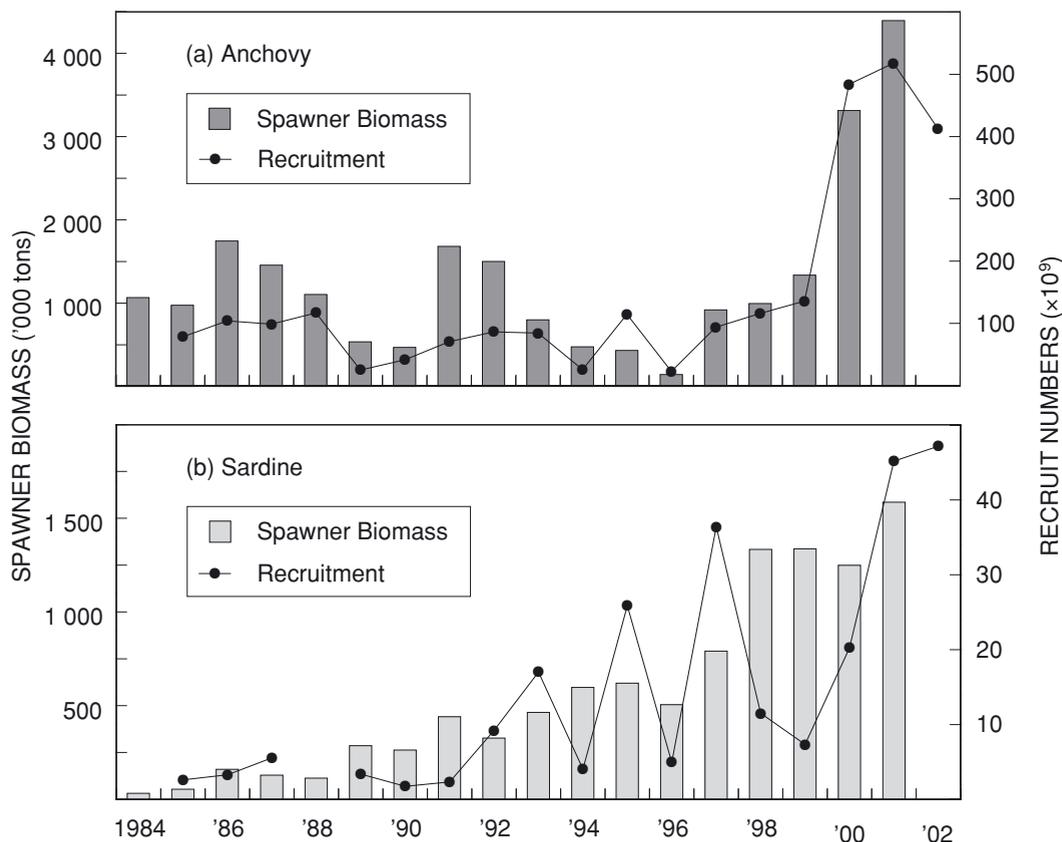


Figure 3: Time-series of spawner biomass and recruitment strength (numbers of 0+ fish) of anchovy and sardine off South Africa derived from hydroacoustic surveys, 1984-2002 (updated from Barange et al. 1999).

Early ichthyoplankton surveys. The rapid expansion of purse seine fishing for pelagic species during the 1940s prompted the introduction of a variety of research programmes, and in 1950 a sampling programme was initiated to locate the major spawning areas of sardine. A further aim was to determine both the extent of spawning and the survival of the larvae, with a view to forecasting future commercial catches (Davies 1954; de Jager 1954). From 1950-1953 the survey area comprised a small grid of stations off the west coast that were sampled monthly (Table 1). This sampling grid was gradually extended farther offshore and along the south coast (see Fig. 4). By 1960 a total of 80 stations over 16 lines between Lamberts Bay and St Sebastian Bay were being sampled quarterly, this sampling programme continuing until 1967. In 1964 sampling for the smaller, elliptical anchovy eggs was initiated, using a finer mesh net in conjunction with the coarser mesh used for sampling sardine eggs (Table 1).

The earliest surveys (1950-1953) showed dense concentrations of sardine eggs in the nearshore region (<20 miles) during early summer (Davies 1956). Extension of the survey grid between 1953

Table 1: Comparison of temporal and spatial coverage, sampling gear and tow profiles for ichthyoplankton surveys conducted off the coast of South Africa from 1950 to present.

| Survey Programme | Period and frequency | Spatial coverage | Gear used | Tow profile | Reference |
|--------------------------------------|---|---|--|--|--|
| Early surveys | March 1950 – April 1969 (monthly). East coast: several cruises during 1951-1969; Jan, Jul, Aug & Dec 1973. | West and South-west coasts. Initially Lamberts Bay to Saldanha Bay, extended to Quoin Point in 1959, and to Cape Infanta in 1961 (80 stations). East coast: Algoa Bay to Tugela. | N100 net (950µm mesh; 100cm diameter; 0.78m ² area). From 1964: N100/70 net (200µm mesh; 100cm diameter; 0.78m ² area). | Horizontal tow (N100H) at surface for 10 mins; vertical haul (N70V) from 100-0m; oblique haul (N100B) from 150-0m. | Davies (1954, 1956); Anders (1965, 1975); King and Robertson (1973). |
| Drogue survey | October 1976 (68 hours). | 10 stations off Cape Peninsula. | Array of 8 Miller nets (300µm mesh; 14.5cm diameter; 0.017m ² area). | Horizontal tows (60 minutes at 3 knots); vertically stratified sampling from 85-0m. | Shelton and Hutchings (1981, 1982). |
| Cape Egg and Larval Programme (CELP) | August 1977 – August 1978 (monthly). | 120 stations on West and South-west coasts extending 50 nm offshore. | Bongo (300µm mesh; 57cm diameter; 0.5m ² area). | Double-oblique tows from 0-100m. | King and Robertson (1973); Shelton (1986); Shelton and Hutchings (1990). |
| Spawner Biomass surveys | November/December 1984 onwards (annually). | ~300 stations on West and South coasts, from Hondeklip Bay to Port Alfred. | CalVET net (300µm mesh; 25cm diameter; 0.05m ² mouth area). | Vertical haul from 200-0m (earlier surveys) or 100-0m (recent surveys). | Armstrong et al. (1988); van der Lingen et al. (2001). |
| East coast larval surveys | May/June 1990, October 1990, February 1991. | 36 stations from Algoa Bay to Tugela. | Bongo (500µm mesh; 57cm diameter; 0.5m ² area). | Stepped oblique tows from 80-0m; 10 min tows; 5m spans. | Beckley and Hewitson (1994). |
| South African SARP | August 1993 – March 1994; September 1994 – March 1995 (monthly). | 17-189 stations on West and Southwest coasts (Olifants River to Cape Agulhas). | CalVET net (as above) for eggs; Paired-bongo net (300µm mesh; 57cm diameter; 0.5m ² area) for larvae. | CalVET: vertical haul from 70-0m. Bongo: oblique haul from 70-0m. | Fowler and Boyd (1998); Painting et al. (1998). |
| SARP Monitoring Line | August 1995 onwards (~ bimonthly). | 12-20 stations, 3 n miles apart, off Cape Peninsula. | Paired mini-bongo net (200 and 300µm; 18cm diameter; 0.025m ² area). | Oblique haul from 70-0m. | Huggett et al. (1998); Huggett (2002). |
| Pre-recruit surveys | March 1991 onwards (annually). | West coast (usually Orange River to Cape Point). | Method frame trawl (5mm mesh; 0.9mm codend; 5m ² area). | Stepped, oblique hauls from 35-0m. | Methot (1986); O'Toole and Crous (1989). |

and 1961 revealed the occurrence of eggs up to 50 n.miles offshore of Cape Columbine, as well as high concentrations off Saldanha Bay and on the south coast.

Between 1961 and 1963 two main foci of sardine spawning were identified, one near Cape Columbine, and one between Cape Point and Danger Point (Crawford 1981a). From 1963 to 1967, sardine spawning activity declined off the west coast, coincident with the disappearance of adults in this region, but spawning was still evident on the south coast as far east as Cape Agulhas (Fig. 4a,b). This shift in spawning was thought to have resulted either from the disappearance of the west coast spawning population, had two separate populations previously existed, or else, a southward contraction of the range of adult sardine. Between 1951 and 1967 most sardine spawning took place between September and February (Crawford 1981a), with greatest densities of sardine eggs encountered during January and February, declining thereafter until June or August when egg production began to rise again (Fig. 4e). Most sardine eggs were collected at temperatures between 15 and 18 °C, with few eggs encountered at temperatures colder than 12 °C or warmer than 23 °C (Fowler et al. 1996).

Following the switch to smaller meshed sampling nets, anchovy eggs were found to be concentrated on the south coast between Cape Point and Cape Infanta, with few eggs collected on the west coast. Greatest concentrations of eggs occurred between Cape Point and Cape Agulhas (Fig. 4c,d) and were associated with relatively warm waters (Crawford 1981b). The absence of anchovy eggs off the west coast was considered to be a result of their low tolerance to cold water, anchovy eggs having a lower lethal limit of 14°C as opposed to 13°C for sardine (King et al. 1978). Anchovy spawning was most intense during early summer (November/December; Fig. 4f), declining rapidly with the advent of autumn (Anders 1965; Crawford 1981b).

Cape Point drogue study. Oceanographic studies conducted during the late 1960s and early 1970s indicated the presence of a strong northward-flowing jet current running from Cape Point along the shelf-edge, associated with a strong thermal front between cold upwelled water and warm oceanic water (Bang and Andrews 1974). The north-westward drift of anchovy eggs and early larvae toward the west coast was thought to be linked to this frontal jet current, and the effect of this water movement on the transport of anchovy eggs and larvae was investigated through the tracking of a free-drifting parachute drogue placed within a dense patch of anchovy eggs south of Cape Hangklip (see Fig. 1) in October 1976 (Shelton and Hutchings 1981, 1982).

The drogue drifted at a mean speed of 0.55 m.s⁻¹, and followed a northwesterly trajectory just offshore of the front (Shelton and Hutchings 1982). Whereas the wind speed decreased towards the end of the drogue track, the speed of the drogue increased, indicating that it was entrained by the jet current. The consistency of physical properties and zooplankton species composition along the drogue path supported the likelihood that the drogue closely approximated the drift of water in which high densities of anchovy eggs were initially located. Anchovy eggs and larvae (mostly <5 mm notochord length) were present throughout the drogue path, although they decreased in abundance with time, which was attributed to an expected high mortality rate. A nocturnal spawning rhythm was suggested by the incidence of newly-spawned eggs on two of the three nights, although early-stage eggs were abundant before sunset on the first night (Shelton and Hutchings 1981, 1982). Most anchovy eggs and larvae occurred above the thermocline, with larvae

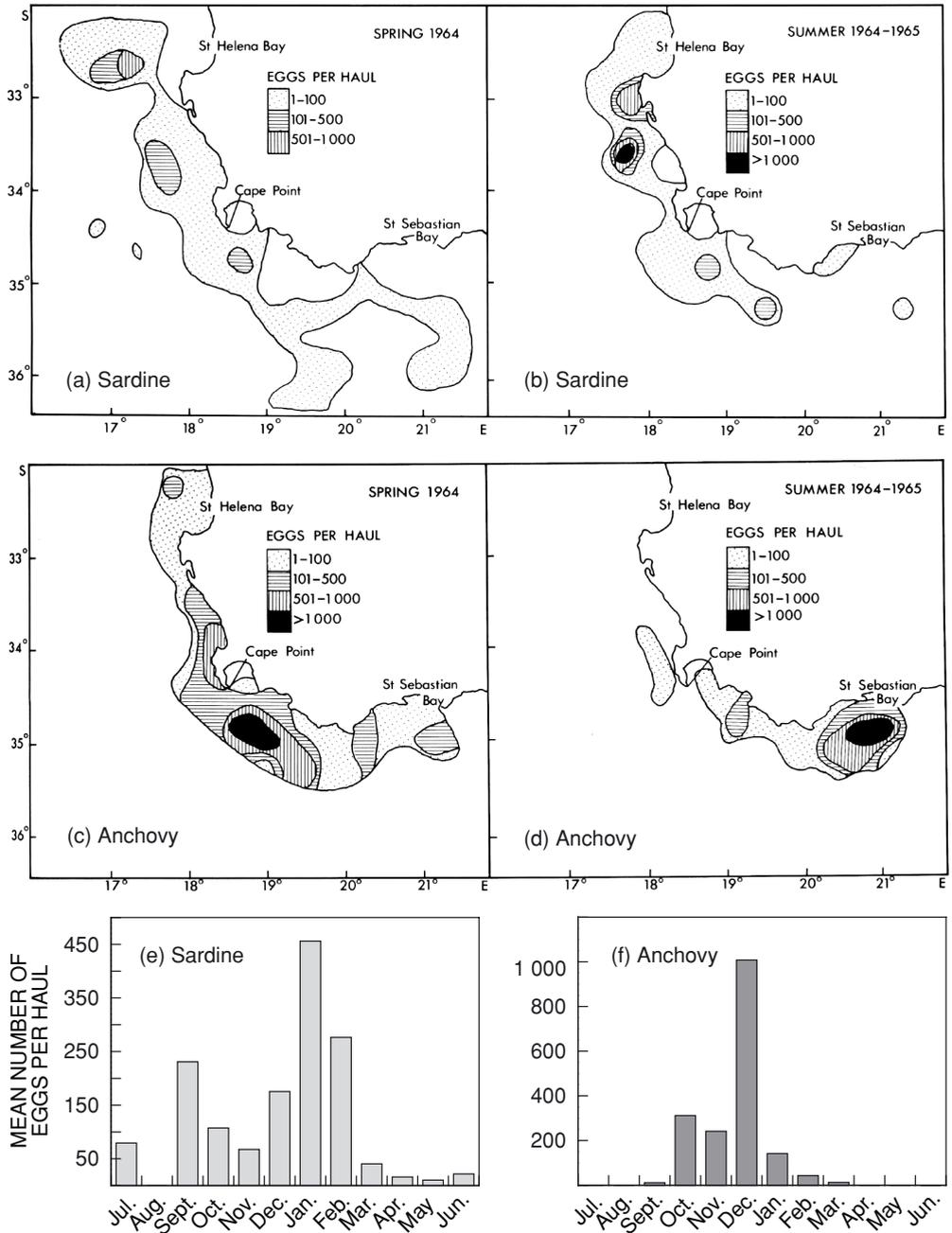


Figure 4: Distribution of sardine eggs during (a) spring 1964 and (b) summer 1964/65, anchovy eggs during (c) spring 1964 and (d) summer 1964/65 (from Crawford 1981a, b), and monthly mean egg abundance for (e) sardine over the period 1955-1967 (redrawn from Crawford 1981a) and (f) anchovy over the period 1965-1967 (redrawn from Crawford 1981b).

more dispersed than eggs in the water column and more abundant near the thermocline. Larvae displayed vertical migration, being closer to the surface at night. This study confirmed the ability of the jet current to entrain and transport eggs and larvae from the Agulhas Bank spawning ground towards the west coast recruitment ground, particularly during periods of southeasterly winds, the dominant wind direction during the anchovy spawning season. Although sustained northwesterly winds could reverse the direction of near-surface currents and hence transport eggs and larvae eastwards, the persistence of subsurface frontal features made it likely that the system could rapidly shift back to the more normal summer pattern of northerly transport (Shelton and Hutchings 1982).

The Cape Egg and Larval Programme. In 1977 the Cape Egg and Larval Programme (CELP) was initiated in order to investigate the composition, distribution and temporal variability of ichthyoplankton in the southern Benguela, thereby increasing understanding of the recruitment mechanisms of important fish species. Environmental and plankton sampling was conducted at monthly intervals from August 1977 to August 1978 over an extensive survey grid of 120 stations (Fig. 5a; Shelton 1986; Shelton and Hutchings 1990; also see Table 1).

Anchovy eggs were absent or rare in autumn and winter (June-August), but both egg and larval abundance increased rapidly with the onset of spring and peaked in late spring and early summer (Fig. 5b). Anchovy spawning was most intense on the Agulhas Bank, with eggs restricted almost entirely to the upper 50m above the thermocline. Anchovy eggs extended up the south-west coast whereas the larvae were dispersed more widely over the Agulhas Bank and extended further up the west coast than the eggs (Fig. 6a, b). Transects across the jet current showed that both anchovy eggs (Fig. 6c, d) and larvae were concentrated within the frontal zone. Sardine eggs and larvae were present during each month of the survey, but were more abundant from mid-winter to early summer (Fig. 5c), and both eggs and larvae were generally widespread over the survey grid (Shelton 1986).

The findings of the CELP surveys and the Cape Point drogue study, combined with commercial catch patterns of recruits on the west coast, provided new and significant insight into the life cycle of the Cape anchovy, and suggested the existence of separate spawning, transport and recruitment areas for this species. Current knowledge indicated spawning on the Agulhas Bank between October and January and transport of eggs and larvae to west coast nursery grounds via the jet current. Juvenile anchovy of approximately 4 months old appeared in commercial catches made off the west coast from March onwards, up to 250 km north of the spawning grounds. Juveniles then began a southerly return movement, against a food gradient, to the Agulhas Bank spawning grounds in spring (Fig. 7). At this time knowledge of the sardine life-cycle was less well understood, but indicated maximum spawning in warm water during spring and summer, subsequent recruitment to the west coast, and a return of mature fish to the Agulhas Bank in spring and summer, with an eastward migration beyond Cape Point in autumn (Crawford 1981a).

The implications of this knowledge of the anchovy life history in terms of understanding recruitment variability were substantial, since it highlighted the critical role of the transport process in determining recruitment success. Advective loss of anchovy eggs and early larvae through offshore divergence of the jet current was postulated as having a substantial, negative impact on re-

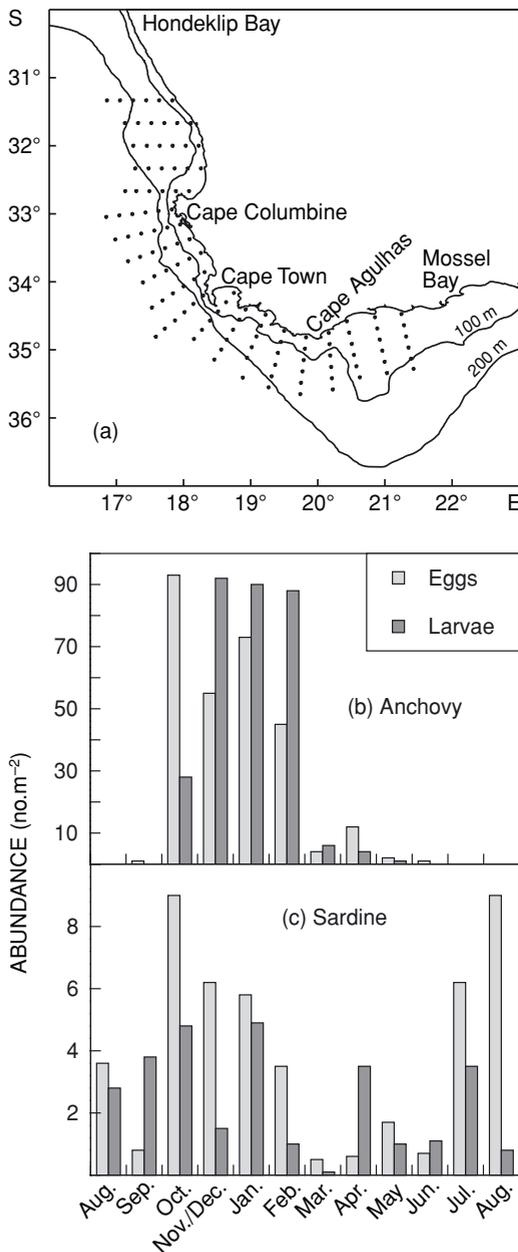


Figure 5: Schematic of Cape Egg and Larval Programme (CELP) surveys showing (a) the location of stations sampled each month using an oblique Bongo net, and monthly mean egg and larval abundance for (b) anchovy and (c) sardine over the period August 1977 to August 1978 (redrawn from Shelton 1986).

recruitment success (Shelton and Hutchings 1982, 1990). With respect to management, understanding of the anchovy life history strategy, derived in part from ichthyoplankton surveys, was later used to set the timing of the pelagic spawner biomass surveys, which at that time (1984) were directed primarily at anchovy since sardine biomass was very low

Spawner biomass surveys. Pelagic spawner biomass surveys conducted annually since 1984 off South Africa's west and south coasts have provided a substantial amount of information on the distribution and abundance of anchovy and sardine eggs. Timed to coincide with peak anchovy spawning (November/December), as determined during the CELP survey, the spawner biomass surveys use hydro-acoustics to estimate the adult biomass of anchovy and sardine required for the setting of Total Allowable Catch (TAC) limits (Barange et al. 1999). The surveys cover an area of 100-150 000 km^2 extending from Hondeklip Bay on the west coast (31°S) to Port Alfred on the southeast coast (27°E), and sample a grid consisting of transects positioned perpendicular to the coastline and extending over the continental shelf. Sampling for biological and physical parameters is conducted at stations positioned 10 n.miles apart along transects, where approximately 300 ichthyoplankton samples are collected with a CalVET (California Vertical Egg Tow) net (Smith et al. 1985; see Table 1). Substantially more anchovy than sardine eggs are collected by the CalVET net during spawner biomass surveys, and anchovy eggs occur in a markedly higher percentage of CalVET samples than do sardine eggs.

The distribution of anchovy eggs in early surveys corroborated acoustically-derived spawner distribution maps, with high egg

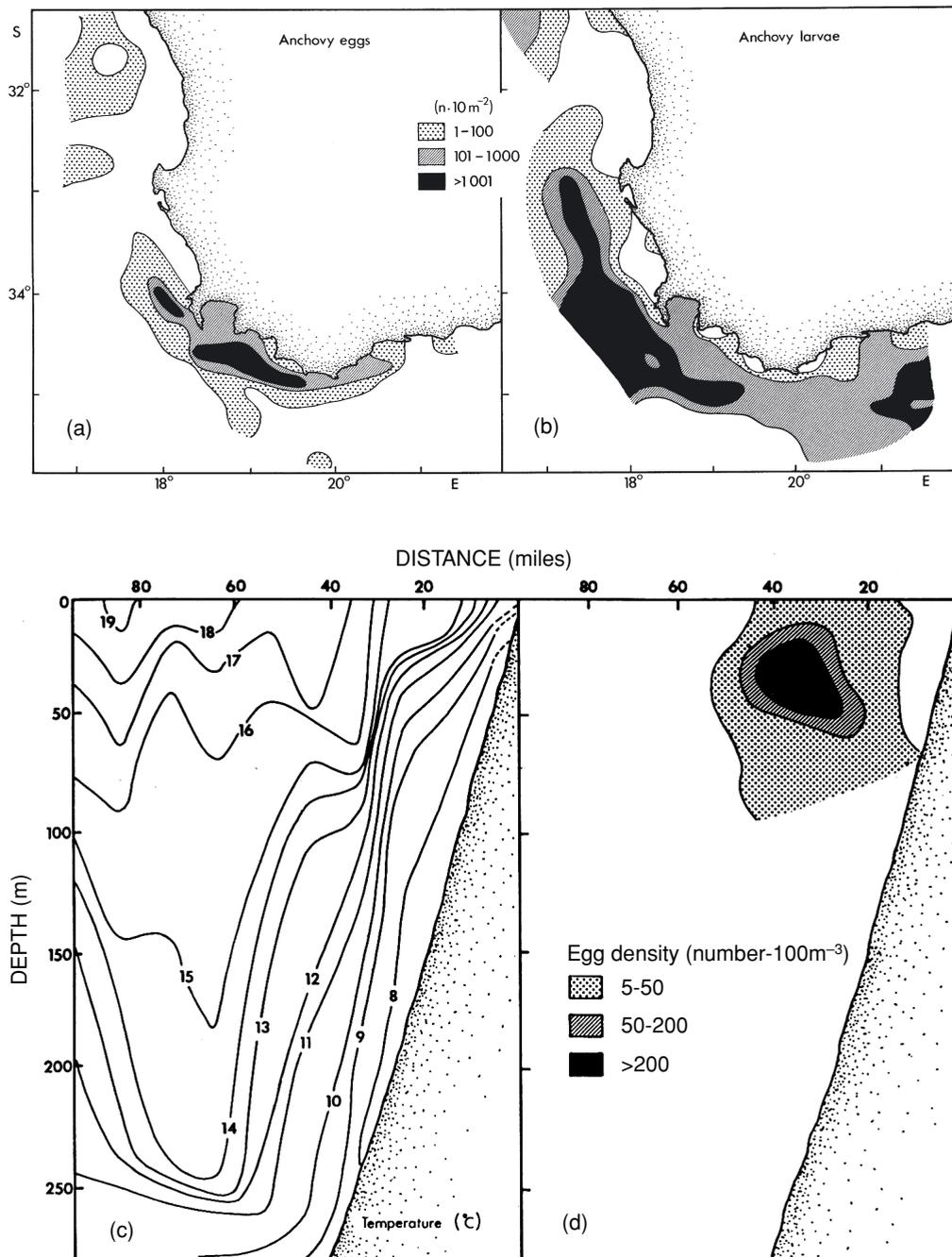


Figure 6: Distribution of anchovy eggs (a) and larvae (b) in January 1978 (from Shelton and Hutchings 1990), and steeply shelving isotherms of the frontal region (c) off the southwest coast with which anchovy eggs (d) are closely associated (from Hutchings 1992; reproduced with permission).

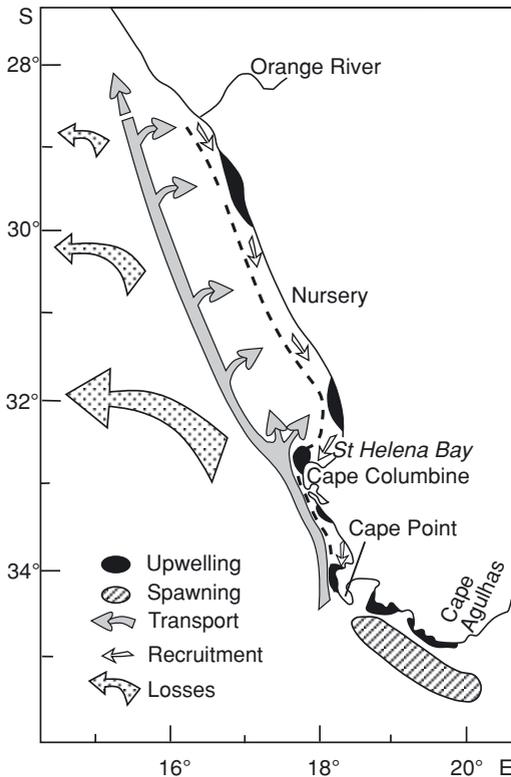


Figure 7: Schematic of the present hypothesis on anchovy life history (from Hutchings et al. 1992; reproduced with permission).

estimates during a period when there was no reliable information on the accuracy or applicability of the target-strength expression used (Hampton 1996). Because of its labour-intensive nature and the fact that the acoustic method is now considered to provide sufficiently robust estimates of anchovy biomass however, the DEPM is no longer used directly in management of the anchovy resource, and has yet to be used to estimate sardine spawner biomass off South Africa. Given that aspects of the behaviour of this species such as a patchy distribution, the formation of dense schools and near-surface schooling, all impact adversely on acoustically-derived biomass estimates, application of the DEPM for providing absolute estimates of the South African sardine population is imperative.

Daily egg production by anchovy measured during spawner biomass surveys was one of a suite of biological and environmental indicators investigated for use in forecasting subsequent anchovy recruitment (Cochrane and Hutchings 1995). Those authors reported a weakly-correlated, dome-shaped relationship between daily egg production and begin-year anchovy recruitment strength back-calculated from recruit survey data, but the relationship was insufficiently strong for daily egg production to be further considered for forecasting recruitment strength. Instead, parameters

densities coinciding with high fish densities (Hampton 1987). Egg data therefore validated newly-developed acoustic methodology. Additionally, anchovy egg data collected during the surveys between 1984 and 1993 were used in the estimation of spawner stock size through the daily egg production method (DEPM; Armstrong et al. 1988; Shelton et al. 1993). Direct estimates of anchovy spawner biomass obtained using the DEPM compared well with indirect estimates derived from acoustic methods for most of the time series (Fig. 8). A combination of estimates from both methods produced a more accurate and reliable estimate than either method alone, with the two estimates being used together with catch data to set TAC levels (Shelton et al. 1993). The closer correspondence between the mean of the egg production estimates and the mean of acoustic estimates derived using a locally-developed anchovy target strength expression (Barange et al. 1996) compared to that derived using a target strength expression for Icelandic herring (Reynisson 1983; see Fig. 8) provided support for the former expression. Hence egg production estimates of anchovy biomass proved useful in scaling acoustic esti-

or variables that could influence anchovy egg production (copepod biomass and production on the spawning grounds) or its cessation (incidence of gonad atresia), and the transport of eggs and larvae to the nursery grounds (the incidence of southerly winds at Cape Point and the distance of the 16°C isotherm from the coast at Cape Columbine during early summer, both of which are used as proxies for transport success) were considered more likely to be useful in predicting anchovy recruitment strength.

Despite Cochrane and Hutchings' (1995) reservations regarding the utility of anchovy daily egg production as a predictor of subsequent recruitment strength, it was incorporated into an expert system designed to provide a qualitative forecast of anchovy recruitment (Korrübel et al. 1998). The expert system linked recruitment to environmental and biological parameters through a set of rules using predictor variables assumed to influence recruitment via the processes shown in Figure 9a. Four differentially-weighted indicator variables, including daily egg production by anchovy estimated during pelagic spawner biomass surveys, were used to provide forecasts of either average/above-average or three categories of below-average (possible, likely, or very likely) anchovy recruitment. Previous simulation studies (Cochrane and Starfield 1992) had indicated that management actions based on correct forecasts of either "below average" or "average or above" recruitment made at the start of the fishing season would result in a 16% increase in mean annual anchovy catches. Data collected from 1985 to 1993 were used to construct the expert system and a prediction of recruitment success was made for 1994. Hindcast and observed anchovy recruitment were closely matched (Fig. 9b), and the prediction of very likely below-

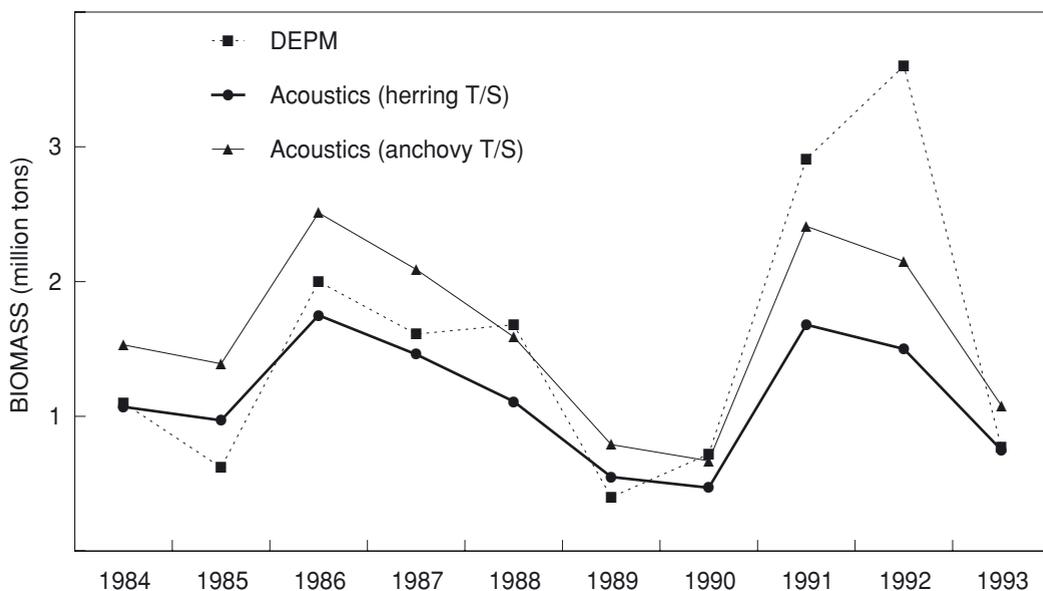


Figure 8: Time-series of estimates of anchovy spawner biomass obtained using the daily egg production method (squares), and the hydro-acoustic method using the Icelandic herring target strength expression (circles) and the anchovy target strength expression (triangles; redrawn from Hampton 1996).

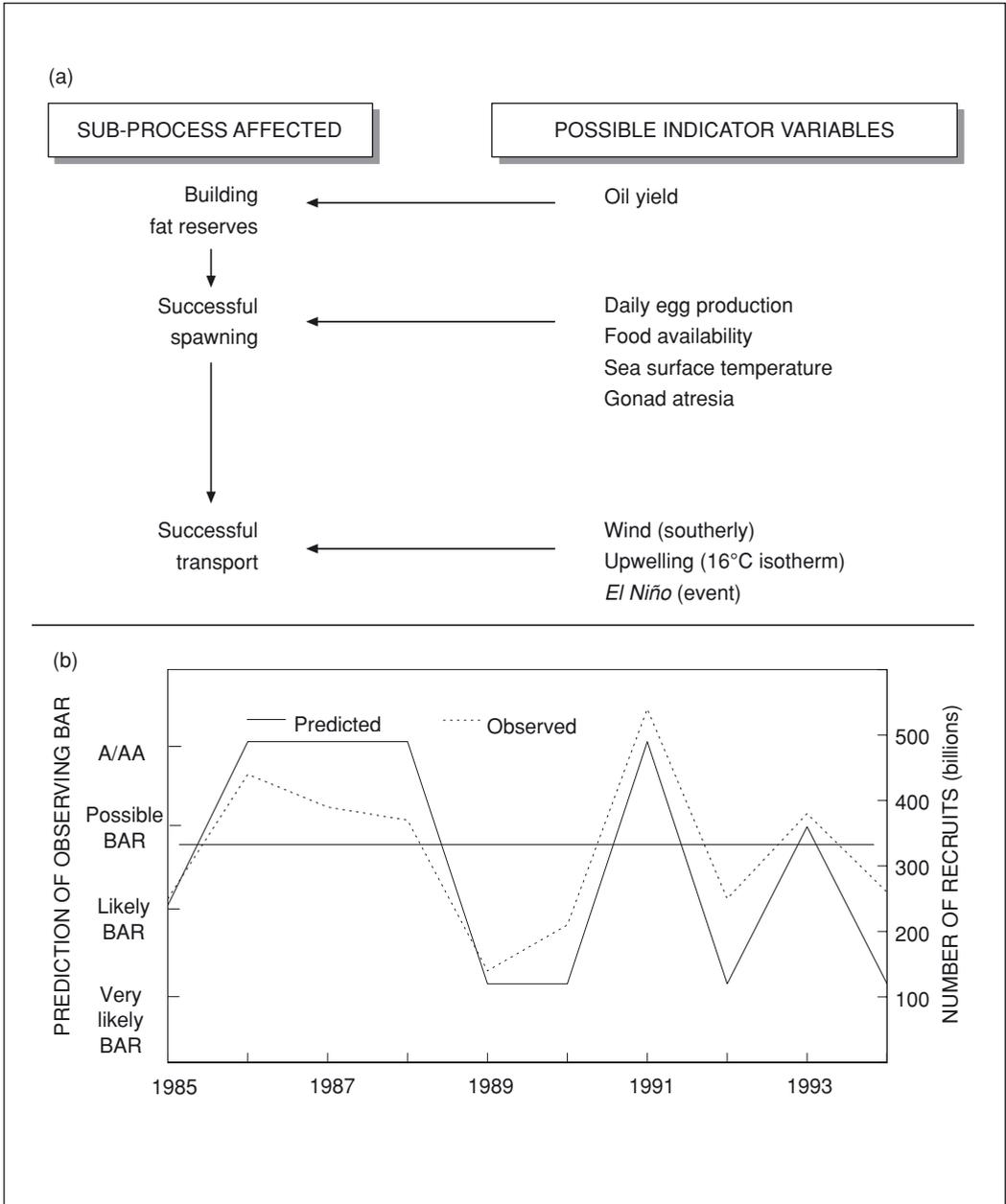


Figure 9: (a) Conceptual model of the major sub-processes and possible indicator variables thought to influence anchovy recruitment used in an expert system; and (b) observed (in begin-year numbers derived from an anchovy population assessment model) and predicted (qualitative predictions of average/above-average [A/AA] and three categories of below-average recruitment [BAR]) anchovy recruitment from the expert system that used daily egg production and three other indicator variables measured during the anchovy spawning season to hindcast recruitment strength (redrawn from Korrúbel et al. 1998).

average recruitment for 1994 was validated by the observation of recruitment that was slightly below long-term average recruitment. Unfortunately, validation of the model was only conducted for one year, owing to the loss of experienced personnel, a situation that has yet to be redressed. Nonetheless, this expert system is considered to have great potential for supporting future management of the anchovy fishery in the dynamic environment of the Benguela upwelling ecosystem (Korrübel et al. 1998).

The relatively long time series of abundance and distribution data on anchovy and sardine eggs has facilitated a spatial comparison of the spawning habitats of these two species. Composite egg distribution maps derived from CalVET net samples collected during spawner biomass surveys over the period 1984–2000 have shown that anchovy eggs are typically found in a continuous band between Cape Point and Algoa Bay, with high concentrations occurring over most of the western Agulhas Bank, the offshore portion of the central Agulhas Bank and over much of the eastern Agulhas Bank (Fig. 10a). Sardine eggs are found from Hondeklip Bay to Algoa Bay, and probably extend farther east (Fig. 10b), with dense concentrations being found offshore of the west coast and over the shelf-edge region of the central Agulhas Bank.

Anchovy egg distributions have been relatively stable through time, showing consistent spawning east of Cape Point with occasional extensions up the west coast in years when Agulhas Bank water intruded north of Cape Point (van der Lingen et al. 2001). However, recent data have indicated an eastward shift in anchovy spawning habitat over the Agulhas Bank, with the offshore regions of the central and eastern Banks appearing to have become the primary anchovy spawning grounds in contrast to the previously dominant western Agulhas Bank (van der Lingen et al. 2002). The fate of eggs spawned in this region is uncertain, since they could either be (1) transported shorewards onto the western Agulhas Bank and so into the jet current to the west coast, (2) retained on the central and eastern Agulhas Banks and recruit on the south coast, or (3) entrained by the Agulhas Current and advected into the southern Indian or Atlantic Oceans where they would be unlikely to contribute to recruitment (Hutchings et al. 2002). In contrast to anchovy, sardine eggs have been more patchily distributed through time and have shown large-scale shifts in their distribution, with both the south and the west coasts comprising the major spawning grounds during different periods. Anchovy and sardine showed a broad overlap in spawning habitat during the 1980s and early 1990s, but their spawning habitats have diverged since 1994, with sardine spawning mainly off the west coast and anchovy still spawning off the south and east coasts (van der Lingen et al. 2001). This change in spawning habitat has been accompanied by a steady increase in sardine population size (from 34 000 tons in 1984 to 1.6 million tons in 2001; see Fig. 3), and the spawning by sardine off the west coast is reminiscent of the high egg concentrations observed there during the 1950s and early 1960s, a time of high sardine biomass and an expanded age composition (Beckley and van der Lingen 1999). Whilst the impact on recruitment of this shift in spawning habitat success is unclear, sardine eggs spawned off the west coast in summer will be subject to offshore Ekman drift during periods of strong equatorward winds and could be displaced far offshore. Hence the west coast may constitute a sub-optimal spawning habitat and the persistence of spawning there by older fish is considered unusual (Hutchings et al. 2002). Nonetheless, sardine recruitment has been higher since 1994 than before (see Fig. 3), although this is most likely a result of increased population size.

Egg data from spawner biomass surveys has also permitted a characterization of anchovy and sardine spawning habitats in terms of physical and biological parameters, such as sea surface temperature (SST). The preferred spawning range of anchovy ranges from 17.4 to 21.1°C, is unimodal, and peaks between 19.5 and 20.5°C (van der Lingen et al. 2001; Fig. 11). The preferred spawning range of sardine is greater than that of anchovy, ranging from 15.2 to 20.5°C, and is essentially bimodal with a major peak from 15.5 to 17.5°C and a secondary peak between 18.7 and 20.5°C. Anchovy therefore spawn preferentially in warmer waters than do sardine, and the broader SST range for sardine reflects spawning by that species on both the west and south coasts. Such characterization of spawning habitat will permit monitoring of the location and magnitude of suitable spawning habitat in the absence of egg data, through, for example, remote sensing of SST via satellite.

A recent addition to ichthyoplankton sampling during pelagic spawner biomass surveys has been the introduction of a continuous underway fish egg sampler (CUFES), first developed to sample eggs of Atlantic menhaden *Brevoortia tyrannus* (Checkley et al. 1997). The CUFES consists

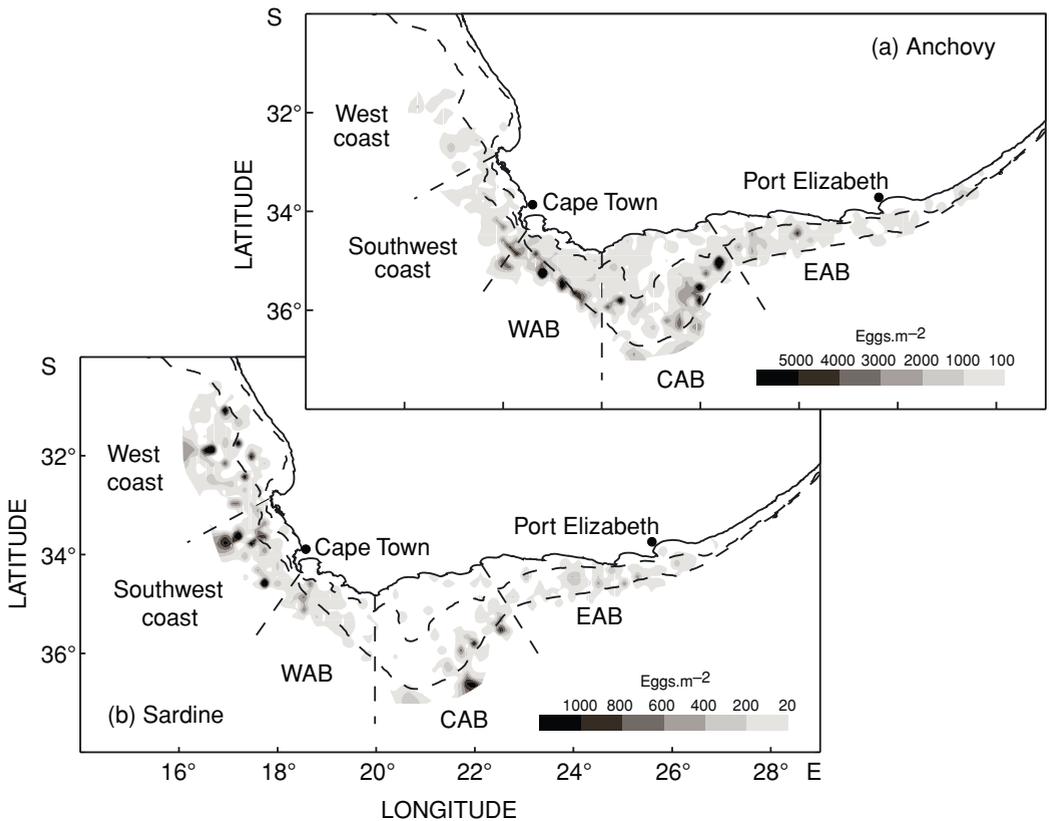


Figure 10: Composite distribution maps for eggs of (a) anchovy and (b) sardine derived from CalVET net samples collected during spawner biomass surveys over the period 1984–2000 (CDvdL, unpublished data). The five survey strata are shown.

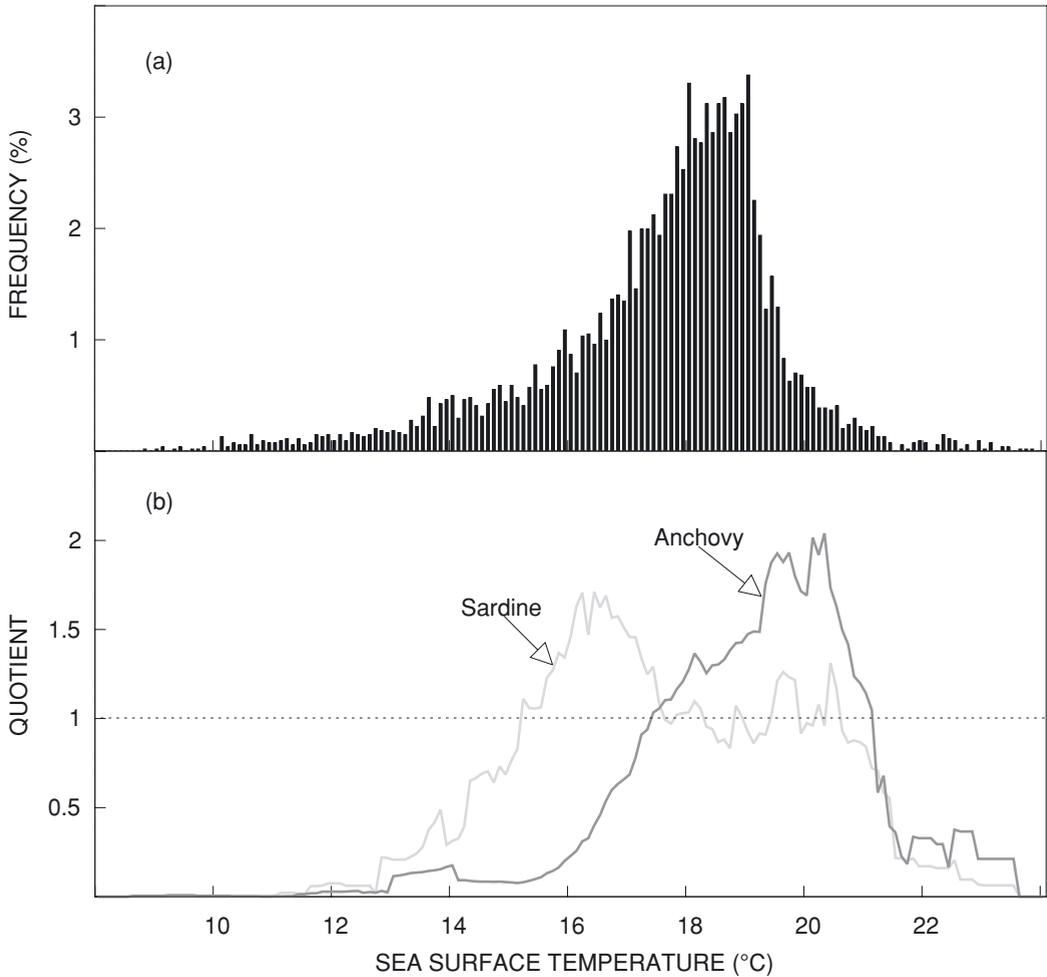


Figure 11: (a) Frequency distribution of sea surface temperature at all CalVET stations sampled during pelagic spawner biomass surveys conducted over the period 1984-1998 and (b) egg abundance/sea surface temperature quotients (the 11-point running means are shown) for anchovy and sardine egg samples collected during those surveys (redrawn from van der Lingen et al. 2001).

of a submersible, high volume pump that can be used whilst the vessel is both on-station and underway; hence the CUFES collects many more samples and provides much higher spatial resolution than is possible using a CalVET net. In contrast to the CalVET net which collects a vertically-integrated sample at a single location however, the CUFES only samples at a fixed depth (usually 3m) and thus collects horizontally-integrated samples at that depth when used whilst underway. The CUFES is now used in many of the world's oceans, particularly for small pelagic species such as anchovy and sardine (see Checkley et al. 2000), and was first used off South Africa in 1996 (van der Lingen et al. 1998). The highly significant correlations observed between CUFES- and CalVET

Table 2: Total numbers of anchovy and sardine eggs collected and numbers of samples taken by the CalVET net, on-station (O-SCUFES) and underway (U-W_{CUFES}) CUFES during pelagic spawner biomass surveys, 1998-2000.

| Year | No. of CalVET net and O-SCUFES samples | No. of U-W _{CUFES} samples | No. of anchovy eggs | | | No. of sardine eggs | | |
|-------|--|-------------------------------------|---------------------|----------|----------------------|---------------------|----------|----------------------|
| | | | CalVET | O-SCUFES | U-W _{CUFES} | CalVET | O-SCUFES | U-W _{CUFES} |
| 1998 | 373 | 1 118 | 3 595 | 427 | 1 147 | 856 | 4 273 | 29 148 |
| 1999 | 314 | 716 | 6 025 | 1 804 | 4 752 | 577 | 2 488 | 22 321 |
| 2000 | 300 | 1 197 | 3 782 | 1 718 | 11 574 | 1 492 | 6 673 | 43 802 |
| Total | 987 | 3 031 | 13 402 | 3 949 | 17 473 | 2 925 | 13 434 | 95 271 |

net-derived density estimates for eggs of sardine and round herring proved the accuracy and demonstrated the suitability of the CUFES as a sampler of fish eggs under local conditions. Additionally, collecting CUFES samples while underway increased the precision of the estimate of mean egg density for sardine eggs, which was attributed to increased sampling effort and the consequent improved coverage of the highly patchy distribution of sardine eggs (van der Lingen et al. 1998). No increase in precision was observed for round herring eggs, most likely due to their less-patchy distribution.

The CUFES has been used on spawner biomass surveys since 1998 and collects substantially more sardine eggs than does the CalVET net during these surveys (van der Lingen and van der Westhuizen 2000; Table 2), indicating that it is particularly well-suited to sampling eggs of this species. This is in contrast to anchovy eggs which are under sampled by the CUFES, most likely due to the 500µm mesh of the concentrator which, because of their elliptical shape and small size, does not retain anchovy eggs with 100% efficiency. For both anchovy and sardine eggs however, the increased small-scale resolution provided by the CUFES will improve our understanding of fish spawning processes, and of the dispersion of eggs following spawning. The high spatial resolution afforded by the CUFES resolves the problem of a highly patchy egg distribution characteristic of sardine, hence this methodology shows substantial promise for incorporation into the DEPM for this species, possibly being used to adaptively establish sampling strata for CalVET net hauls. Such an approach has been used for sardine off California, where it reduced the number of CalVET samples taken (which reduced ship-time costs per survey mile) and increased the percentage of egg-positive CalVET hauls (Lo et al. 2001). Hence the CUFES is likely to prove useful to the DEPM in that it should increase the precision of biomass estimates, reduce survey costs, and, because of the near-real-time information it provides, reduce the potential bias of not enclosing the entire population within the survey grid.

East coast larval surveys. Several surveys were conducted along the east coast between 1951 and 1969 (Anders 1975). Sardine eggs were not found there until 1960, but the time of year of these cruises is uncertain. Subsequent cruises timed to coincide with the annual "Sardine Run" off Durban in winter (June/July) revealed concentrations of sardine eggs south of Durban (~27-29°E),

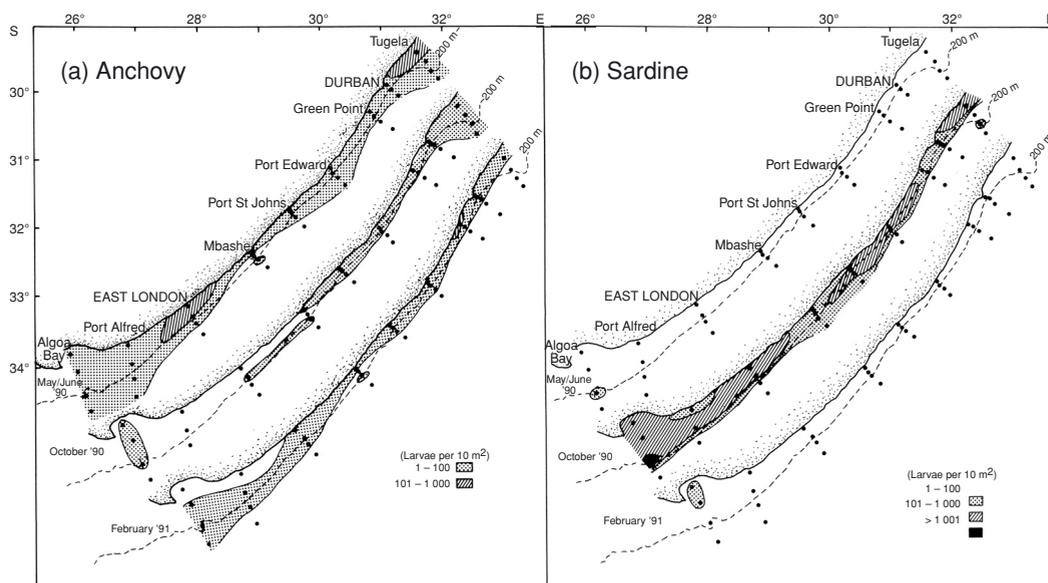


Figure 12: Abundance of (a) anchovy and (b) sardine larvae along the east coast of South Africa in the period 1990/91 (from Beckley and Hewitson 1994; reproduced with permission).

but only in the vicinity of Durban itself immediately following the sardine run. No anchovy eggs were found during these cruises (Anders 1975). Additional surveys along the east coast during January, July, August and December 1973 revealed dense concentrations of sardine eggs in August and of anchovy eggs in December, although eggs of both species were found during both months (Anders 1975). Greatest concentrations of both species were found in the coastal waters north of East London, and anchovy eggs were also collected north of Durban, where the continental shelf widens. These findings of widespread spawning by both anchovy and sardine along the east coast provided the first real indication of the suitability of this region as a spawning habitat.

No further ichthyoplankton surveys were conducted along the east coast until 1990/1991, when three cruises (May/June 1990, October 1990 and February 1991) were undertaken to investigate the distribution and abundance of clupeoid larvae in this relatively under explored part of the coastline (Beckley and Hewitson 1994).

Anchovy larvae were fairly widespread on all three cruises, with greatest abundance off East London and Tugela (north of Durban) during the May/June cruise (Fig. 12a). Sardine larvae were only abundant during the October cruise, when they were found along the entire survey area, with greatest densities near the shelf-break off Algoa Bay (Fig. 12b; Beckley and Hewitson 1994). In addition to these surveys, studies of seasonal variability in sardine egg abundance south of Durban have shown peaks in sardine egg abundance in June/July and again in November/December, which were attributed to the northward and southward (return) migrations associated with the sardine run (Connell 1996). Very few sardine eggs were found between January and May. Together, these

findings complement the earlier discovery of anchovy and sardine spawning along the east coast by Anders (1975), and support the hypothesis of Armstrong et al. (1991) that this region forms an extension of the spawning environment of South African temperate clupeoids.

South African Sardine/Anchovy Recruitment Project surveys. In the late 1980s, the international Sardine/Anchovy Recruitment Project (SARP) was initiated by IOC/UNESCO and FAO in order to investigate biological and physical oceanographic processes governing recruitment fluctuations in marine fish stocks, in particular, factors causing mortality of early life stages (IOC/UNESCO 1990). South African SARP (SA SARP) was initiated in 1993 and comprised monthly cruises from spring through autumn to investigate within-season variability in factors thought to affect sardine and anchovy recruitment (Painting 1993). Sampling details are given in Table 1. SA SARP extended over 2 consecutive spawning seasons, namely 1993/1994 and 1994/1995 (Painting et al. 1998).

A comparison of egg and larval distributions with water current features during individual cruises corroborated the important role of the frontal jet current in transporting eggs and early larvae from the south coast spawning grounds to the west coast nursery area (Fig. 13), supporting the hypothesis originally proposed by Shelton and Hutchings (1982). Convergent flow south of the Cape Peninsula merged into the fast-flowing jet current, the location of which was found to vary between the 200 and 500 m isobaths. Generalized flow patterns indicated that transport was favoured in spring compared to summer, suggesting that eggs spawned earlier in the spawning season would be more likely to reach the nursery grounds than those spawned later (Fowler and Boyd 1998). Currents beyond the 500 m isobath had a much stronger offshore component in some months during the first season (e.g. November 1993; Fig. 13c) compared to the second, suggesting that offshore advection of the spawning products of both species may have been higher in the first season. The increased anchovy and sardine recruitment in 1995 compared to 1994 (see Fig. 3) appears to be consistent with this observed disparity in advective processes. Despite the potential for losses of spawning products through offshore divergence of the jet current, flow patterns observed during some of the SA SARP surveys indicated that eggs and larvae advected offshore could be returned to the system farther north via onshore flow. Hence, whereas some eggs and larvae could be lost by offshore flow, others could reach the nursery grounds via a delayed route farther offshore (Fowler and Boyd 1998).

Richardson et al. (1998) used data collected during the SA SARP programme to examine the effect of SST and food availability (copepod biomass, consistency of copepod biomass, and daily copepod production) on anchovy spawning success. Total anchovy egg abundance over the western Agulhas Bank (WAB) was used as an index of spawning intensity, and was substantially higher during spring and early summer compared to winter or late summer. Total anchovy egg abundance was also significantly related to the area of 16–19°C water over the WAB (Fig. 14a). The disparity between this SST range and that estimated from spawner biomass surveys (17.4–21.1°C) is a result of the extension farther east of the spawner biomass surveys compared to the SARP surveys, which only extended as far as Cape Agulhas. Richardson et al's (1998) results led to the conclusion that the WAB was a more suitable spawning area than the southwest coast because of its greater thermal stability, a larger area of 16–19°C water, and a more consistent food environment having high copepod production, and to the hypothesis that anchovy spawning success was dependent on the extent of such suitable spawning habitat.

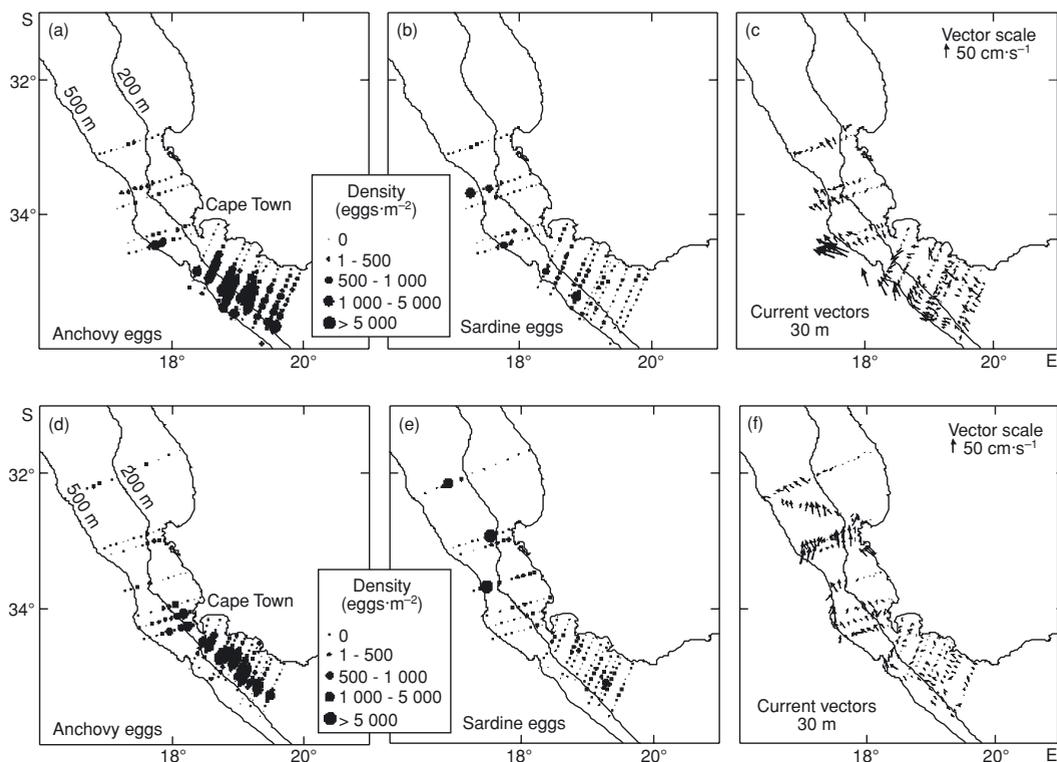


Figure 13: Density of (a) anchovy and (b) sardine eggs, and (c) current vectors at 30m depth during the SA SARP survey of November 1993; and (d) anchovy and (e) sardine eggs, and (f) current vectors at 30m depth during the SA SARP survey of November 1994. Redrawn from Fowler and Boyd (1998).

The seasonal intensity of sardine spawning varied independently of the area of 16-19°C water, suggesting that the thermal conditions that define suitable spawning habitat for sardine are not necessarily the same as for anchovy, or that sardine spawning is less restricted by temperature than is that of anchovy (Fowler 1998). However, intensity of sardine spawning was positively correlated with integrated chlorophyll *a* on the WAB, with highest egg abundance values occurring during early spring (Fig. 14b). This coincidence between spawning intensity and the spring bloom over the WAB, during which there is enhanced phytoplankton, microzooplankton and mesozooplankton production over the inner shelf (Mitchell-Innes et al. 1999), suggests that sardine spawning activity may be related to feeding conditions (Fowler 1998). The decrease in sardine spawning intensity on the WAB during summer when chlorophyll *a* concentrations are low, coupled with the second sardine spawning peak in late summer (February) that coincides with maximal upwelling (Fowler 1998) and increased primary production, lend support to this hypothesis. Hence the temporal pattern of sardine spawning behaviour may be related to the availability of food for spawners, but in terms of phytoplankton as opposed to zooplankton for anchovy.

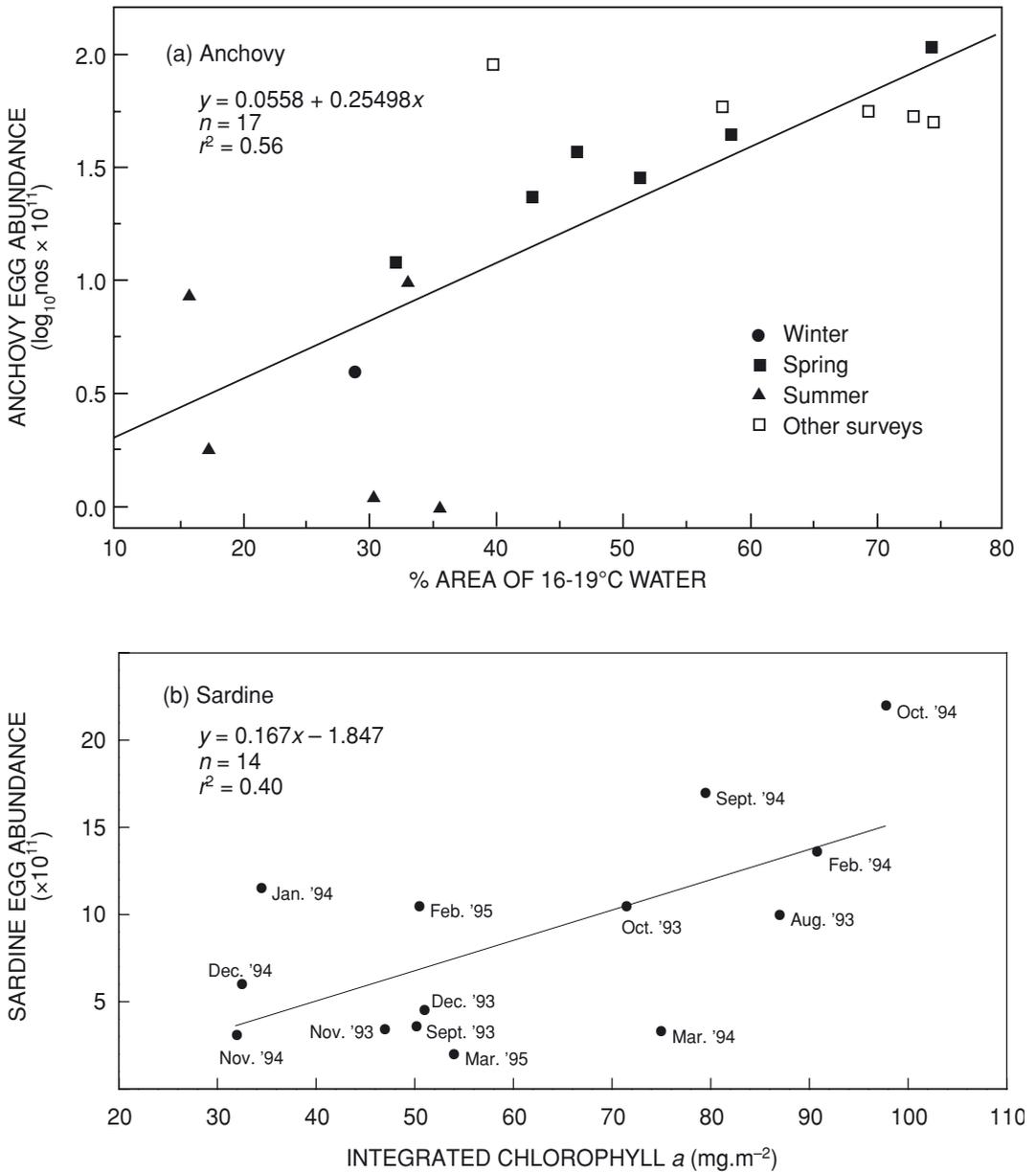


Figure 14: Relationships between (a) total anchovy egg numbers on the western Agulhas Bank and the area of 16-19°C water (arcsine transformed to improve normality) during SA SARP surveys (winter, spring and summer) and five pelagic spawner biomass surveys (November 1988-1992; from Richardson et al. 1998; reproduced with permission), and (b) total sardine eggs numbers on the western Agulhas Bank and the concentration of chlorophyll a (integrated over the WAB) during SA SARP surveys (redrawn from Fowler 1998).

A modified expert system was examined for suitability in forecasting anchovy recruitment strength from data collected during the monthly surveys of SA SARP (Painting and Korrúbel 1998). Since construction of the original expert system model was not based on SARP field data, the use of those data provided an independent test of the expert system and also allowed for a comparison between predictions based on data from a single annual survey (e.g. Korrúbel et al. 1998; see section on pelagic spawner biomass surveys) and those resulting from surveys conducted at regular intervals throughout the spawning season. In this instance six indicator variables were considered in the analysis: (1) distance offshore of the 16°C isotherm, (2) southerly wind stress, (3) anchovy egg abundance (no.m⁻²), (4) the incidence of oocyte atresia in adult females, (5) an index of adult fish starvation derived from estimates of fish density and copepod biomass, assuming a P:B ratio of 0.2 day⁻¹ for copepods and a fish daily maintenance ration of 2% dry body mass.day⁻¹, and (6) oil : meal ratios obtained from anchovy processing factories that were used as an index of fish condition (Painting and Korrúbel 1998). Since forecasts of anchovy recruitment would be required at the start of the fishing season (January) in order to make the results useful for management, only those SA SARP data from the first part of the spawning season (August/September up to and including December) were used in the procedure. In the model, data for each variable were assessed against a threshold value; “active” variables were assumed to contribute towards below-average recruitment (BAR) by being either above or below threshold values, as defined by model rules. If there were no active variables then “average/above-average recruitment” was forecast; if the number of active variables was between one- and two-thirds of the number of variables used then “likely below-average recruitment” was forecast; and if the number of active variables was greater than two-thirds then “very likely below-average recruitment” was forecast.

Egg abundance (daily egg production was not estimated during the SA SARP surveys) was used as a predictor of recruitment based on the assumptions that this index was one step shorter in the causal chain than the classic stock-recruit relationship, and that a low egg abundance implied an increased chance of a BAR event (Painting and Korrúbel 1998). Mean monthly egg abundance during the SA SARP surveys was less than the threshold value of 300 eggs.m⁻², and hence indicative of a BAR event, in only three of the nine surveys from which data were used. Nonetheless, forecasts of anchovy recruitment made from the SA SARP data were for below-average recruitment for both 1994 and 1995. For 1994, all the monthly forecasts fell in the BAR category (including two possible, two likely and one very likely forecast of BAR), and both observed (direct survey estimate; see Fig. 3) and estimated (from an anchovy assessment model) recruitment was below average. For 1995, three of the four monthly forecasts fell into the BAR category (two possible and one very likely) and the other into the “average/above-average recruitment” category; observed recruitment that year was above average but recruitment estimated using the assessment model was below average.

Painting and Korrúbel (1998) found that predicted and observed/estimated anchovy recruitment compared favourably, and suggested that field measurements of biological and environmental variables could thus be used in a structured manner to obtain timely forecasts. They emphasized that recruitment may be impacted by a suite of variables, which could vary in importance from year to year and which could act in isolation or conjunction. Nonetheless, those authors deemed two of their six variables to be essential, namely the position of the 16°C isotherm (which they used as a proxy for upwelling intensity and strength of the frontal jet current, and considered to be

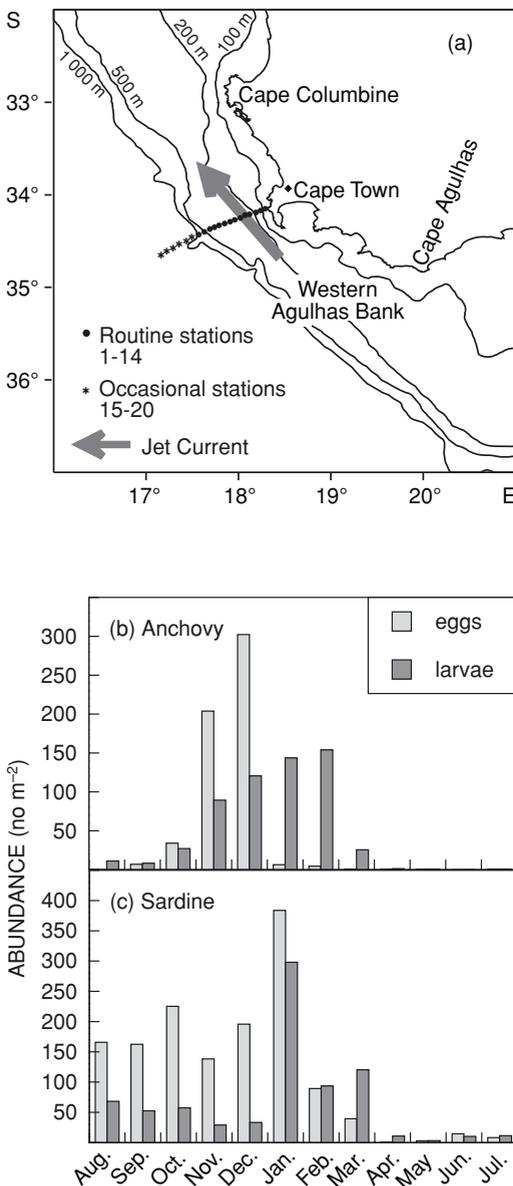


Figure 15: Schematic of the SARP Monitoring Line showing (a) the location of stations sampled along the line using a mini-Bongo net and the approximate position of the shelf-edge jet current (from Huggett 2002), and monthly mean egg and larval abundance for (b) anchovy and (c) sardine over the period August 1995 to July 2001 (JAH, unpublished data).

a driving variable in the recruitment process) and the index of southerly wind stress. Given this complexity in the processes impacting on recruitment, Painting and Korrübel (1998) concluded that rule-based models provided a particularly appropriate system, since rules may be added easily with little disturbance to the system.

SARP Monitoring Line. The monthly SA SARP surveys provided valuable insight into processes affecting recruitment but were too labour intensive to maintain, leading to the decision to constrain regular ichthyoplankton sampling to a more focused objective. The SARP Monitoring Line programme was initiated in August 1995, with the aim of providing increased temporal coverage of the transport of early life history stages by the Benguela jet current. Given the role of the shelf-edge jet current in the concentration and transport of eggs and larvae from the western Agulhas Bank spawning grounds to the west coast nursery area (Shelton and Hutchings 1990), regular monitoring of this critical gateway during the spawning season was considered useful in attempting to predict recruitment success (Hutchings 1992). Sampling along the SARP Monitoring Line, which crosses the jet current off the Cape Peninsula (Fig. 15a), began in August 1995 and is ongoing; sampling frequency is effectively bimonthly (Table 1; Huggett et al. 1998). Ichthyoplankton collected along this transect primarily reflect spawning on the western Agulhas Bank, as the relatively small mouth area of the mini-Bongo net (Table 1) only samples eggs and small larvae (mainly <20mm TL), and larger larvae advected from farther east would be able to avoid the net.

Spawning seasonality inferred by egg and larval abundance along the SARP monitoring line has largely supported the findings of the

earlier studies. Monthly averages from 1995 to 2001 indicate that anchovy eggs were most abundant from November to December, and anchovy larvae from November to February (Fig. 15b). Sardine eggs and larvae were most abundant from August to March, peaking in January, but were found throughout the year (Fig. 18c). Eggs and larvae of both species showed different patterns of abundance from year to year, emphasizing the within-season variability in spawning patterns observed during the monthly SA SARP surveys. Similar variability was observed in the position and strength of the jet current, which was usually located close to the coast during spring, moving farther offshore in early summer (Huggett et al. 1998; Huggett 2002). This observation led Boyd and Nelson (1998) to suggest that the first part of the spawning season (October-December) was more favourable for transport to the nursery area than the second part (January-March), supporting the findings of Fowler and Boyd (1998) for the SA SARP surveys.

An important objective of the SARP Monitoring Line originating from the initial aims of the SA SARP surveys was to investigate within-season variability in spawning and transport success by comparing monthly egg and larval abundance along the SARP line to the birth date distributions of subsequent recruits. Mean monthly anchovy (August-March) and sardine (September-February) egg abundances from 1995/1996 were significantly correlated with the estimated birth date distribution of the 1996 recruits (Huggett et al. 1998; Painting et al. 1998), suggesting promise in this approach. However, the birth date distributions were only rough estimates derived from length frequency distributions of the 1996 recruits and average growth rates from 1993/94, and the lack of routine daily ageing and growth studies in the region since 1994 has hampered the further pursuit of such relationships. This has been a lost opportunity, as discrepancies between recruit or pre-recruit birth dates/hatch dates and monthly egg abundance along the SARP line would have facilitated the identification of event-related differential survival or mortality in relation to the transport process.

The within-season variability in the timing, duration and extent of spawning on the western Agulhas Bank, as indicated by the SARP Monitoring Line, invites comparison between years of low and high anchovy recruitment. Low anchovy recruitment in the winter of 1996 was preceded by a brief warming period along the monitoring line during the summer of 1995/1996, and a narrow window of anchovy eggs and larvae from late October to early December (Fig. 16a). In contrast, high anchovy recruitment in winter 2001 was preceded by an extended period of warm water along the monitoring line, and greater and more persistent abundance of anchovy larvae in particular, as well as eggs, which were associated with the warm water (Fig. 16b). Annual indices of mean egg and larval abundance (no.m^{-2}) along the monitoring line (Fig. 17a, b) have yielded positive linear correlations between eggs, larvae, and eggs and larvae combined, and the subsequent number of recruits for both anchovy and sardine over the 7 years sampled. Whereas most correlations were not significant, there was a significant correlation between anchovy eggs and subsequent numbers of recruits (Fig. 17c), indicating the potential of this index for use in forecasting anchovy recruitment strength before the final TAC revision. Nevertheless, it should be cautioned that the time-series is still very short, and correlations could change with the addition of future data points. The usefulness of these data for forecasting sardine recruitment is doubtful (Fig. 17d), most likely due to the considerable portion of sardine spawning that has taken place in recent years on the west coast, to the north of the SARP Monitoring Line (van der Lingen et al. 2001; see section on pelagic spawner biomass surveys).

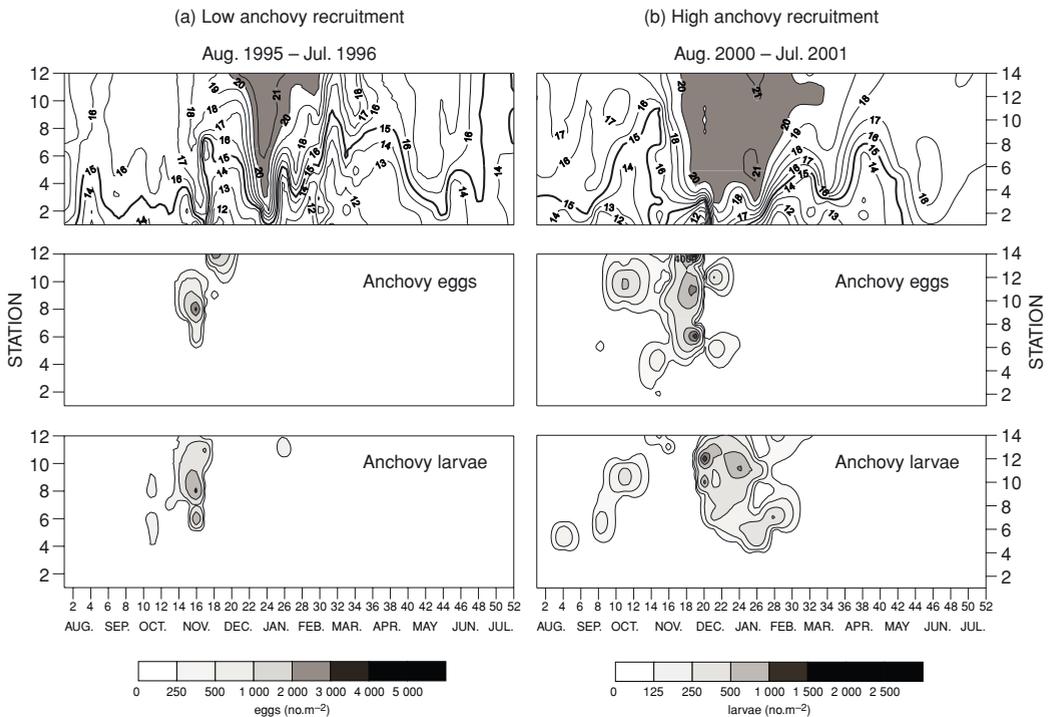


Figure 16: Contoured plots of SST ($^{\circ}\text{C}$), and anchovy eggs and larvae (no.m^{-2}) across the SARP Monitoring Line (station numbers are shown on the y-axis) during (a) 1995/1996, a year of low anchovy recruitment, and (b) 2000/2001, a year of high anchovy recruitment. Week number is shown on the x-axis (from Huggett 2002).

Post-larval and pre-recruit surveys. Extensive datasets now exist concerning intra- and interannual changes in the abundance and distribution of eggs and early larvae of anchovy and sardine, but information for late and post larvae is relatively limited. Boyd and Hewitson (1983) described the distribution of anchovy larvae of a wide size range (3 to 42 mm standard length; *SL*) off the west coast between Lüderitz and Cape Columbine over the period 1978 to 1982, derived from oblique Bongo net tows made at 15 and 60 n.miles offshore at intervals approximately 30 n.miles apart along the coast. Their results showed that there was a rapid decrease in the abundance of larvae $<20\text{mm } SL$, and an increase in larval mean length, with decreasing latitude (Fig. 18). Their results supported the existence of a nursery ground north of Cape Columbine for larvae spawned off the south coast as proposed by Shelton and Hutchings (1982), and further suggested that there was some degree of northward transport or migration of larvae.

Surveys to assess the abundance and distribution of pre-recruit anchovy and sardine have been conducted off South Africa's west coast since 1991. Whilst not strictly ichthyoplankton surveys, since sampling is aimed at larval and juvenile fish that are active swimmers as opposed to passive drifters, the pre-recruit surveys have provided useful knowledge on these early life history stages of

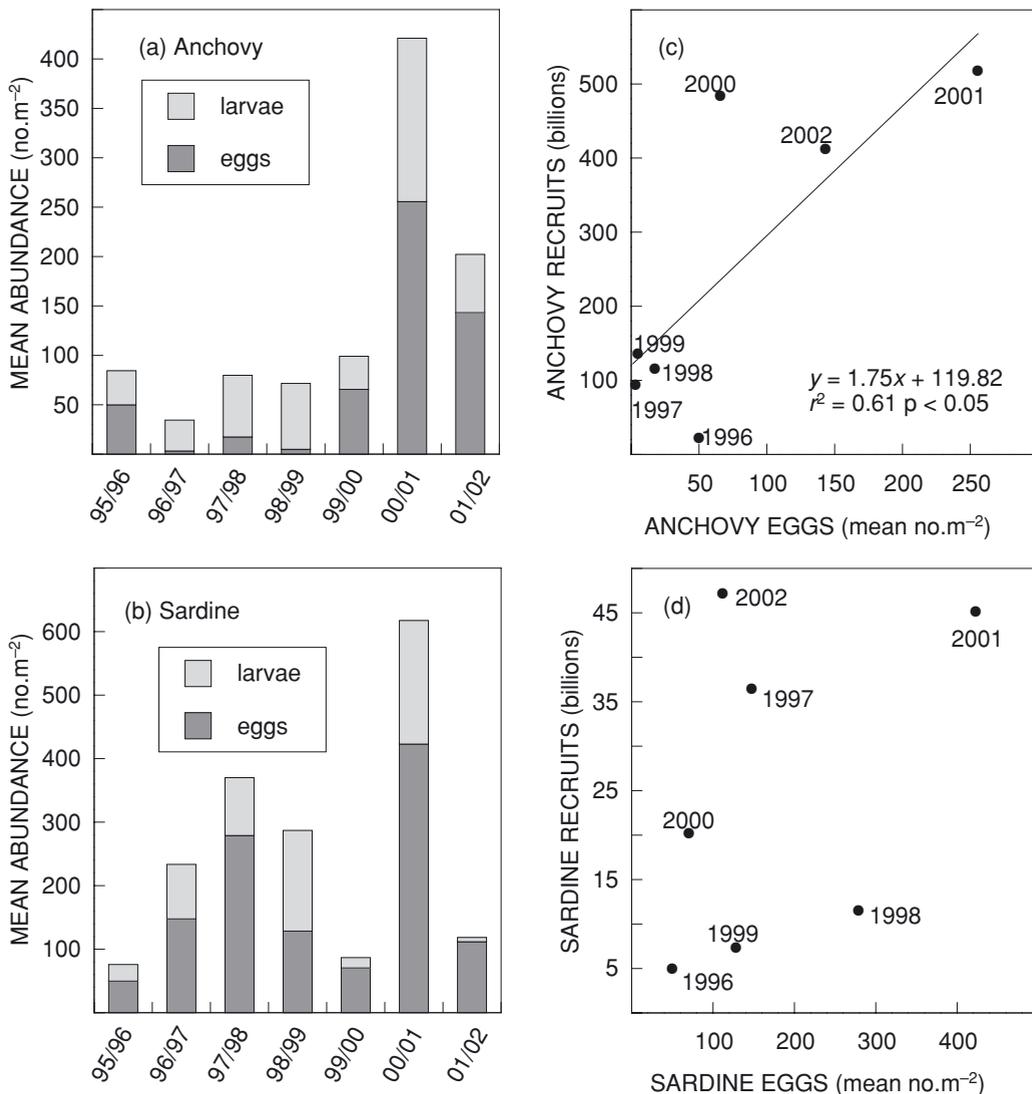


Figure 17: Indices of mean (a) anchovy and (b) sardine egg and larval abundance (no.m⁻²) along SARP Monitoring Line between September and March each year from 1995/96 to 2001/02, and scatterplots between mean abundance of (c) anchovy and (d) sardine eggs across the SARP Monitoring Line and subsequent numbers of recruits (billions; JAH, unpublished data).

anchovy and sardine. Designed to obtain an indication of forthcoming recruitment strength, primarily for anchovy (O’Toole and Hampton 1989), and timed so that such information would be obtained sufficiently early in the fishing season to be useful for management (Cochrane and Starfield 1992), pre-recruit surveys are conducted during March, when surviving fish from eggs spawned in summer are 2-5 months old.

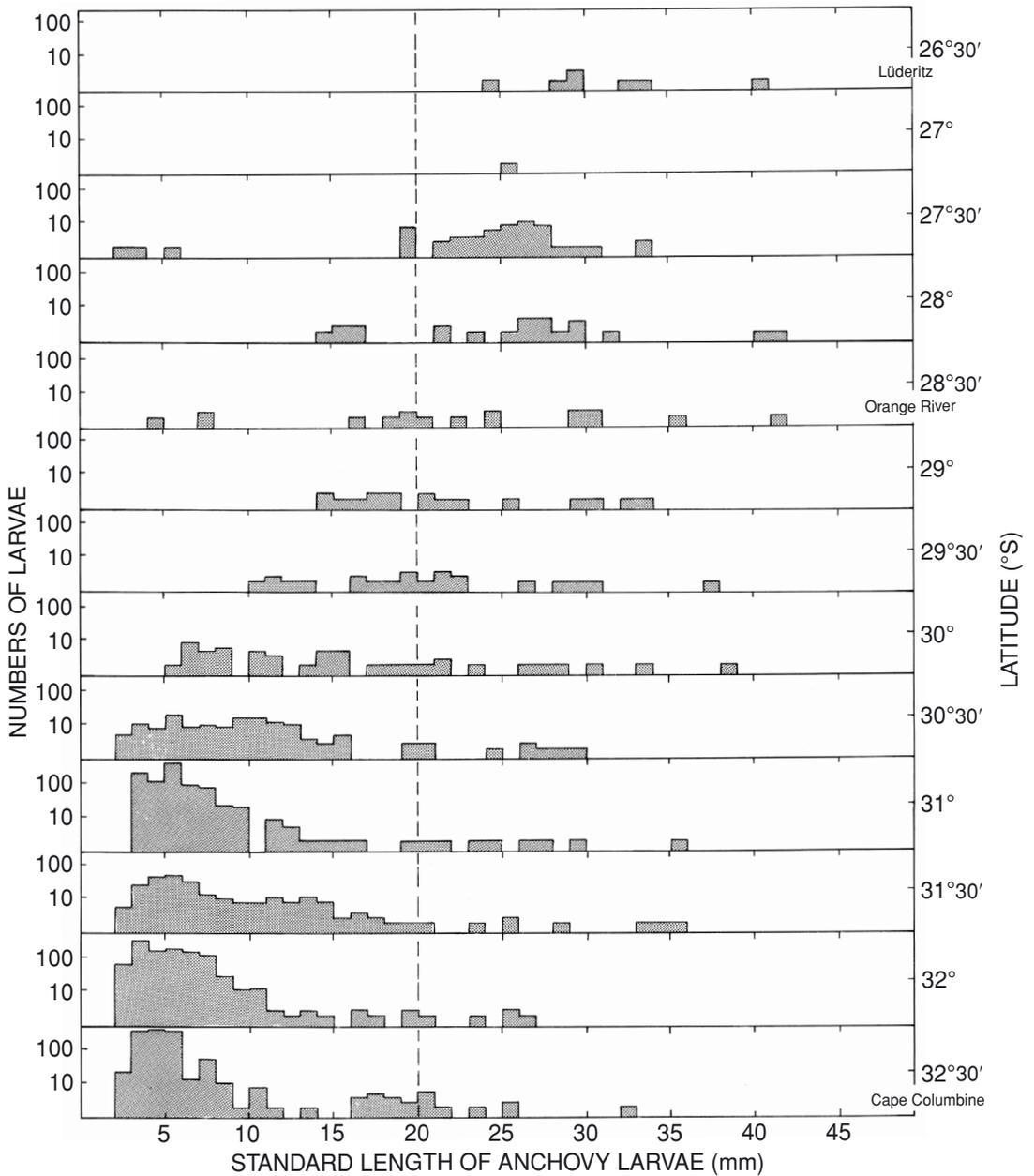


Figure 18: Size frequency distributions of anchovy larvae and caught with an obliquely-towed Bongo net per half degree of latitude between 26°30' and 32°30'S, 1979-1982 (from Boyd and Hewitson 1983; reproduced with permission).

Surveys sample a grid of randomly-positioned transects, orientated perpendicular to the coast and extending across the continental shelf over the west coast nursery grounds between the Orange River mouth and Cape Point. Stepped, oblique hauls of a modified Methot frame trawl (Methot 1986; O’Toole and Crous 1989) from 35m depth to the surface are performed at 5-9 stations along survey transects, sampling being conducted at night when fish are dispersed near the surface and avoidance of the sampling gear is likely to be substantially reduced compared to day-time sampling.

Anchovy pre-recruits have been collected more frequently and at substantially higher densities than sardine pre-recruits during all surveys (Fig. 19). Survey mean density for anchovy pre-recruits has shown a 35-fold variation over the time-series, with peak densities observed in 1991 and 2001. Survey mean density for sardine pre-recruits has varied by 10-fold, and sardine have shown a slight increase in their frequency of occurrence during the time-series.

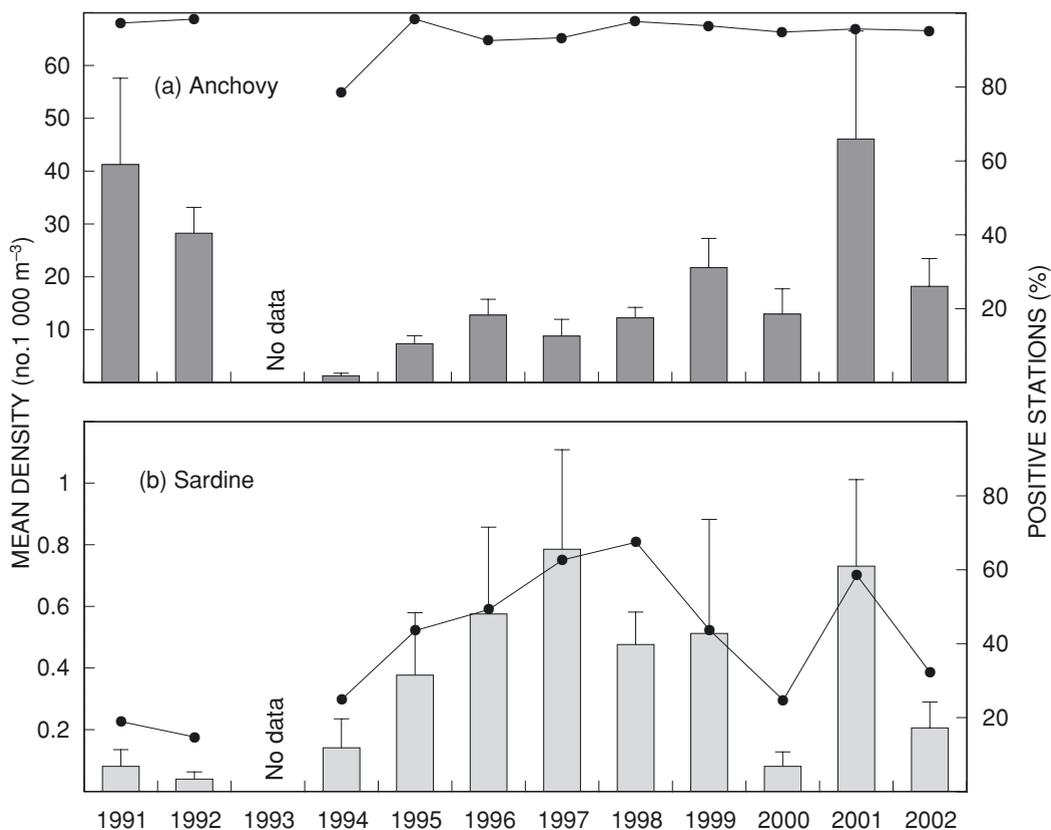


Figure 19: Survey mean density (number.1000m⁻³) and frequency of occurrence (percent positive stations) for anchovy and sardine pre-recruits surveyed between the Orange River mouth and Cape Columbine over the period 1991-2002. Vertical bars indicate the coefficient of variation (CV) of the mean; note that the density axes for anchovy and sardine are not to the same scale (CDvdL, unpublished data).

Both anchovy and sardine pre-recruits show distinct distribution patterns according to size, although the picture is clearer for anchovy as a result of the greater numbers captured. Smaller (<35mm total length; *TL*) anchovy pre-recruits are found offshore of the continental shelf, predominantly in the south of the survey area (Fig. 20a, b), whereas larger (>35mm *TL*) pre-recruits are found closer inshore (Fig. 20c, d). This cross-shelf gradient in pre-recruit size is annually consistent, and indicates that early anchovy larvae are not transported by the jet current directly to the inner continental shelf of the west coast. However, the mechanisms by which larvae are transported shoreward, growing as they go, are unclear, and require further research. Active swimming seems probable, since larvae longer than 7mm *TL* can swim sufficiently well to avoid a bongo net during daylight (Badenhorst and Boyd 1980). An anchovy larva of 20mm *TL* swimming at a cruising speed of 1 body length s^{-1} (Hunter 1972) for 12 h.d $^{-1}$ would take 105 days to swim the 180 km from offshore of the continental shelf to the coast, if water movement either toward or away from the coast were zero. However, this is probably too long a period for active swimming to be solely responsible for onshore movement of larvae. Hence, passive transport is likely too, particularly in the region where the shelf narrows (approximately 31°S) and onshore movement of surface waters could transport larvae and pre-recruits shoreward. Similarly, the relaxation of upwelling-favourable southeasterly winds during late summer could promote a shoreward movement of the upwelling front.

Estimates of mean pre-recruit density are not correlated with subsequent estimates of recruit numbers made two months later during winter (May) recruit surveys for either anchovy or sardine. The lack of significant relationships between pre-recruit abundance and subsequent recruitment strength may be indicative of inadequate sampling, most likely due to insufficient temporal coverage, but may also be due to the short time-series of available data. However, whilst the pre-recruit surveys may not have provided the forecast of anchovy recruitment strength anticipated of them, they have provided a wealth of new information regarding these early life history stages, raised new ecological questions, and indicated new avenues of research.

Discussion

Ichthyoplankton surveys off South Africa have underpinned understanding of the life history cycles of anchovy and sardine in the southern Benguela. These surveys have also played an important role in the management of the anchovy fishery through, for example, application of the DEPM to estimate spawner stock size, and in identifying key mechanisms thought to impact on anchovy recruitment success. The latter is an important management consideration since most of the catch consists of young of the year. Management of the anchovy fishery consists primarily of the setting of total allowable catch (TAC) levels (Geromont et al. 1999). An initial anchovy TAC is set at the beginning of the year (Fig. 21), derived from a constant proportion formula that uses estimates of adult biomass obtained from the spawner biomass survey conducted in November of the preceding year and assumes that forthcoming recruitment will be the median of observed values. To counter possible poor recruitment, a scale-down factor of approximately 0.7 is applied to the derived value. Following the May/June recruit survey during which anchovy recruitment strength

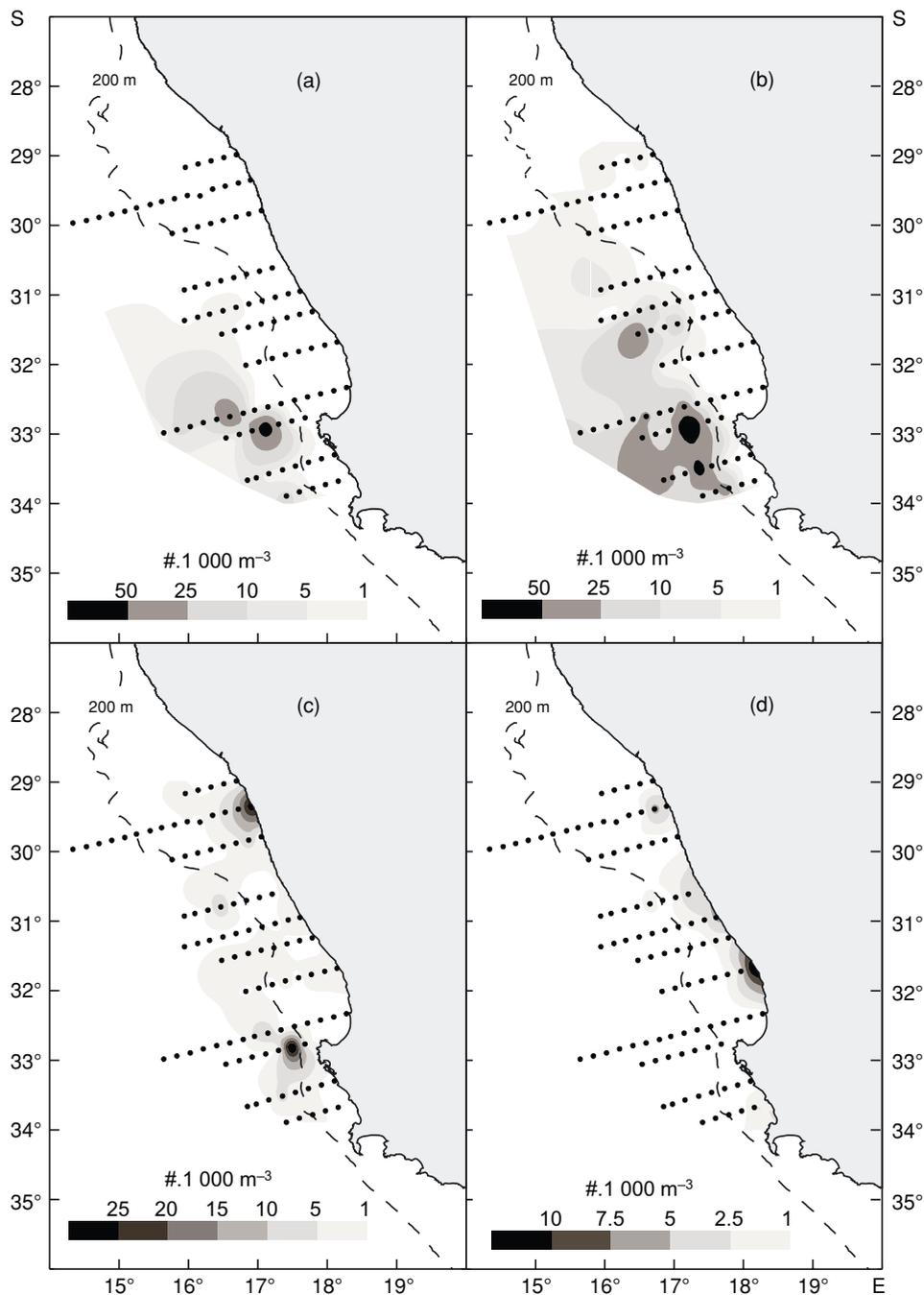


Figure 20: Distribution by size class of anchovy pre-recruits collected using a Methot frame trawl during the March 1998 survey; (a) <20mm TL, (b) 20-34mm TL, (c) 35-50mm TL and (d) >50mm TL. Dots indicate the positions of Methot stations. Note that the density scales differ between size classes (CDvdL, unpublished data).

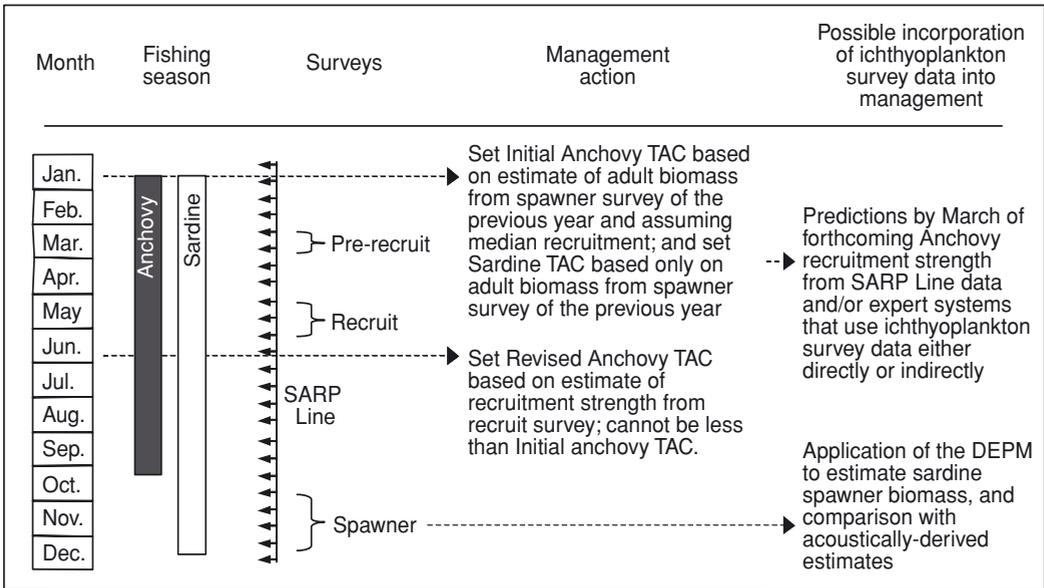


Figure 21: Schematic indicating the annual sequence of fishing, surveys, and management actions pertinent to management of South Africa's pelagic fishery, and how and when ichthyoplankton survey data can be incorporated into management procedures.

is estimated acoustically, a revised (or final) anchovy TAC is set, depending on recruitment strength. If recruitment is higher than median the TAC is increased; if recruitment is median or lower the TAC remains unchanged.

Only one of the current ichthyoplankton survey programs, the SARP Monitoring Line, shows promise as a predictor of anchovy recruitment success. Simulation studies (Cochrane and Starfield 1992) have shown that predictions of anchovy recruitment that are correct 70% of the time and are made at the start of the fishing season could allow an increase in mean annual anchovy catch for the same level of risk under which the fishery is managed. Although some fishing for adult anchovy takes place during January and February, recruits are only caught in significant quantities towards the end of March. Hence predictions of anchovy recruitment strength would have to be made by the end of March at the latest in order to be of benefit to management (Fig. 21). The SARP Monitoring Line is also the only ichthyoplankton survey that provides an index that explains over 50% of the total variation in recruitment, the minimum percentage for the incorporation of such indices into management procedures for South African anchovy to show benefits in terms of risk and average catch (Barange 2001).

Despite substantial development of expert systems to forecast anchovy recruitment in the southern Benguela, and indications of their likely usefulness, these systems have yet to be incorporated into management procedures. The most recent expert system (Miller and Field 2002) is a deterministic model that uses five predictors that cover the spawning, transport and nursery areas of the anchovy life cycle to provide a qualitative (below median, median, and above median) forecast

of forthcoming anchovy recruitment strength. Two of the predictors are related to ichthyoplankton, namely an index of thermally suitable (16-19°C; Richardson et al. 1998) spawning area on the Agulhas Bank, and an index of wind velocity over the transport area between Cape Point and Cape Columbine. This system correctly predicted recruitment for 89% of the 18 year time-series, including two test years that were not used in model construction. The high success rate of this, and other expert systems, motivates strongly for their inclusion into management procedures.

Ichthyoplankton survey data have not provided substantial input into management of the South African sardine fishery. The DEPM has yet to be used to estimate sardine spawner biomass off South Africa, despite concerns regarding current acoustically-derived estimates of biomass, and its use in estimating population sizes of this species elsewhere (e.g. off California [Lo et al. 1996] and Peru [Alheit 1993]). Since only one sardine TAC is set annually (Fig. 21), predictions of recruitment strength for this species are not as important for management as they are for anchovy, but would be useful for predicting bycatch levels of juvenile sardine in the anchovy fishery.

A substantial amount of research effort has gone into attempts to understand variability in anchovy recruitment, using data derived from ichthyoplankton surveys as well as other sources (Hutchings et al. 1998). A decade ago, a multidisciplinary South African research program called the Benguela Ecology Program (BEP; Moloney et al. in press) that was primarily focused on pelagic resources was credited with having a “level of understanding of the ecosystem [that] is as good as anywhere in the world”, and as being “close to resolving the recruitment problem of the anchovy” (Rothschild and Wooster 1992). A breakthrough in anchovy recruitment prediction has yet to be achieved, despite further research and promising empirical relationships. For example, observations that higher than average southeasterly (SE) winds during the October-March spawning season were followed by poor anchovy recruitment over the period 1984-1994 (Boyd et al. 1998) led to the development of a statistically significant relationship relating anchovy recruitment success to the cumulative SE wind anomaly at Cape Point (Fig. 22a). This inverse relationship was thought to occur because more wind resulted in an offshore displacement of the jet current, which increased advective loss of anchovy eggs and early larvae. However, the addition of subsequent data to this relationship removed its significance (Fig. 22b), with high SE anomalies (in 2000 and 2001) associated with exceptionally high recruitment success. This suggests a more complex relationship between seasonal wind patterns and recruitment strength than previously thought. Roy et al. (2001) proposed that a succession of weak and strong upwelling periods, and the appropriate timing of these events within the anchovy life cycle, represents the canonical pattern of environmental variability for maximizing anchovy recruitment success. This premise reconciled previous findings and provided a plausible explanation for the exceptional anchovy recruitment in 2000, but does not explain the high recruitment success recorded since 2001.

The collapse of such empirically-derived relationships both here in the Southern Benguela and elsewhere (Myers 1998) is not surprising, considering that many factors may affect the recruitment process, and any one variable may vary in relative importance from year to year in a dynamic system. For example, variability in spawning success and productivity of the west coast nursery area are both likely to contribute significantly to recruitment variability. Additionally, non-stationarity in the responses of fish populations to fishing or environmental pressure, such as regime shifts (Schwartzlose et al. 1999), changes in spawning habitat location (van der Lingen et al.

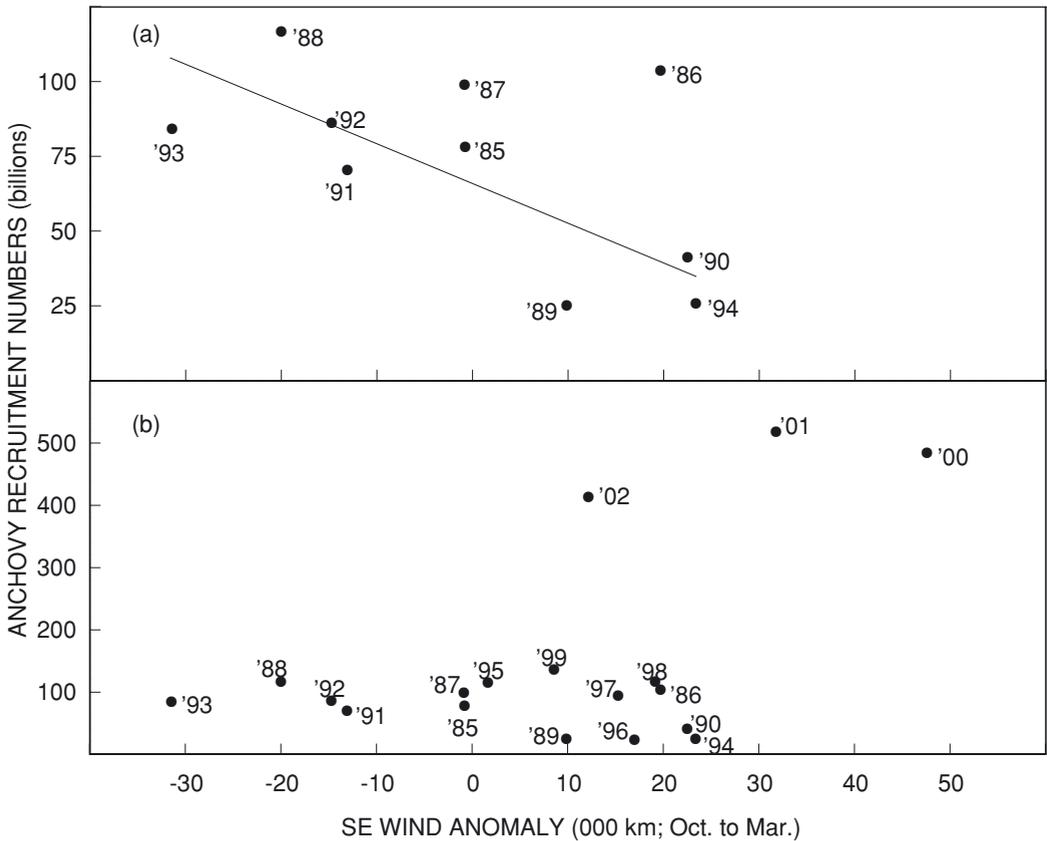


Figure 22: Relationship between anchovy recruitment numbers and the southeast wind anomaly at Cape Point for (a) the period 1985-1994 (note that the data-point for 1986 was not included in generating the linear regression [see Boyd et al. 1998]), and (b) the period 1985-2002.

2001), and other behavioural responses may invalidate the assumptions of long-term system stationarity that underlie conventional empirical analyses (Bakun 2002). A further complicating factor is that the understanding of micro- and mesoscale physical processes and their impact on recruitment variability in the southern Benguela is poor. For example, turbulence has been shown to have a substantial impact on survival of clupeid larvae elsewhere (see references in Bakun 1996), and offshore mesoscale eddies have been identified as areas of exceptional pre-recruit survival and production for sardine off California (Logerwell et al. 2001). A firm adherence to empirical relationships between physical and/or biological parameters and anchovy recruitment in the southern Benguela may thus be misplaced; such relationships may be spurious or transitory.

These problems suggest that a more integrative approach is required to address the “recruitment problem”, such as that provided by expert systems or modeling. Ecosystem and holistic modeling

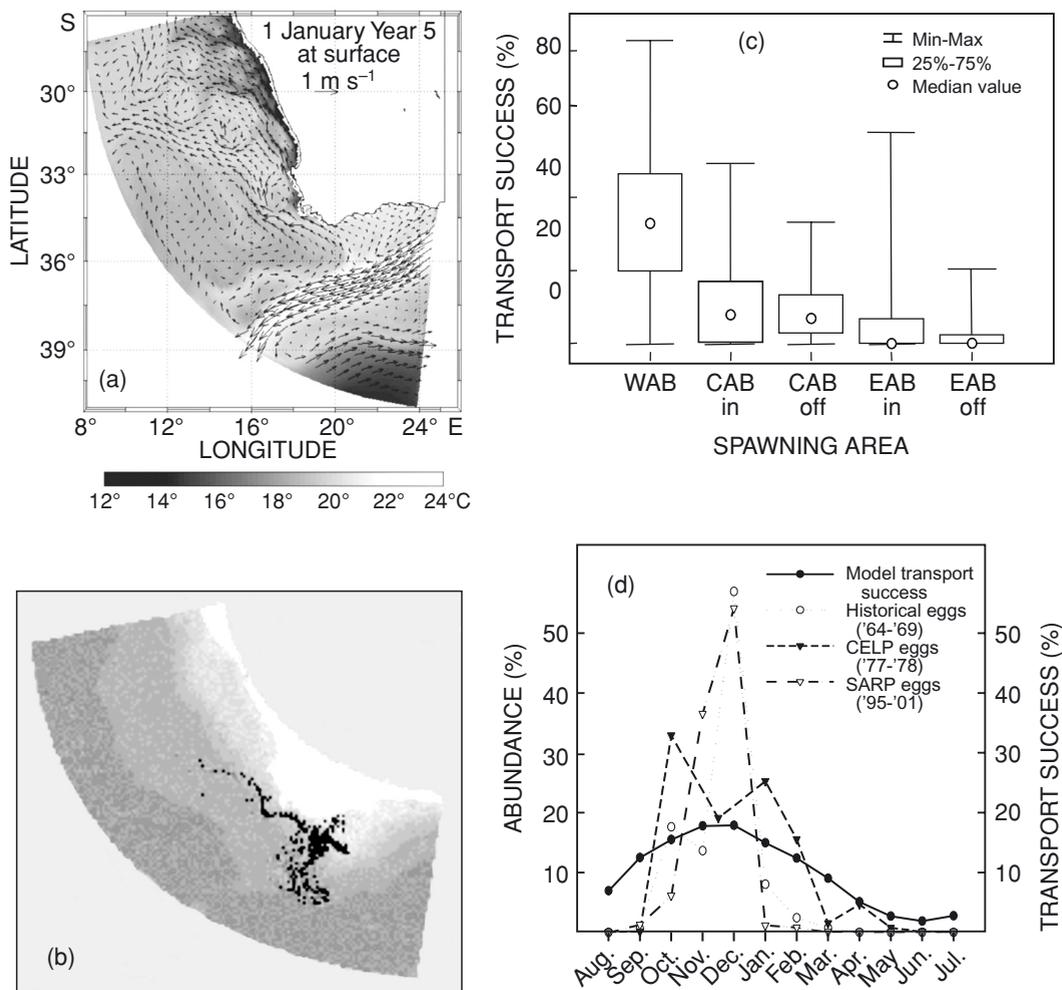


Figure 23: Examples of (a) the surface structure of the temperature and currents (arrows, only drawn every 3rd vector) in the Southern Benguela region simulated by a 3D hydrodynamic model (the snapshot of the 1st January of Year 5 shows the main oceanographic features of the region, including the warm, westward-flowing Agulhas Current and coastal upwelling off the West Coast; from Parada et al. 2003), and (b) output from an IBM experimental simulation showing the position of individual particles in the 3D hydrodynamic model (courtesy of C. Parada, IDYLE, University of Cape Town); results from an IBM showing transport success of particles representing anchovy eggs and early larvae as a function of (c) spawning area (the western Agulhas Bank [WAB], and the inshore [in] and offshore [off] regions of the Central (CAB) and Eastern Agulhas Banks [EAB]); and (d) a comparison between modeled transport success and observed patterns in anchovy egg abundance from three ichthyoplankton survey programmes (c and d from Huggett et al. 2003).

studies have recently been initiated for the southern Benguela, and provide an opportunity for improved understanding of recruitment variability through computer simulation. An example of this is the individual based model (IBM) approach, whereby the output (temperature, salinity and velocity fields) of a 3D hydrodynamic model of the sub-region is coupled to a model that tracks particles representing anchovy eggs and early larvae that have been allocated biological properties (Fig. 23a, b). Such coupling has enabled quantification of the effect of varied physical and biological parameters on the transport of particles from spawning to nursery grounds. In the southern Benguela, IBMs have been used to assess the effect of spatio-temporal variability in anchovy spawning on transport success (Huggett et al. 2003), to examine the influence of egg buoyancy on transport success (Parada et al. 2003), and to explore environmental constraints that select for observed spatial and temporal anchovy spawning patterns on an evolutionary time-scale (Mullon et al. 2002). Results from these IBMs have matched our knowledge of the spawning habits of anchovy in the southern Benguela to a large degree (Fig. 23c, d). Such agreement provides confidence that inferences made from these IBMs are meaningful, and that these experimental simulations are likely to improve our understanding of the key processes responsible for anchovy recruitment success in relation to their spawning strategy, a major step in predicting fluctuations in stock size.

Ichthyoplankton survey data from the southern Benguela have been used for management purposes to a lesser extent compared to other eastern boundary current systems where large populations of clupeids occur. Ichthyoplankton data from the California Cooperative Oceanic Fisheries Investigations (CalCOFI) surveys were used to develop the DEPM to estimate spawner biomass of anchovy *Engraulis mordax*, in response to perceived problems with traditional methods of stock assessment, such as acoustic surveys (Lasker 1985). It has since been used to estimate population size of clupeids in many other areas, such as Peru and the Bay of Biscay (Alheit 1993). However, more research aimed at relating clupeoid early life history stages to recruitment variability appears to have been conducted in the southern Benguela than in other eastern boundary systems. This is probably due to the large separation between anchovy spawning and nursery areas, a feature that is unique to the southern Benguela, and the consequent pivotal role of the transport process to recruitment variability in this region.

Future research concerning ichthyoplankton surveys off South Africa should be directed at obtaining a better understanding of the life history strategy of sardine, including an assessment of the south coast as a nursery area and an examination of spatio-temporal variability in spawning over the central and eastern Agulhas Banks. Additionally, research for both anchovy and sardine should be directed at assessing the effects of parental condition on egg quality and subsequent recruitment success; examining offshore retention and onshore movement of pre-recruits off the west coast; determining annual hatch date distributions; examining vertical distribution of eggs, larvae and pre-recruits off the west coast; and determining larval diets and the effects of frontal concentration mechanisms. Such research will help to improve our knowledge of factors influencing recruitment variability of these species and may assist in management of the fisheries they support.

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The arrival of late-stage coral reef fish larvae in near-shore waters in relation to tides and time of night

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Abstract

This study examined the influence of tidal state (low, flood, high, ebb), flow and time of night of sampling on catches of larval fishes in light traps. Light traps were sampled at 2 hr intervals for 13 consecutive nights centred on the new moon in each of three lunar months. Highest catches coincided with the mid-point of the ebbing tide and lowest catches with the flooding tide, although relationships between tidal flow and nightly catches were complex. This result contrasts with previous studies and probably reflects the relatively small tidal range in the San Blas region. Weak off-reef tidal currents are likely to be insufficient to prevent the active onshore migration of pre-settlement stage coral reef fishes. There were no significant changes in catch rates during the night (all taxa combined), although there was a slight trend of increasing catches towards dawn. Catches of Pomacentridae composed 57.35% of the total catch, and increased throughout the night. These results suggest that the abundance of late-stage larvae in catches is likely to be determined by factors other than the weak tidal currents experienced in the San Blas region.

Introduction

The spatial and temporal variability in the replenishment of populations of coral reef fishes has been the focus of considerable research over the past two decades (reviewed in Doherty and Williams 1988; Doherty 1991; Leis 1991; Caley et al. 1996). This has largely been due to the realisation that variations in the supply of competent larvae to reef habitats (hereafter defined as replenishment) is an important determinant of the dynamics of adult populations of reef fishes (Victor 1986; Doherty and Fowler 1994a, 1994b; Booth and Brosnan 1995). Until recently however, most

studies that have examined the processes underlying patterns of replenishment have focused on those factors that are likely to influence the transport and dispersal of larvae in the plankton. Theories of long distance dispersal are based on the concept that most populations of coral reef fishes are demographically 'open', where new individuals arrive from a source population that is at a distance from the sink population (but see Jones et al. 1999; Cowen et al. 2000; Paris et al. 2002). Consequently, a number of studies have attempted to correlate nightly patterns of replenishment with environmental variables (wind, tides and currents) that are likely to influence the transport of larvae as they arrive in nearshore reef environments (Milicich 1994; Sponaugle and Cowen 1996*a*, 1996*b*; Kingsford and Finn 1997; Wilson and Meekan 2001). In this paper, I examine the relationships among the number of pre-settlement coral reef fishes arriving in coral reef habitats, the time of night, and changes in tides in the San Blas Archipelago, Caribbean Panama, where tidal flows are much reduced in comparison to flows on the Great Barrier Reef and the North-West Shelf of Australia.

Typically, correlations between environmental variables and patterns of replenishment are weak and variable in time and space (Wilson and Meekan 2001). The lack of strength of these correlations may be a result of the heightened mobility of late larval stages. For example, recent work has indicated that late-stage larvae are far from passive particles in the water column (Leis and Carson-Ewart 1997; Stobutzki and Bellwood 1997; Fisher and Bellwood 2002). The active behaviour of these individuals in the form of directional swimming and vertical migration among water masses (Hendriks et al. 2001) due to a well developed swimming ability (Leis et al. 1996; Stobutzki and Bellwood 1997) and visual capabilities (McCormick and Shand 1992; Job and Bellwood 2000) will result in complex interactions between larvae and environmental processes to control both the dispersal distance (Cowen et al. 2000, Paris et al. 2002) and the timing of replenishment (Kingsford et al. 1991; Sponaugle and Cowen 1996*b*; Kingsford and Finn 1997; Wolanski et al. 1997; Wilson and Meekan 2001, 2002). For example, Kingsford and Finn (1997) suggested that late-stage larvae around One Tree Reef (central GBR) may be using a range of sensory stimuli derived from reef habitats that are carried into nearshore waters on tidal currents, to actively select and migrate to suitable settlement habitats. If fish larvae prove to be capable of using reef derived stimuli to initiate the replenishment process, then this will further decouple any simple relationship between environmental variables and replenishment patterns. Thus, the first objective of this study is to examine the influence of tidal state on replenishment patterns of coral reef fish larvae. This was achieved by sampling light traps at 2 hour intervals throughout the night and comparing catches to tidal flow patterns.

In addition, the majority of studies that have examined replenishment patterns of coral reef fishes have examined patterns at either lunar monthly and/or nightly scales (Robertson 1992). To date, the only studies that have measured replenishment patterns at finer temporal scales (within night) have used crest nets (French Polynesia: Dufour 1991; Dufour and Galzin 1993, GBR: Doherty and McIlwain 1996, NW-Shelf Australia: McIlwain 1997). These studies have identified conflicting patterns in the timing of replenishment within each night. To date, no study has examined if such fine-scale temporal patterns of replenishment occur in the Caribbean. Thus, the second objective of this study is to determine the extent to which movement patterns of replenishing larvae are influenced by time of night factors. This was achieved by the sampling of larval arrival in nearshore waters at intervals throughout the entire night.

Materials and Methods

Study site. Field sampling was conducted around the fringing reefs of the San Blas Archipelago, which extends up to 5 km offshore ($9^{\circ}34'N$, $78^{\circ}58'W$) (Figure 1). The region undergoes distinct wet (May-December) and dry (January-April) seasons (Cubit et al. 1989). Sampling was conducted during the wet seasons months, characterized by light and variable winds and intense periods of rainfall which peak during October-November each year (Wilson 2001; Wilson and Meekan 2001). The maximum tidal range in San Blas is 0.6 m (Panama Canal Commission 1998, M.E.S.P. 1999). The tidal regime experienced during the wet season months around San Blas Point is complex with varying combinations of diurnal and semidiurnal tides (Wilson and Meekan 2001). Additionally, tidal heights, amplitudes and flow rates varied considerably during the study period. For greater oceanographic detail, including local current patterns, see D'Croze and Robertson (1997), Robertson et al. (1999) and Wilson (2001).

Sampling device. The light trap was chosen as the sampling device due to a number of important features. Firstly, the automation of the light trap technique lends itself to providing a synoptic picture of replenishment at a range of spatial and temporal scales of interest to the researcher

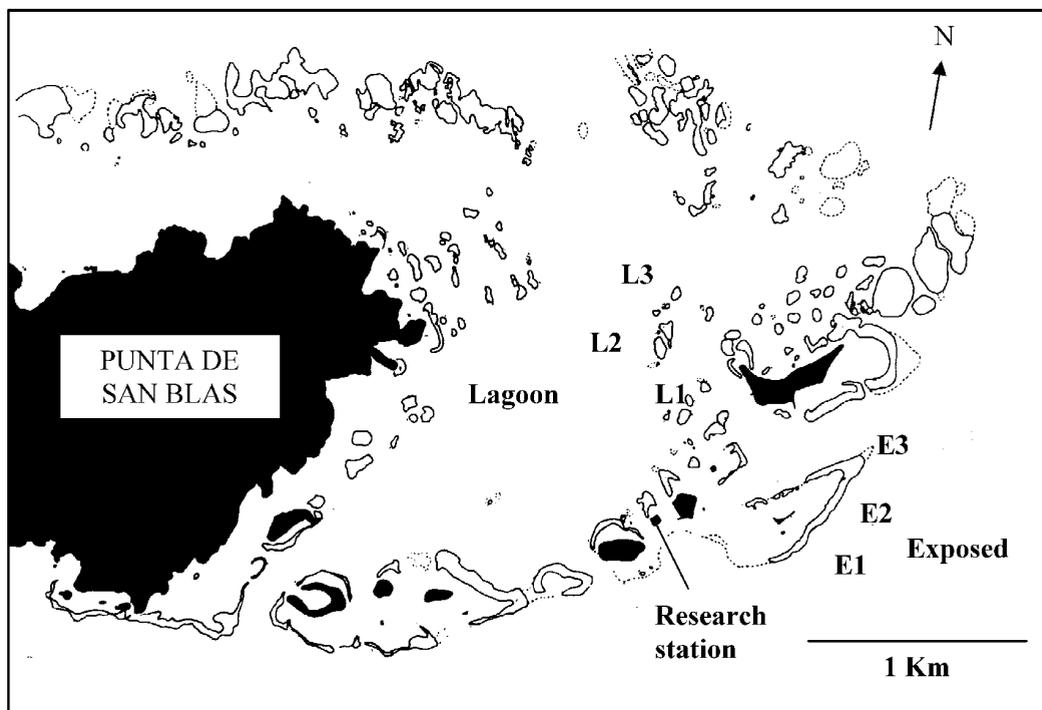


Figure 1. Location of sampling sites at Punta de San Blas located on the Caribbean coast of Panama ($9^{\circ}34'N$, $78^{\circ}58'W$). E1-E3 = exposed traps, L1-L3 = lagoon traps.

(Doherty 1987). Secondly, they can be operated in nearly all reef environments. This allows sampling to be stratified by depth (e.g. Doherty and Carleton 1997; Hendriks et al. 2001) or by habitat (Milicich and Doherty 1994; Wilson 2001; Wilson and Meekan 2001, 2002). In contrast, techniques such as crest or channel nets can only be used in a limited sampling environment, such as reef crests exposed to strong and constant wave action and current flows. Disadvantages of the light trap technique include taxonomic biases related to the degree of phototactic response by individual larvae. The ability of larvae to enter a trap once attracted to the light then depends upon the swimming ability and/or body size of the individual, as the entrance to traps is likely to be size selective (Choat et al. 1993; Brogan 1994). However, the advantages of automation and ease of replication means that this technique is well suited to the study of replenishment (see Milicich 1988; Choat et al. 1993; Wilson 2001).

The light trap design used throughout the study is described in Wilson (2001). Briefly, the trap consisted of a single plexi-glass chamber with a tube running through its centre. The chamber was open to the exterior by four horizontal slits (7 cm high by 25 cm wide), through which photopositive organisms entered the trap. The slits were tapered to a height of 1.5 cm inside the trap to inhibit escapement. An 8W DL (Day-Light) fluorescent tube was used as a light source. The light was encased within a central tube of clear plexi-glass, while the power pack was housed in a plastic case above the light. The plexi-glass chamber was protected by an aluminum frame. When the trap was removed from the water after fishing, catches accumulated in a detachable plastic collection box at the trap base. Mesh sides (500 μm) permitted water movement through the collection box to maintain the catches in good condition.

Field sampling. The effects of tidal state and time of night on catches were examined during 13 consecutive nights centred on the new moon in each of three lunar months, 30 May to 11 June 1997, 28 June to 10 July 1997 and 19 May to 31 May 1998. During the first and third of these sampling periods, three traps were deployed in the lagoon, while in the second sampling period, three traps were deployed in the exposed habitat. On each night of sampling catches were removed from traps at 2 hr intervals from 18:00 to 06:00 hrs. Traps within a habitat were moored ca 100 m apart in order to avoid any overlap in the sampling fields between traps, and at a distance of 50 to 100 m from the reef margin. Traps were anchored on the reef slope in water depths of 20 m in the exposed habitat and 12 m in the lagoon. Entrance slits were 1-1.5 m below the surface. Samples were immediately sorted in the laboratory and preserved in 75% ethanol. Most fishes could be identified to species level or type, although some very small specimens were grown out in aquaria to confirm identifications. After catches had been cleared from the final 2 hrs of sampling, the traps were rinsed and left to dry, before being randomly re-deployed to fixed moorings in the evening. Measurements of tidal height were obtained on-site at the Smithsonian's San Blas Research Station using a tidal logger (M.E.S.P. 1999).

Data analysis. Initially, catches from each of the 13 consecutive nights of sampling in three lunar cycles were concatenated giving a time series of 234 abundance measurements recorded at 2 hr intervals. Each data point (2 hrs) was the mean catch record from the three light traps operating during each 2 hr sampling period. An estimate of tidal flow was calculated as the change in tidal

Table 1. Catch composition of light traps during three lunar months of sampling (13 consecutive nights per month) in the San Blas Archipelago, Caribbean Panama. Shown as raw and percentage abundance. $n = 3,801$.

| Taxa | Raw abundance | % catch | Taxa | Raw abundance | % catch |
|-----------------------------------|---------------|-------------|----------------------------------|---------------|--------------|
| Acanthuridae | 10 | 0.26 | Labrid type 2 | 2 | 0.05 |
| <i>Acanthurus chirurgus</i> | 6 | 0.16 | <i>Thalassoma bifasciatum</i> | 2 | 0.05 |
| <i>Acanthurus coeruleus</i> | 4 | 0.11 | Labrisomidae | 72 | 1.89 |
| Apogonidae | 34 | 0.89 | <i>Labrisomus nuchipinnis</i> | 6 | 0.16 |
| <i>Apogon maculatus</i> | 6 | 0.16 | <i>Malacoctenus macropus</i> | 63 | 1.66 |
| <i>Astrapogon puncticulatus</i> | 24 | 0.63 | <i>Malacoctenus triangulatus</i> | 3 | 0.08 |
| <i>Phaeoptyx pigmentaria</i> | 4 | 0.11 | Lutjanidae | 232 | 6.10 |
| Aulostomidae | 1 | 0.03 | <i>Lutjanus apodus</i> | 130 | 3.42 |
| <i>Aulostomus maculatus</i> | 1 | 0.03 | <i>Lutjanus chrysurus</i> | 5 | 0.13 |
| Blenniidae | 326 | 8.58 | <i>Lutjanus cyanopterus/joci</i> | 4 | 0.11 |
| Blenniid type 1 | 1 | 0.03 | <i>Lutjanus mahogani</i> | 93 | 2.45 |
| <i>Hypseurochilus bermudensis</i> | 4 | 0.11 | Monacanthidae | 49 | 1.29 |
| <i>Hypsoblennius exstochilus</i> | 1 | 0.03 | <i>Aluterus scriptus</i> | 1 | 0.03 |
| <i>Ophioblennius atlanticus</i> | 308 | 8.10 | <i>Monacanthus setifer</i> | 48 | 1.26 |
| <i>Parablennius marmoratus</i> | 11 | 0.29 | Mugilidae | 2 | 0.05 |
| <i>Scartella cristata</i> | 1 | 0.03 | <i>Mugil cephalus</i> | 2 | 0.05 |
| Bothidae | 5 | 0.13 | Mullidae | 4 | 0.11 |
| <i>Bothus lunatus</i> | 4 | 0.11 | <i>Pseudupeneus maculatus</i> | 4 | 0.11 |
| <i>Bothus</i> spp. | 1 | 0.03 | Polynemidae | 32 | 0.84 |
| Bregmacerotidae | 5 | 0.13 | <i>Polydactylus virginicus</i> | 32 | 0.84 |
| <i>Bregmaceros atlanticus</i> | 5 | 0.13 | Pomacanthidae | 1 | 0.03 |
| Carangidae | 25 | 0.66 | <i>Pomacanthus arcuatus</i> | 1 | 0.03 |
| <i>Chloroscombrus chrysurus</i> | 23 | 0.61 | Pomacentridae | 2,180 | 57.35 |
| <i>Selar crumenophthalmus</i> | 2 | 0.05 | <i>Abudefduf saxatilis</i> | 5 | 0.13 |
| Chaetodontidae | 19 | 0.50 | <i>Abudefduf taurus</i> | 1 | 0.03 |
| <i>Chaetodon capistratus</i> | 18 | 0.47 | <i>Chromis insolata</i> | 2 | 0.05 |
| <i>Chaetodon ocellatus</i> | 1 | 0.03 | <i>Chromis multilineata</i> | 1 | 0.03 |
| Congridae | 9 | 0.24 | <i>Microspathodon chrysurus</i> | 17 | 0.45 |
| Congridae spp. | 9 | 0.24 | <i>Stegastes diencaeus</i> | 58 | 1.53 |
| Dactylopteridae | 1 | 0.03 | <i>Stegastes dorsopunicans</i> | 109 | 2.87 |
| <i>Dactylopterus volitans</i> | 1 | 0.03 | <i>Stegastes leucostictus</i> | 36 | 0.95 |
| Diodontidae | 10 | 0.26 | <i>Stegastes partitus</i> | 485 | 12.76 |
| <i>Chilomycterus</i> spp. | 7 | 0.18 | <i>Stegastes planifrons</i> | 1,415 | 37.23 |
| <i>Diodon histrix</i> | 3 | 0.08 | <i>Stegastes variabilis</i> | 51 | 1.34 |
| Elopidae | 79 | 2.08 | Scaridae | 1 | 0.03 |
| <i>Megalops atlanticus</i> | 79 | 2.08 | <i>Scarus iserti</i> | 1 | 0.03 |
| Gerreidae | 236 | 6.21 | Scombridae | 9 | 0.24 |
| <i>Eucinostomus melanopterus</i> | 236 | 6.21 | <i>Scomberomorus regalis</i> | 9 | 0.24 |
| Gobiidae | 7 | 0.18 | Scorpaenidae | 14 | 0.37 |
| Gobiid type 1 | 1 | 0.03 | <i>Scorpaena plumeri</i> | 14 | 0.37 |
| Gobiid type 3 | 6 | 0.16 | Serranidae | 121 | 3.18 |
| Haemulidae | 1 | 0.03 | <i>Epinephelus cruentatus</i> | 120 | 3.16 |
| Haemulidae spp. | 1 | 0.03 | Serranidae type 1 | 1 | 0.03 |
| Holocentridae | 7 | 0.18 | Sphyraenidae | 32 | 0.84 |
| <i>Holocentrus vexillarius</i> | 1 | 0.03 | <i>Sphyraena barracuda</i> | 13 | 0.34 |
| <i>Holocentrus coruscus</i> | 6 | 0.16 | <i>Sphyraena picudilla</i> | 19 | 0.50 |
| Labridae | 14 | 0.37 | Syngnathidae | 1 | 0.03 |
| <i>Halichoeres bivittatus</i> | 1 | 0.03 | <i>Cosmocampus elucens</i> | 1 | 0.03 |
| <i>Halichoeres pictus</i> | 1 | 0.03 | Synodontidae | 262 | 6.89 |
| <i>Halichoeres poeyi</i> | 8 | 0.21 | Synodontidae spp. | 262 | 6.89 |

Table 2. Cross-correlation coefficients (r) from the comparison between catches of pre-settlement reef fish larvae collected at 2 hour intervals in each of three lunar sampling periods and tidal flow components (directional flow, absolute flow and tidal height). Directional flow rate = average change in tide height each sampling period expressed as positive for flooding tides or negative for ebb tides; Absolute flow rate = average change in tide height each sampling period; Tidal height = actual tidal heights in cm. Number in brackets = lag in 2 hr intervals.

| Taxa Flow-Rate | Sampling period Flow-Rate | Directional Height | Absolute | Tidal |
|----------------|------------------------------|--------------------|-----------|------------|
| All taxa | 30 May 1997 to 11 June 1997 | -0.24 (3) | -0.26 (0) | n.s. |
| | 28 June 1997 to 10 July 1997 | n.s. | -0.24 (2) | 0.24 (0) |
| | 19 May 1998 to 31 May 1998 | -0.32 (0) | n.s. | 0.26 (0) |
| | Combined months | -0.18 (3) | n.s. | -0.18 (2) |
| Pomacentridae | 30 May 1997 to 11 June 1997 | -0.29 (0) | 0.36 (0) | 0.25 (1) |
| | 28 June 1997 to 10 July 1997 | n.s. | n.s. | n.s. |
| | 19 May 1998 to 31 May 1998 | -0.37 (0) | 0.35 (0) | 0.33 (0) |
| | Combined months | -0.27 (0) | 0.27 (0) | -0.19 (-1) |

height (in cm) during each of the 2 hrs of sampling by the traps. This was calculated both with directional components (i.e. flood tides were +ve and ebb tides were -ve flows) and as an absolute value (i.e. ignoring the +ve or -ve sign). Tidal heights every 2 hrs were also measured. Time series of catches and tidal flow (directional and absolute) and tidal heights were analyzed using time series analysis (Chatfield 1997). The presence of autocorrelation in each time series was examined using autocorrelation function (ACF) plots (Chatfield 1997) and where present, this was removed. Data sets were transformed to $\ln(x+1)$ values in order to stabilize the variance. Auto Regressive Integrated Moving Average (ARIMA) models were then fitted to the data. The number of autoregressive (AR) and moving average (MA) parameters were determined by inspecting the autocorrelogram. A model was then fitted to the series and its reliability confirmed by examining the autocorrelogram to ensure that serial dependency had been removed. In addition, the residuals from the model were plotted to examine normality. The residuals computed from the ARIMA model were then used in the subsequent cross-correlation analysis. Temporal coherence of 2 hourly replenishment and tidal patterns (directional and absolute tidal flow, and tidal height) were calculated using cross-correlation functions (CCF's). These were generated by computing Pearson correlation coefficients between two time series as one series was progressively shifted night by night across the other series (see Milicich et al. 1992; Wilson and Meekan 2001).

Nightly catches were pooled into low, flood, high and ebb tide categories for each lunar cycle. Levene's test (Zar 1996) indicated that these did not conform to the assumptions of normality required by parametric analysis, even after transformation. Consequently, Kruskal-Wallis tests were used to determine if catches varied between flood and ebb tidal states and Tukey-type multiple comparisons used where appropriate. For similar reasons, catches in each of the 2 hr sampling periods from 18:00 to 06:00 hrs were also compared using the non-parametric Kruskal-Wallis test to determine if larvae were more abundant at a particular time of the night.

Results

A total of 3,801 pre-settlement reef fishes from 32 families were collected during the three lunar cycles of sampling (Table 1). Pomacentrids were the most abundant taxa in catches comprising 57.35% of the total catch. The remaining larvae were dominated by the Blenniidae – 8.58%, Synodontidae – 6.89%, Gerreidae – 6.21%, Lutjanidae – 6.10% and the Serranidae 3.18% (Table 1). Due to the abundance of pomacentrids in catches, they were analyzed both separately, and as part of the total catch pooled among taxa. There were complex relationships between tidal flow (change in tide height 2 h^{-1}) and nightly catches for the pooled (Fig. 2) and the pomacentrid catches separately (Fig. 3). The cross-correlation of the larval abundance time series measured at 2 hr intervals and those of directional and absolute tidal flow rates and tidal height measurements for pooled and pomacentrid catches produced a number of significant correlations at short lags (Table 2).

All taxa combined. Larval catches were negatively correlated to directional flow rates in the third cycle of sampling with an r -value of -0.32 at a lag of 0 (Table 2, Fig. 2c). In the analysis, flood tide flows were assigned positive values and ebb tide flows negative values, thus a negative correlation indicates that high larval abundance occurred during ebb tides (-ve flows) (Table 2). Larval abundance was also negatively correlated to directional flow rates in the first cycle and summed time series at a lag of 3 (6 hrs) (Table 2, Fig. 2a, d). Thus, tidal flows during the ebb tides were associated with high catches and tidal flows during flood tides were associated with low catches. There were no significant correlations between flow rates and catches in the second cycle, however low catches may have affected the power of analyses to detect correlations (Fig. 2b). A negative correlation between absolute tidal flow rates and larval abundance was identified in the first and second lunar cycles of sampling at lags of 0 and 2 (4 hrs) respectively. Finally, larval abundance showed no consistent positive or negative patterns with tidal height measurements for the three sampling periods (Table 2).

Average catch per 2 hr sampling period in each of the 4 tidal phases varied across all three sampling cycles (Fig. 4). Analysis of catches during flood and ebb tides found that greater numbers of larvae arrived in nearshore waters during the ebb tidal flows each night in the second cycles of sampling (Table 3). Although there is a suggestion of peak larval arrival during the ebb tide in comparison to the flood tide for the first, third and combined cycles, the variance in catches prevented any significant differences being identified (Fig. 4). The null hypothesis of no difference in catches with tidal state (flood and ebb only) could not be rejected for these samples (Table 3). Table 4 shows a family level breakdown of total catch per 2 hrs of sampling during each of the 4 tidal states for all cycles combined. Catches appeared to peak during the low and high tidal phases for 4 and 2 families respectively (Table 4), although the sporadic appearance of larvae in traps prevented any formal analysis of these data (except for the Pomacentridae, analyzed below). In comparison, 5 families peak in abundance during the flood tides, while 7 families peaked in abundance during ebb tidal flows (Table 4).

There was a tendency for catches to increase during the night, so that the greatest abundance of larvae occurred just prior to dawn (Figs. 2, 5), however, no significant effects of time of night on catch rates were found for pooled taxa, and the null hypothesis of no difference in arrival time was unable to be rejected (Kruskal-Wallis test, Table 5).

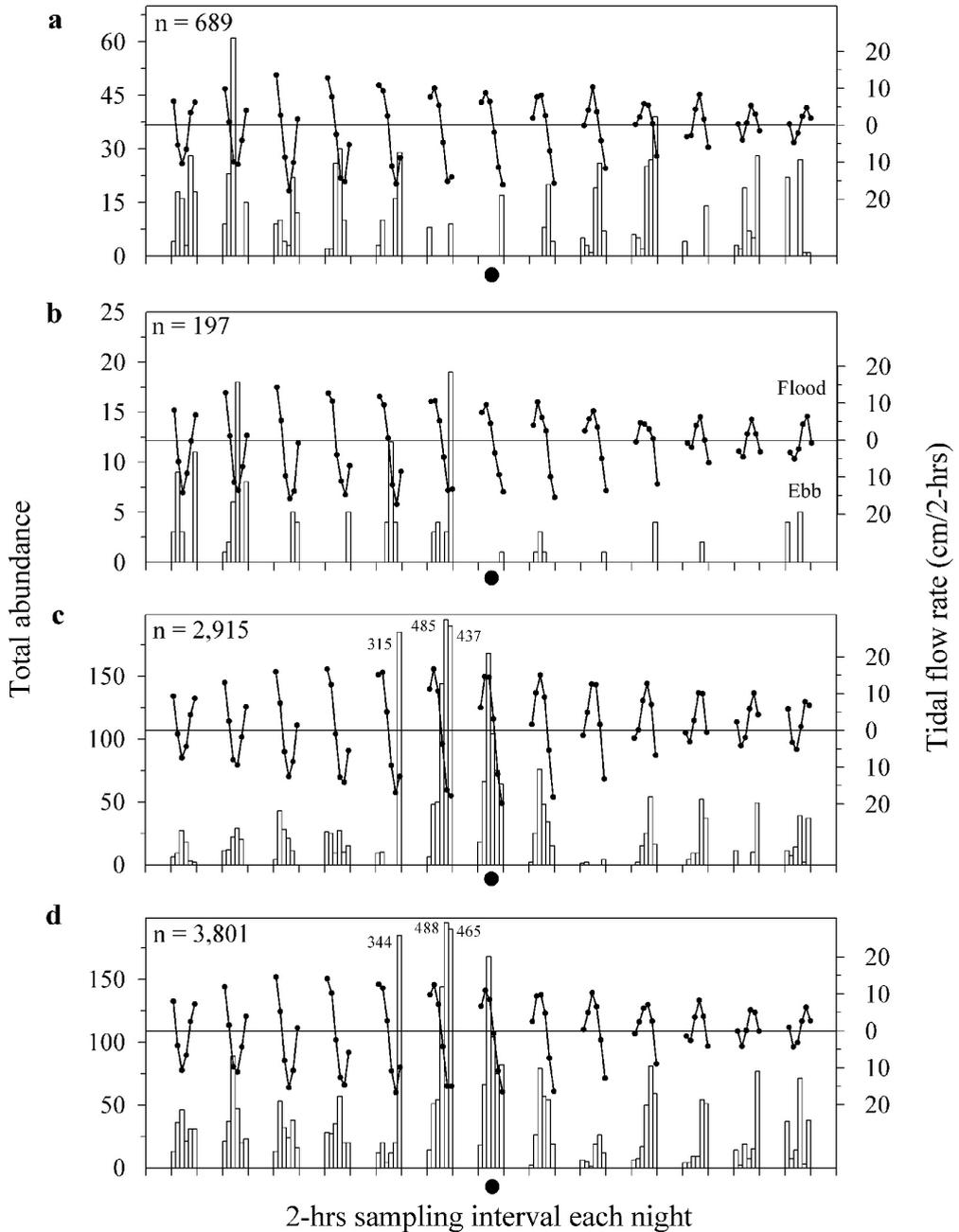


Figure 2. Comparison of total larval abundance (bars) and tidal flow rates (lines). Samples taken at 2 hr intervals from 18:00 to 06:00 hrs. (a) lagoon – 30 May to 11 June 1997, (b) exposed – 28 June to 10 July 1997, (c) lagoon – 19 May to 31 May 1998 inclusive and (d) all three sampling cycles combined. Horizontal line represents zero flow. Above line = flood tide flows, below line = ebb tide flows.

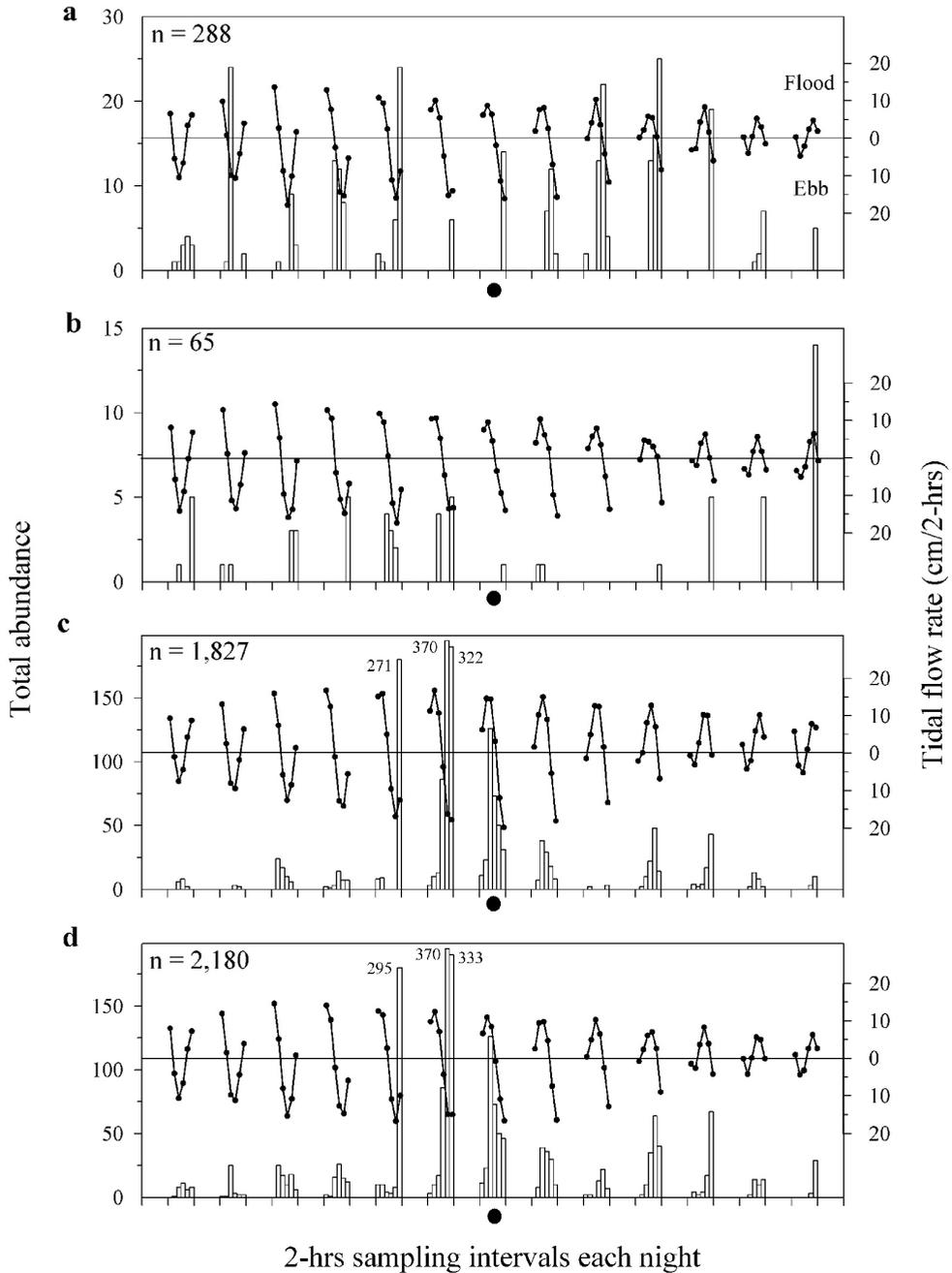


Figure 3. Comparison of larval abundance for the Pomacentridae (bars) and tidal flow rates (lines). Samples taken at 2 hr intervals from 18:00 to 06:00 hrs. (a), (b), (c) and (d) as shown in Figure 2. Horizontal line represents zero flow. Above line = flood tide flows, below line = ebb tide flows.

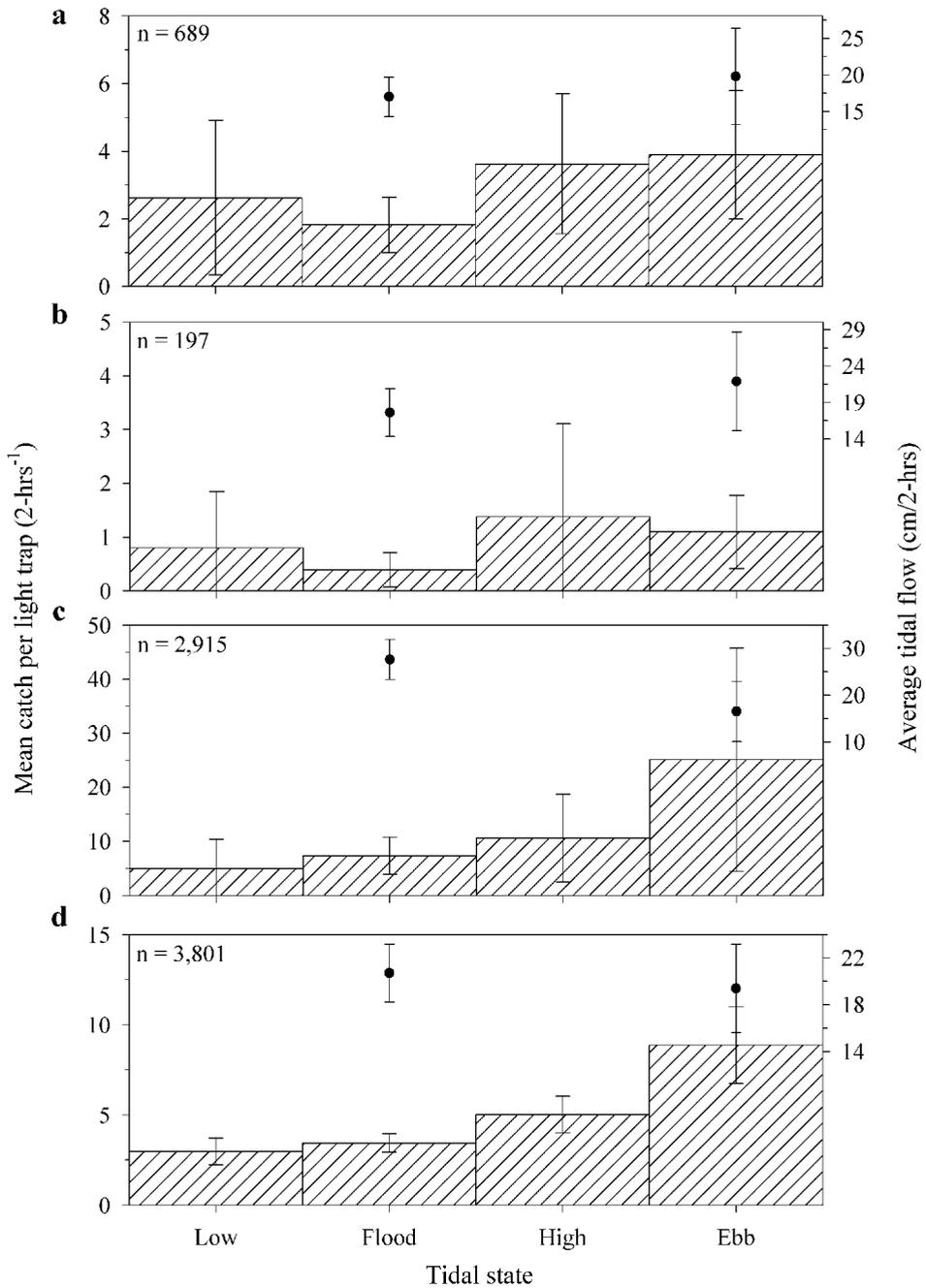


Figure 4. Mean catch of pre-settlement reef fishes (pooled) per 2 hr sampling interval for each tidal state (bars) (+/- 95% CI's) and average tidal flow for flood and ebb tides (dots) (+/- 95% CI's). (a), (b), (c) and (d) as shown in Figure 2.

Table 3. Comparison of total catches during flood and ebb tides in each of the three lunar sampling periods and the combined samples. Kruskal-Wallis H_{adj} statistics.

| Taxa | Sampling period | H_{adj} | df | p | Difference |
|---------------|------------------------------|-----------|-----------|-------|------------|
| All taxa | 30 May 1997 to 11 June 1997 | -0.24 (3) | -0.26 (0) | n.s. | |
| All taxa | 30 May 1997 to 11 June 1997 | 0.740 | 1 | 0.390 | n.s. |
| (pooled) | 28 June 1997 to 10 July 1997 | 4.762 | 1 | 0.029 | E > F |
| | 19 May 1998 to 31 May 1998 | 1.346 | 1 | 0.246 | n.s. |
| | Combined months | 1.700 | 1 | 0.192 | n.s. |
| Pomacentridae | 30 May 1997 to 11 June 1997 | 5.183 | 1 | 0.023 | E > F |
| | 28 June 1997 to 10 July 1997 | 4.016 | 1 | 0.045 | E > F |
| | 19 May 1998 to 31 May 1998 | 0.871 | 1 | 0.351 | n.s. |
| | Combined months | 4.153 | 1 | 0.042 | E > F |

Table 4. Total catch per 2 hrs of light trap sampling during each tidal state (low, flood, high, ebb). Families where at least 10 individuals were collected over a minimum of three separate nights are shown (i.e. not in a single pulse). Total number of 2 hr sampling periods = 234. Numbers in brackets represents the number of 2 hr periods sampled during each tidal state over the three months of sampling. Highest catches for each family are shown in bold.

| Taxa | Raw abundance | Low (20) | Flood (98) | High (41) | Ebb (75) |
|----------------|---------------|-------------|-------------|-------------|-------------|
| Acanthuridae | 10 | 0.05 | 0.00 | 0.12 | 0.05 |
| Apogonidae | 34 | 0.10 | 0.06 | 0.20 | 0.24 |
| Blenniidae | 326 | 0.60 | 0.73 | 1.78 | 2.25 |
| Carangidae | 25 | 0.00 | 0.09 | 0.00 | 0.21 |
| Chaetodontidae | 19 | 0.15 | 0.07 | 0.05 | 0.09 |
| Diodontidae | 10 | 0.00 | 0.06 | 0.00 | 0.05 |
| Elopidae | 79 | 0.25 | 0.47 | 0.39 | 0.16 |
| Gerreidae | 236 | 2.75 | 0.65 | 0.66 | 1.20 |
| Labridae | 14 | 0.15 | 0.05 | 0.02 | 0.07 |
| Labrisomidae | 72 | 0.35 | 0.44 | 0.20 | 0.19 |
| Lutjanidae | 232 | 0.70 | 0.45 | 0.61 | 1.99 |
| Monacanthidae | 49 | 0.25 | 0.24 | 0.24 | 0.15 |
| Polynemidae | 32 | 0.00 | 0.12 | 0.34 | 0.08 |
| Pomacentridae | 2180 | 2.10 | 4.90 | 8.39 | 17.52 |
| Scorpaenidae | 14 | 0.05 | 0.08 | 0.03 | 0.05 |
| Serranidae | 121 | 0.20 | 0.66 | 0.27 | 0.55 |
| Sphyraenidae | 32 | 0.15 | 0.06 | 0.12 | 0.24 |
| Synodontidae | 262 | 0.75 | 0.95 | 1.39 | 1.2 |

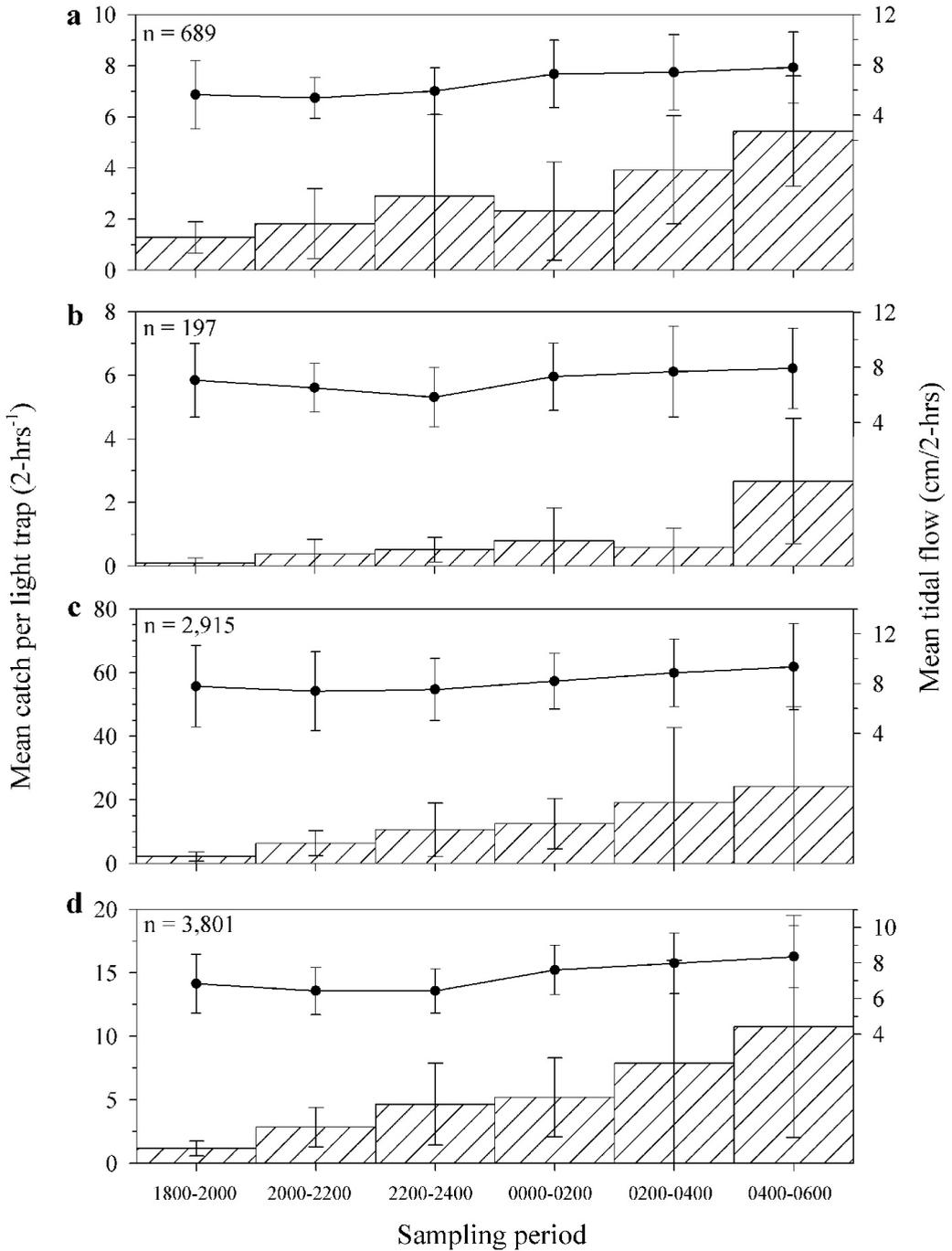


Figure 5. Mean catch of pre-settlement reef fishes (pooled) per 2 hr sampling interval (bars) (+/- 95% CI's) and average tidal flow for each sampling period (lines) (+/- 95% CI's). (a), (b), (c) and (d) as shown in Figure 2.

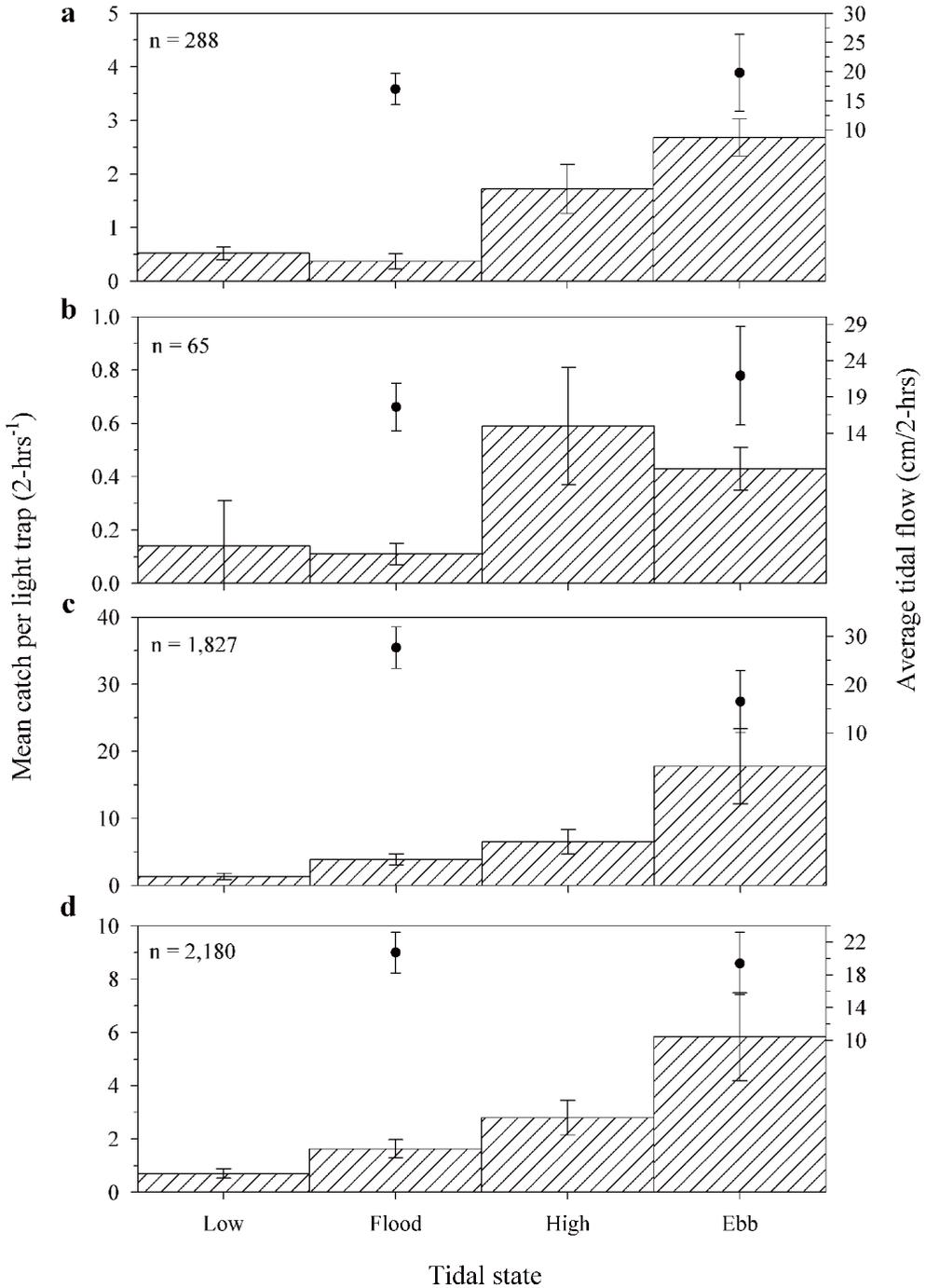


Figure 6. Mean catch of pre-settlement pomacentrids per 2-hrs sampling interval for each tidal state (bars) (+/- 95% CI's) and average tidal flow for flood and ebb tides (dots) (+/- 95% CI's). (a), (b), (c) and (d) as shown in Figure 2.

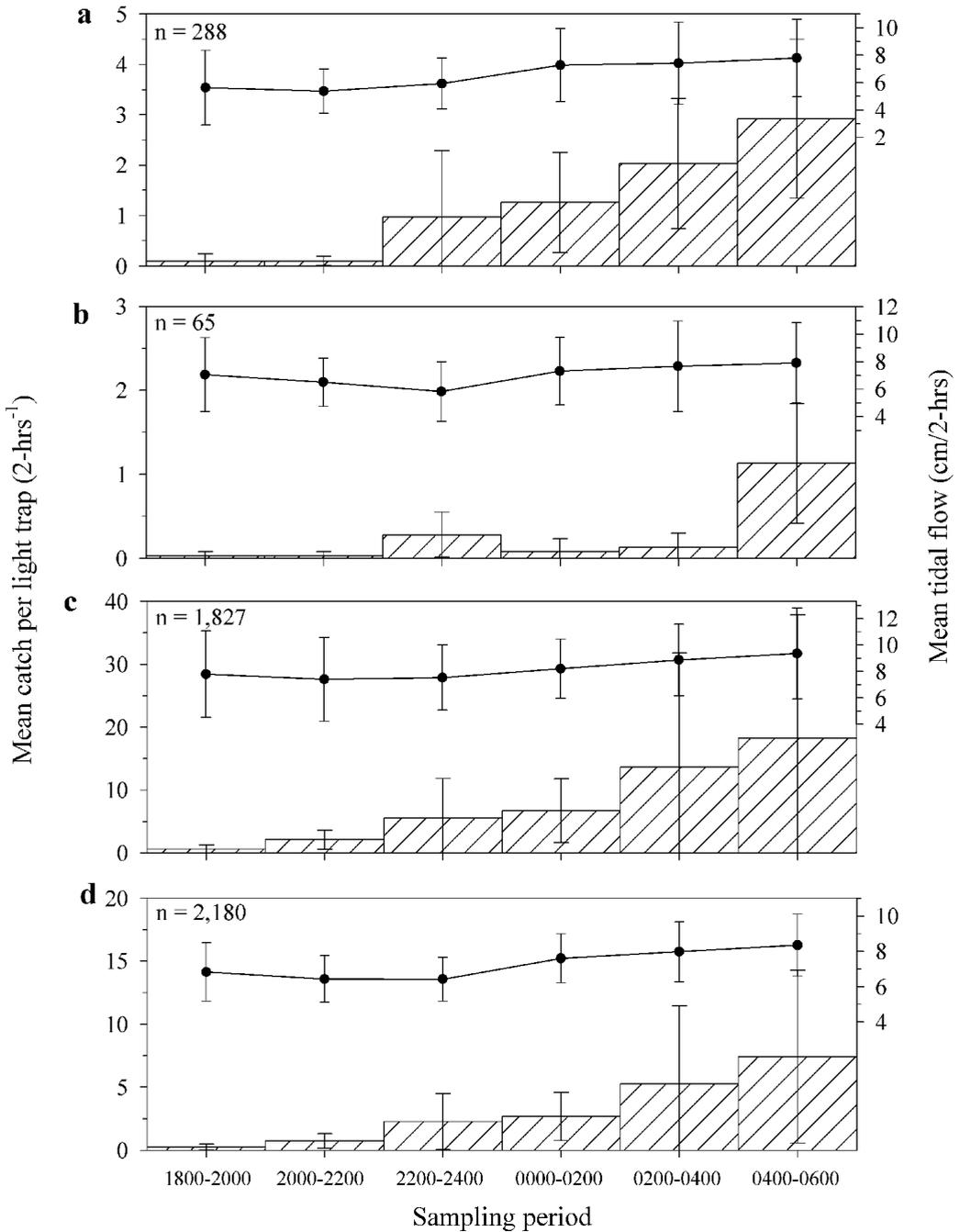


Figure 7. Mean catch of pre-settlement pomacentrids per 2-hrs sampling interval (bars) (+/- 95% CI's) and average tidal flow for each sampling period (lines) (+/- 95% CI's). (a), (b), (c), and (d) as shown in Figure 2.

Table 5. Comparison of total catches during 2 hour sampling periods (I = 18:00-20:00 hrs, II = 20:00-22:00, III = 22:00-24:00, IV = 24:00-02:00, V = 02:00-04:00, VI = 04:00-06:00 hrs) each night for the three lunar sampling periods and the combined samples. Kruskal-Wallis H_{adj} statistics.

| Taxa | Sampling period | H_{adj} | df | p | Difference |
|---------------|------------------------------|-----------|----|--------|-------------------------|
| All taxa | 30 May 1997 to 11 June 1997 | 7.103 | 5 | 0.213 | n.s. |
| | 28 June 1997 to 10 July 1997 | 6.296 | 5 | 0.278 | n.s. |
| | 19 May 1998 to 31 May 1998 | 5.600 | 5 | 0.347 | n.s. |
| | Combined months | 7.737 | 5 | 0.171 | n.s. |
| Pomacentridae | 30 May 1997 to 11 June 1997 | 25.296 | 5 | <0.001 | VI > I, II, IV |
| | 28 June 1997 to 10 July 1997 | 24.860 | 5 | <0.001 | VI > I, II, III |
| | 19 May 1998 to 31 May 1998 | 12.403 | 5 | 0.03 | - |
| | Combined months | 36.455 | 5 | <0.001 | VI > I, II, III ; V > I |

Pomacentridae. Abundance of pomacentrid larvae was negatively correlated to directional flow rates in the first, third and combined cycles of sampling (-0.27 to -0.37 at lags of 0) (Table 2), indicating that for these sampling series, highest larval abundance occurred during the ebb tide each night (Fig. 3a, c, d). There were no significant correlations between directional flow rates and catches in the second cycle, however low catches may have affected the power of analyses to detect correlations (Fig. 3b). Positive correlations between the abundance of pomacentrids and absolute tidal flow rates were identified in the first, third and combined lunar cycles of sampling at lags of 0 (Table 2). No significant correlation was identified in the second cycle of sampling (Table 2). Catches showed no consistent positive or negative patterns with tidal height measurements for the 3 sampling periods (Table 2).

Average catch per 2 hr sampling period varied in each of the four tidal phases across all three sampling cycles (Fig. 6). Analyses of larval abundance and tidal state (flood and ebb only) indicated that catches from the first, second and combined cycles were greatest during the ebb tide each night (Kruskal-Wallis test, Table 3). While there is a suggestion of peak larval arrival during the ebb tide in the third sampling cycle in comparison to the flood tide (Fig. 6), the null hypothesis of no difference in catchability with tidal state was unable to be rejected (Table 3).

There was a tendency for catches of pomacentrid larvae to increase during the night, so that the largest catches occurred just prior to dawn in all sampling months (Figs. 3 and 7). Significant effects of time of night on catch rates in all sampling cycles was found (Kruskal-Wallis test, Table 5), with the greatest abundance of larvae recorded during the sampling periods immediately prior to dawn each night (Fig. 7). However, due to the variance in 2-hourly catches in the third sampling cycle, the Tukey-type multiple comparisons were unable to identify which periods were different (Table 5).

Discussion

Tidal movement in the San Blas region is much reduced in comparison to the GBR (San Blas: mean tidal change = 40 cm, max = 60 cm; GBR: mean ca 2 m). This reduced flow combined with the swimming capabilities of pre-settlement reef fishes, may help to explain the occurrence of

several large peaks of replenishing larvae during ebbing tides in San Blas, particularly the pomacentrids, blenniids and lutjanids. Larvae may be using the weak flow rates running off the reef on the ebb tide to facilitate a behavioural response. This is consistent with the idea that late-stage reef fish larvae are highly mobile and need only make minor adjustments to their vertical or horizontal position close to reefs in response to a combination of optimal conditions for colonisation, thereby controlling their entry to the reef (Doherty and Carleton 1997; Olivar and Sebates 1997; Stobutzki and Bellwood 1998; Hendriks et al. 2001).

The few studies that have previously examined the nocturnal arrival times of fish larvae to reef habitats in association with tidal flows, have used crest nets as a sampling tool (Society Islands: Dufour 1991; 1994; Dufour et al. 1996; GBR: Doherty and McIlwain 1996; N-W Shelf, Australia: McIlwain 1997). McIlwain (1997) deployed a single crest net at Ningaloo reef for five consecutive nights from 19:00 to 01:00 hrs and showed that larval movement across the crest began soon after dusk and continued through the flood tide each night. She suggested that larvae are likely to be migrating into surface waters in the late afternoon or early evening and are transported by flood tides across the reef crest (McIlwain 1997). Similarly, Doherty and McIlwain (1996) conducted an earlier study at One Tree Reef, GBR, where three replicate crest nets sampled at various intervals from 19:00 to 01:00 hrs for five consecutive nocturnal flood tides. They detected large pulses of larvae within the first hour of the flood tide crossing the crest (Doherty and McIlwain 1996). Despite the differences in sampling times, these two studies detected a consistent pattern of larval arrival during the initial flood tide each night for most taxa. However, due to the restricted temporal sampling period each night (19:00 to 01:00 hrs) no data were available for the second half of each night.

The same pattern has also been recorded in the Bahamas, where channel nets deployed by Shenker et al. (1993) and Thorrold et al. (1994a) collected the majority of larvae on the flood tide using multiple nets cleared at each tide change during ten and eight nights respectively (see also Thorrold et al. 1994b, 1994c). They suggested that pre-settlement stage larvae used nocturnal flood tides to enter the reef complex by positioning themselves in the surface layers in front of reefs during ebb tidal run-off and allowing tidal currents to transport them over the reef. Dufour (1994) sampled all tidal states using a single crest net at Rangiroa and Moorea atolls for two and three nights respectively (16:00 to 08:00 hrs). He showed that when larval arrival times were compared to tidal water flow, no clear link could be identified, except for low water flow, which he suggested could hinder the replenishment process (Dufour 1994). In an earlier study by the same author, nocturnal arrival across the reef crest was found to be continuous throughout the night, irrespective of tidal flows (Dufour 1991).

The different results between these studies and my sampling using light traps in San Blas can largely be attributed to the sampling technique and environment in each case. Crest nets are only efficient at sampling larval replenishment during a narrow period on the flood tide, when the stationary nets filter the water column as it floods over the reef crest. As flows weaken and then reverse, the nets are no longer capable of sampling the water column. Thus, while crest nets are an invaluable tool for determining the initiation of the replenishment process each night, the cessation of larval movement across the crest should not be extrapolated to replenishment times and persistence of larvae in other habitats such as the fore-reef, lagoon or back-reef. It should be noted

however, that in areas where wave action continues to push water over the reef crest, crest nets will continue to sample water masses effectively. Light traps however, are capable of sampling replenishment during all tidal states.

In a recent study using light traps in the near-shore waters around Barbados, Caribbean, the arrival times of crab larvae were found to peak during nocturnal ebb tides (Reyns and Sponaugle 1999). The authors suggested that the use of ebb tidal flows during the replenishment process by crab megalopae, may be due to three primary factors: reduced tidal flow rates due to the small tidal movement in the Caribbean, the off-reef transport of chemical cues that may act as stimuli for replenishing larvae or by tidal eddies, possibly in combination. For larvae to move onshore during ebb tidal flows, settlement stage larvae must be able to actively swim up-current. For fish larvae, this ability is now well documented (Stobutzki and Bellwood 1994, 1997; Leis and Carson-Ewart 1997, 1999; Fisher et al. 2000). For example, Leis and Carson-Ewart (1997) measured the swimming abilities of 55 species from 15 families of reef fishes from the GBR and Society Islands and calculated an average speed of 20.6 cm s^{-1} . Similarly, Stobutzki and Bellwood (1997) measured the sustained swimming abilities of a variety of taxa in a swimming chamber, against a current flow of 13.5 cm s^{-1} (mean current speed in the region). They found that some taxa were able to maintain themselves against the current for extensive periods (e.g. Acanthuridae: mean 194.3 hrs, Pomacentridae: mean 50 hrs). Both studies used pre-settlement larvae collected in light traps immediately prior to settlement.

In conclusion, the replenishment of coral reef fishes has traditionally been thought to occur on the incoming flood tide each night, however, the timing of replenishment in this study suggests a pattern not normally associated with replenishment. This pattern may be unique to San Blas where tides are very small, however further work is required to examine if the replenishment patterns identified in San Blas are consistent at a broader spatial scale in the Caribbean.

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Fish larvae from a Caribbean estuarine system

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Key words: *ichthyoplankton, species richness, diversity, estuaries, Sian Ka'an Biosphere Reserve*

Abstract

This study provides an estimation of the species richness of fish larvae, and a recognition of the main station groups on the basis of physical and biological characteristics in an estuary of the Sian Ka'an Biosphere Reserve, Mexican Caribbean Sea, during two seasons, October 1997 and March 1998. Circular surface tows with a conical plankton net were carried out in 67 sampling stations. Water samples for phytoplankton cell counts were also taken. Location, temperature, salinity, depth and dissolved oxygen were recorded at each sampling station. Zooplankton biomass was estimated as wet weight, and fish larvae were identified to the lowest possible taxon. Physical and biological data were included in a Principal Component Analysis in order to detect similarities among sampling stations. Phytoplankton concentration (~ 400 cell mL⁻¹), fish larvae density (~ 50 ind 100 m⁻³) and zooplankton biomass (~ 30 g 100 m⁻³) were generally low. A total of 26 species were recognized. The dominant species belong to the Gobiidae (40%), Engraulidae (26.9%), Clupeidae (7.7%), Tetraodontidae (5.8%) and Atherinidae (5.2%) families. Multivariate analysis identified three groups of stations along a north-south axis, with salinity being the main factor in the formation of these groups. Species composition of each group revealed an oceanic affinity in the southern group and an estuarine origin for the other two groups. It is postulated that the low species richness observed in this tropical system is due to a high environmental variability and low primary and secondary productions.

Introduction

Estuarine systems are a unique and important part of aquatic environments as a result of high productivity, a high physical variability resulted from the transition between inland and ocean waters, and particular environmental properties determined by local conditions (Ketchum 1983; Kennish 1986). Primary productivity in these ecosystems is dominated by plants in bordering wetlands, rather than the water itself. Thus, phytoplankton concentration, although high compared with that in the open sea, is therefore relatively low (Dobson and Frid 1998). Estuaries play an important part in the development of many marine species, in spite of the low volume they represent in the hydrosphere. These systems are nursery areas for larval stages of estuarine dependent marine fish, as they provide food, refuge and protection against predation.

Fish larvae in estuarine systems can originate either from marine or freshwater environments, or from within the estuary. Seasonal changes in fish larvae abundance are more pronounced in temperate environments (Raynie and Shaw 1994) than in tropical areas (Barletta-Bergan et al. 2002), and their distribution patterns can be explained by the coupling between physical and biological processes acting over similar ranges of time and length (Sanvicente-Añorve et al. 2000). In spite of the diverse origins of fish larvae in estuarine systems, diversity of estuarine animals is relatively low as a consequence of the physiological difficulties that the animals face to tolerate a high environmental variability (Slobodkin and Sanders 1969; Costanza et al. 1993). Diversity at the seaward is usually higher than the up-estuary as a result of the progressive loss of species intolerant of low or fluctuating salinity (Barr et al. 1990; Dobson and Frid 1998).

Knowledge of the ichthyoplankton in the estuaries of the peninsula of Yucatán, Mexico, is limited. Ruiz-Nuño and Toral-Almazán (1982) studied the zooplankton fauna of Laguna Celestún, in the northern area of this peninsula, and emphasized fish larvae. Sanvicente-Añorve et al. (1999) listed fish larvae in the estuary of Campechén-La Ría, in the Sian Ka'an Biosphere Reserve, on the eastern coast of Yucatán, and Sanvicente-Añorve et al. (2002) analyzed temporal ichthyoplankton assemblages from February 1996 to August 1997. Supplementing these studies, this research provides an estimation of species richness of fish larvae in this system, as well as a recognition of the main station groups in the system on the basis of some physical (temperature, salinity, depth, dissolved oxygen) and biological (zooplankton biomass, ichthyoplankton and phytoplankton densities) variables during two seasons of the year, October 1997 and March 1998.

Materials and Methods

Study area. The estuary studied here is a narrow water body located along the eastern coast of the Yucatán Peninsula, Mexico. From north to south, four water bodies compose this estuary: Campechén, Boca Paila, San Miguel and La Ría (Figure 1). This system will be referred to as 'Campechén-La Ría'. This estuarine system lies between 19° 45' and 20° 15' N, and between 87° 25' and 87° 35' W, within the Sian Ka'an Biosphere Reserve. This area is influenced by the Yucatán current that flows into the Gulf of Mexico through the Yucatán channel, as well as by a southward coastal counter-current (Merino 1986). The peninsula is limestone (Gischler and Lomando 1999)

and is provided with a significant subterranean source of water. The eastern coast of the Yucatán peninsula is bordered by three species of mangrove trees: *Rhizophora mangle*, *Avicennia germinans* and *Laguncularia racemosa* (Trejo-Torres et al. 1993). The Campechén-La Ría system has a surface of approximately 60 km², a length of ~15 km, a significant inlet (~3 km) located south of the Ría, and another less important inlet (~100 m) called Boca Paila (Figure 1).

Sampling program. Plankton samples totaled 34 in October 1997 and 33 in March 1998. The sampling grid covered different lagoon environments including freshwater influenced areas, marine influenced areas, and semi-enclosed water bodies. Position, temperature (°C), salinity (psu), depth (cm) and dissolved oxygen (mg L⁻¹; only in October) were recorded at each sampling station. Also, water samples were taken for phytoplankton cell counts (Utermöhl technique), and phytoplankton concentration was expressed as cell mL⁻¹. Plankton samples were collected from circular surface tows during the day using paired conical plankton nets with mesh sizes of 500 and 200 µm equipped with mechanical flowmeters to measure the volume of sampled water. Samples were stored in a 4% neutralized formalin solution. All fish larvae were removed from the 500 µm samples and identified to the lowest possible taxon. Zooplankton biomass was estimated from the 200 µm samples as wet weight. These values were normalized to 100 m³ of water. Additional ichthyoplankton data was added to our results (Sanvicente-Añorve and Chiappa-Carrara 1998) in order to provide reliable information on the species richness in the system. Physical (temperature, salinity, dissolved oxygen and depth) and biological (zooplankton biomass, ichthyoplankton and phytoplankton densities) variables were included in a Principal Component Analysis (PCA) to determine similarities among sampling stations and the main relationships between both types of variables, during March and October. Input data matrix crossing stations and variables were transformed into a correlation matrix, from which eigenvalues and eigenvectors were extracted. The eigenvectors yield the principal axes, and the eigenvalues the length of these axes, *i.e.* a part of the total variance of the data set (Legendre and Legendre 1979). The elements of an eigenvector are also the weights, or *loadings* of the original descriptors, in the linear combination of these descriptors from which the principal components are computed. These give the *coordinates* (*COO*) of the objects in a new system constituted by the principal axes. The *correlation* (*COR*) between descriptors and the axes is given by the angle between descriptors-axes, and *contribution* (*CTR*) of each descriptor is proportional to its mass:

$$COR = \left(\frac{COO}{RHO} \right)^2 ; \quad CTR = m \left(\frac{COO}{\lambda} \right)^2$$

where,

COO = coordinate of an element in the axis,

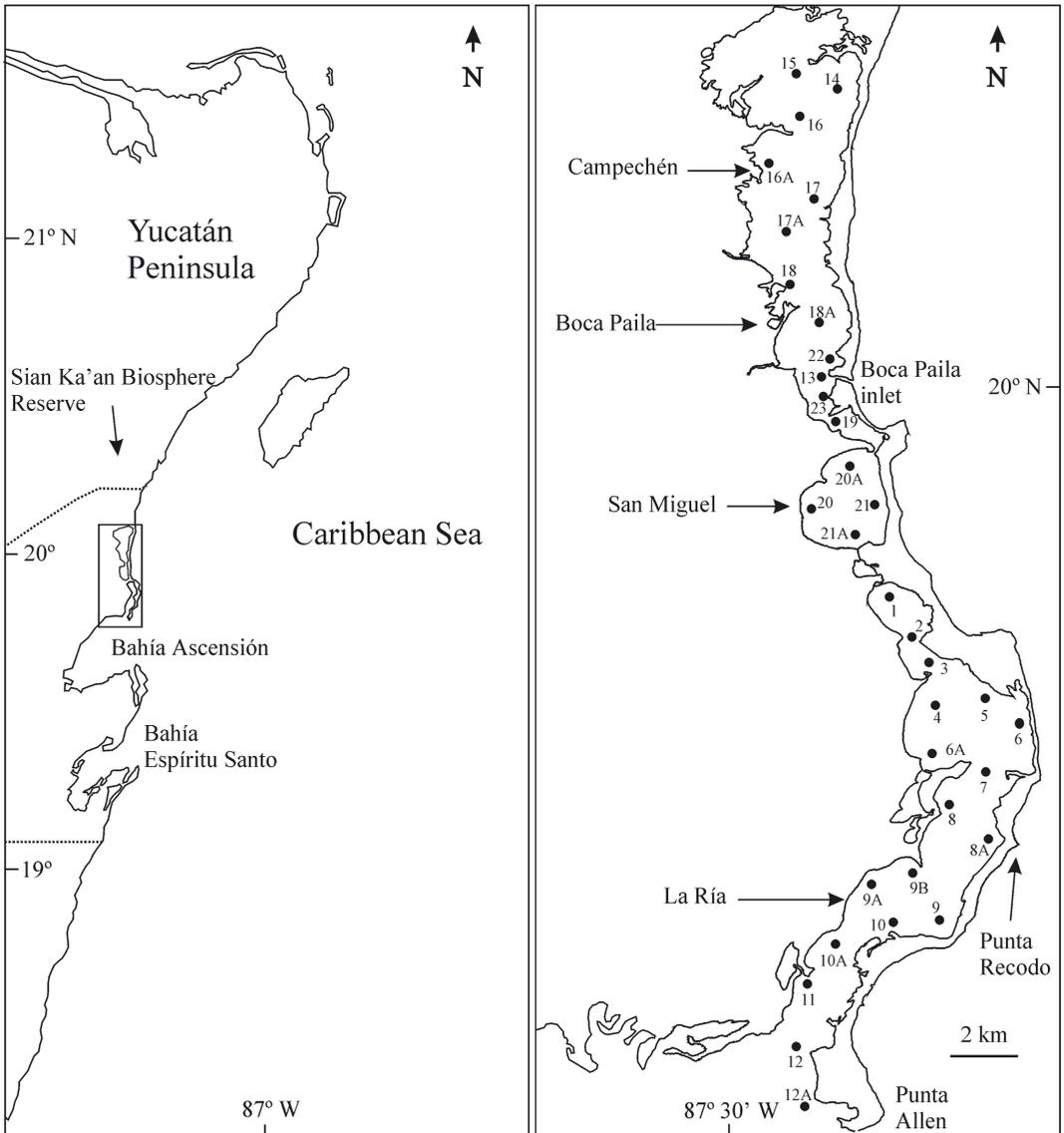
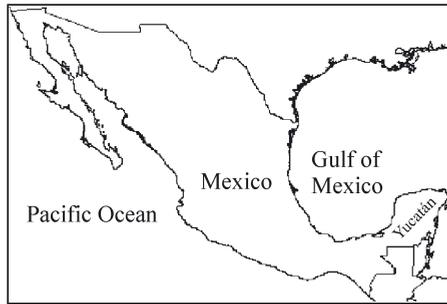
RHO = distance from an element to the gravity center of the factorial plane,

m = mass of the element, and

λ = eigenvalue.

The ADDAD software was used for this purpose. The PCA procedure has also been used by Blanc et al. (2001) and D’Elbee (1995) to analyze environmental variables and zooplankton distribution.

Figure 1.
Location of the
study area.



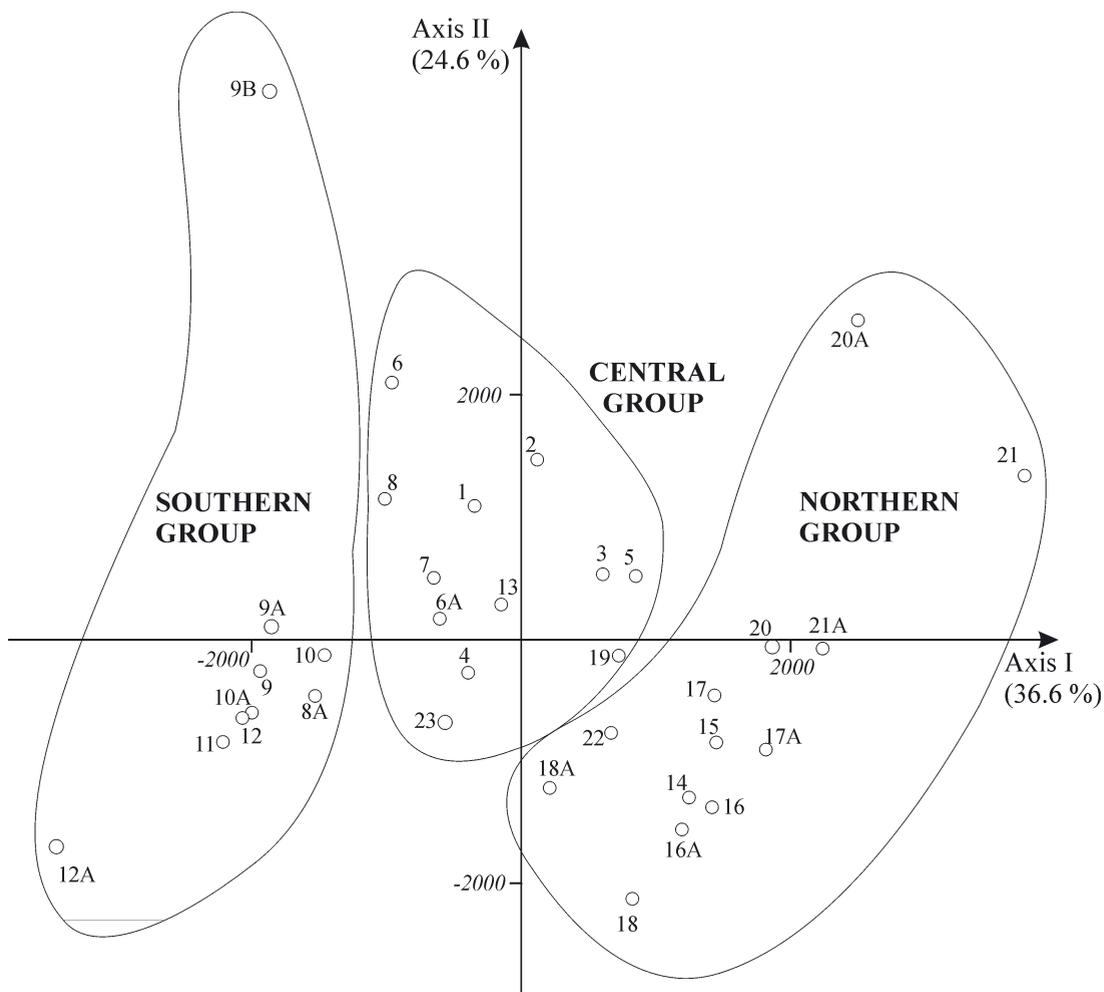


Figure 2. Representation of the sampling stations in the I-II axes of the PCA, October 1997.

Results

The study area is a shallow lagoon, with an average depth of 130 cm. We recorded temperatures of 25.8 to 30.2 °C in October, and of 24.5 to 28.7 °C in March. There was a marked gradient in salinity from south (32.9 psu) to north (6.3 psu) in October, while in March salinities varied from 34.3 psu in the southern region to 16.4 psu in the northern one. Dissolved oxygen, recorded only in October, was lower in areas with marine influence (6.7 to 8.7 mg L⁻¹), and higher in San Miguel and Campechén (10.9 to 12.9 mg L⁻¹).

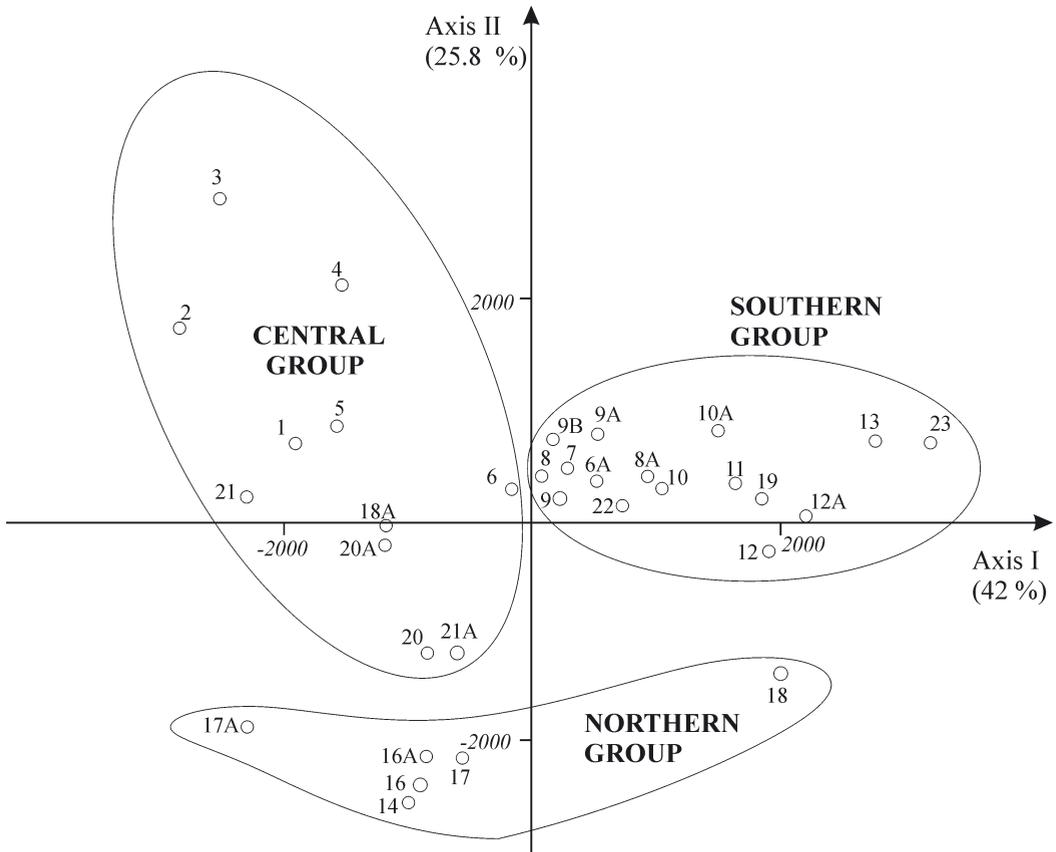


Figure 3. Representation of the sampling stations in the I-II axes of the PCA, March 1998.

Zooplankton biomass, ichthyoplankton density and phytoplankton concentration values were generally low. Most values of phytoplankton cell counts ranged from 28 to 627 cell mL⁻¹ in October, and from 103 to 744 cell mL⁻¹ in March. The highest values were observed in northern Campechén and San Miguel, and the lowest in La Ría and around Boca Paila inlet. Zooplankton biomass was higher in the central part of the system (~27 g 100 m⁻³ in October, >40 g 100 m⁻³ in March), and lower in Campechén (7-9 g 100 m⁻³). Larval densities did not exhibit a well defined spatial pattern and values varied from 0 to 227 ind 100 m⁻³ in October. In March, the highest ichthyoplankton densities (>100 ind 100 m⁻³) were registered in the middle of the study area.

A total of 4,460 fish larvae were collected during the two seasons, and a total of 26 species were registered for 14 families. The presence of at least 35 species included in 26 genera and 20 families (Appendix) can be estimated considering fish larvae information from four previous samplings in the surveyed area (Sanvicente-Añorve and Chiappa-Carrara 1998), collected from 189 paired sampling stations and 14,110 specimens. Only the anchovies *Anchoa mitchilli* and *Anchoviella elongata* represent new records in this estuarine system. The dominant families were Gobiidae (40%),

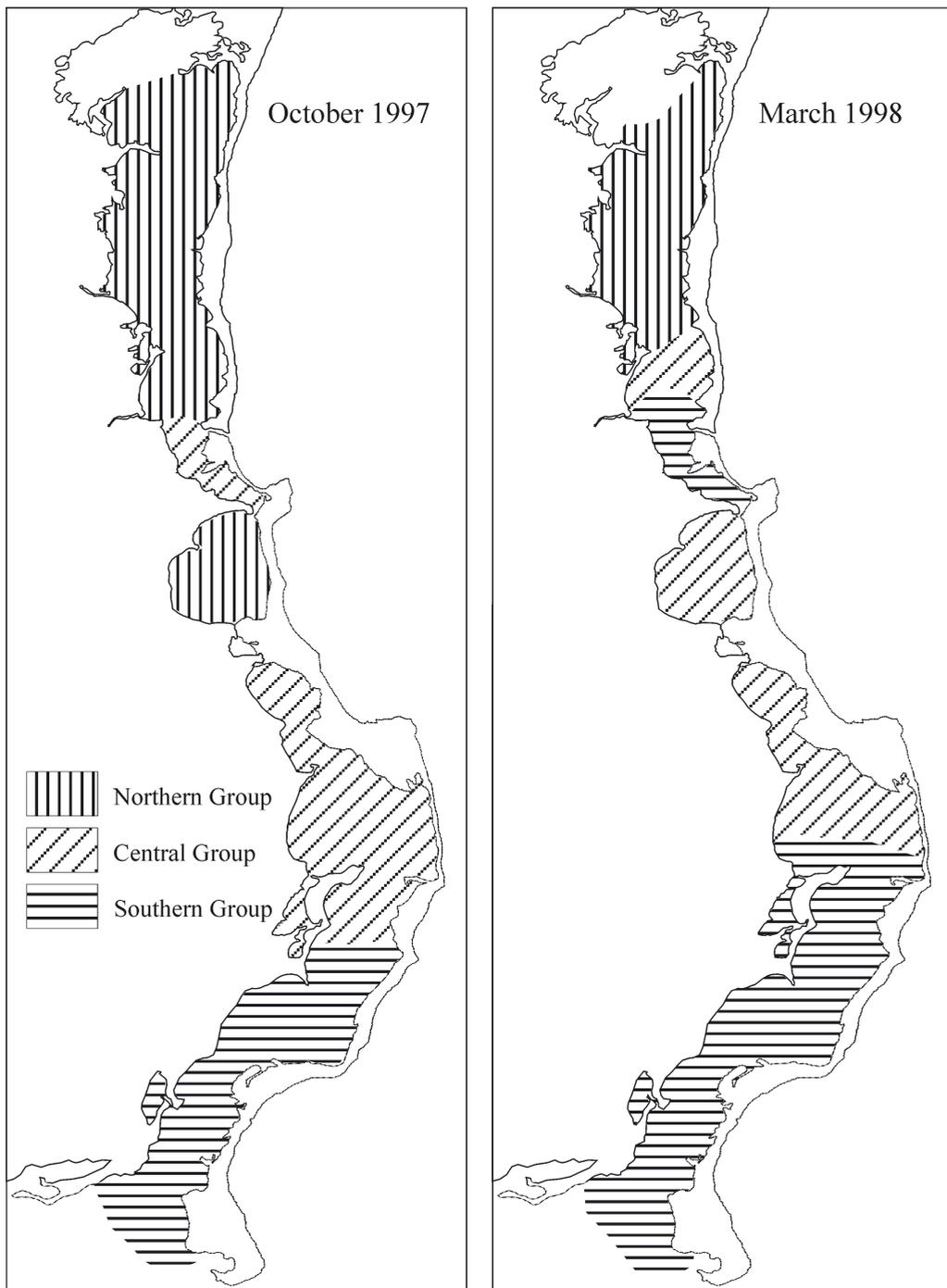


Figure 4. Geographical location of the three groups recognized on the basis of their physico-biological characteristics in the Campechén-La Ría estuary of the Sian Ka'an Biosphere Reserve.

Table 1. Variables contributing to the formation of the main axes of the PCA. COO = coordinate in the axis; COR = correlation between descriptor and axis; CTR = contribution of the descriptor to the axis, * highest contributions.

| | Axis I | | | Axis II | | | Axis III | | |
|---------------------|--------|-----|------|---------|-----|------|----------|-----|------|
| | COO | COR | CTR | COO | COR | CTR | COO | COR | CTR |
| OCTOBER 1997 | | | | | | | | | |
| Temperature | -37 | 1 | 1 | 574 | 329 | 191 | -708 | 501 | *475 |
| Salinity | -880 | 774 | *302 | 73 | 5 | 3 | 153 | 23 | 22 |
| Depth | -521 | 271 | 106 | -358 | 128 | 74 | 386 | 149 | 141 |
| Oxygen | 882 | 778 | *304 | -142 | 20 | 12 | 272 | 74 | 70 |
| Zooplankton | -158 | 25 | 10 | 841 | 708 | *411 | 253 | 64 | 61 |
| Ichthyoplankton | 265 | 70 | 27 | 728 | 530 | *308 | 492 | 242 | 229 |
| Phytoplankton | 801 | 642 | *250 | -44 | 2 | 1 | -26 | 1 | 1 |
| MARCH 1998 | | | | | | | | | |
| Temperature | -79 | 6 | 2 | 857 | 734 | *474 | 69 | 5 | 7 |
| Salinity | 750 | 563 | *224 | 488 | 238 | 154 | 288 | 83 | 120 |
| Depth | 680 | 462 | 184 | 195 | 38 | 25 | -661 | 436 | *630 |
| Zooplankton | -568 | 323 | 128 | 616 | 380 | *245 | -287 | 83 | 119 |
| Ichthyoplankton | -739 | 547 | *217 | 337 | 114 | 73 | 159 | 25 | 37 |
| Phytoplankton | -785 | 617 | *245 | -214 | 46 | 30 | -246 | 60 | 87 |

Table 2. Mean values \pm standard deviation of physico-biological variables registered in the three groups defined by the PCA in the Campechén-La Ría estuary of the Sian Ka'an Biosphere Reserve.

| Group | Zooplankton g 100 m ⁻³ | Ichthyoplankton ind 100 m ⁻³ | Phytoplankton cell mL ⁻¹ | Temperature °C | Salinity psu | Depth cm | Oxygen mg L ⁻¹ |
|---------------------|--------------------------------------|--|--|-------------------|-----------------|------------------|------------------------------|
| OCTOBER 1997 | | | | | | | |
| Northern | 9.4 \pm 14.2 | 61.0 \pm 74.4 | 394.6 \pm 178.8 | 27.6 \pm 1.1 | 9.2 \pm 2.4 | 126.2 \pm 37.5 | 11.1 \pm 1.2 |
| Central | 26.7 \pm 20.2 | 39.2 \pm 42.8 | 257.1 \pm 167.5 | 29.2 \pm 0.6 | 16.0 \pm 3.2 | 126.4 \pm 20.9 | 8.7 \pm 0.8 |
| Southern | 23.1 \pm 44.2 | 57.2 \pm 64.3 | 84.7 \pm 42.4 | 27.9 \pm 0.5 | 27.5 \pm 4.0 | 163.2 \pm 31.6 | 8.0 \pm 0.6 |
| MARCH 1998 | | | | | | | |
| Northern | 7.6 \pm 7.9 | 30.8 \pm 27.4 | 599.5 \pm 272.1 | 25.5 \pm 0.5 | 17.1 \pm 0.8 | 17.1 \pm 0.8 | — |
| Central | 33.5 \pm 25.9 | 101.6 \pm 68.4 | 574.3 \pm 158.1 | 27.5 \pm 1.0 | 22.0 \pm 1.0 | 22.0 \pm 1.0 | — |
| Southern | 14.3 \pm 12.6 | 26.8 \pm 52.2 | 300.3 \pm 204.9 | 27.1 \pm 0.6 | 29.9 \pm 3.5 | 29.9 \pm 3.5 | — |

Engraulidae (26.9%), Clupeidae (7.7%), Tetraodontidae (5.8%) and Atherinidae (5.2%) families. *Microgobius* spp was the most abundant (26.9%) and frequent taxon occurring in the estuary.

Three main stations groups were defined from the PCA analysis: Northern, Central and Southern (Figures 2 to 4; Table 1), but the spatial boundaries varied in accordance with time of year.

During October, the first two factorial axes explained 61% of the total variability (Figure 2). In the first component, phytoplankton concentration and dissolved oxygen provided the most impor-

Table 3. Fish larvae species collected in the Campechén-La Ría estuary of the Sian Ka'an Biosphere Reserve during October 1997 and March 1998 (mean abundance X , ind 100 m^{-3} , frequency of occurrence F , %).

| | OCTOBER 1997 | | | | | | MARCH 1998 | | | | | |
|-----------------------------------|--------------|------|---------|------|----------|------|------------|------|---------|------|----------|------|
| | Northern | | Central | | Southern | | Northern | | Central | | Southern | |
| | X | F | X | F | X | F | X | F | X | F | X | F |
| <i>Anchoa hepsetus</i> | 0.1 | 7.7 | 0.2 | 8.3 | | | 4.7 | 50.0 | 18.8 | 72.7 | 0.1 | 6.3 |
| <i>Anchoa mitchilli</i> | 0.4 | 23.1 | | | | | 1.2 | 50.0 | 31.7 | 72.7 | 0.2 | 6.3 |
| <i>Anchoviella elongata</i> | 0.1 | 7.7 | | | | | 0.8 | 33.3 | 9.1 | 63.6 | 0.1 | 6.3 |
| <i>Bathygobius strumosus</i> | | | | | | | | | | | 0.1 | 6.3 |
| <i>Brevoortia</i> sp. | | | | | | | | | | | 0.3 | 12.5 |
| Clupeidae not determined | | | | | | | | | 3.7 | 27.3 | 0.2 | 12.5 |
| <i>Diplogrammus pauciradiatus</i> | | | 0.6 | 25.0 | 1.5 | 55.6 | | | 0.1 | 9.1 | 1.8 | 37.5 |
| <i>Dormitator maculatus</i> | <0.1 | 7.7 | 0.3 | 8.3 | | | | | | | | |
| Engraulidae not determined | | | | | | | | 6.3 | 9.1 | | | |
| <i>Eucinostomus</i> sp. | 0.1 | 15.4 | 0.1 | 8.3 | 0.2 | 11.1 | | | | | 0.1 | 6.3 |
| Gerreidae Type I | | | 0.1 | 8.3 | | | 1.3 | 33.3 | | | | |
| Gerreidae Type II | | | | | | | 0.9 | 50.0 | | | | |
| <i>Gobiesox strumosus</i> | 1.9 | 30.8 | 0.8 | 33.3 | 2.1 | 66.7 | 0.1 | 16.7 | 0.8 | 18.2 | 0.6 | 18.8 |
| <i>Gobionellus boleosoma</i> | | | | | | | | | | | <0.1 | 6.3 |
| <i>Gobionellus</i> sp. | 0.1 | 7.7 | 0.2 | 16.7 | 0.7 | 33.3 | 0.2 | 33.3 | 4.1 | 54.5 | 1.3 | 37.5 |
| <i>Gobiosoma</i> sp. | | | 0.4 | 8.3 | 7.3 | 44.4 | 3.2 | 16.7 | 13.7 | 54.5 | 1.3 | 18.8 |
| <i>Hyporhamphus unifasciatus</i> | 0.3 | 23.1 | | | 0.1 | 11.1 | 0.4 | 16.7 | 1.7 | 45.5 | 1.6 | 43.8 |
| <i>Jenkinsia lamprotaenia</i> | | | 3.6 | 50.0 | 20.5 | 88.9 | | | 1.0 | 9.1 | | |
| <i>Lupinoblennius nicholsi</i> | 3.6 | 61.5 | 3.6 | 75.0 | 0.7 | 44.4 | 2.7 | 83.3 | 2.6 | 81.8 | 0.8 | 37.5 |
| <i>Membras martinica</i> | 0.1 | 15.4 | 2.7 | 50.0 | 6.6 | 77.8 | 4.3 | 83.3 | 0.3 | 18.2 | 3.1 | 81.3 |
| <i>Microgobius</i> spp. | 54.9 | 84.6 | 10.5 | 75.0 | 4.6 | 66.7 | 8.9 | 83.3 | 3.2 | 36.4 | 0.7 | 31.3 |
| <i>Oligoplites saurus</i> | | | 0.2 | 8.3 | 9.9 | 22.2 | | | 0.3 | 18.2 | | |
| <i>Opisthonema oglinum</i> | | | | | | | | | 3.5 | 27.3 | | |
| <i>Strongylura marina</i> | | | 0.1 | 8.3 | | | | | | | 0.1 | 12.5 |
| <i>Syngnathus scovelli</i> | 0.2 | 23.1 | 0.4 | 25.0 | 0.5 | 33.3 | 0.7 | 66.7 | 1.3 | 45.5 | 0.7 | 50.0 |
| Tetraodontidae Type I | 2.8 | 38.5 | 12.3 | 58.3 | 0.4 | 33.3 | | | 0.4 | | | 18.2 |

tant contributions in the positive part, and salinity in the negative part (Table 1). This indicates a south-north gradient for salinity, and an inverse gradient for oxygen and phytoplankton. Zooplankton biomass and ichthyoplankton density were positively correlated to the second component (Table 1). During this month, the Northern Group comprised Campechén and San Miguel, where the highest values of dissolved oxygen ($\bar{X} = 11\text{ mg L}^{-1}$) and phytoplankton density ($\bar{X} = 395\text{ cell mL}^{-1}$), and the lowest of salinity ($\bar{X} = 9.2\text{ psu}$) and zooplankton biomass ($\bar{X} = 9.4\text{ g }100\text{ m}^{-3}$) were recorded. The highest temperatures ($\bar{X} = 29\text{ }^{\circ}\text{C}$) and the lowest ichthyoplankton densities ($\bar{X} = 39\text{ ind }100\text{ m}^{-3}$) were observed in the Central Group, located in shallow areas ($\bar{X} = 126\text{ cm}$) of La Ría and near Boca

Paila inlet. The Southern Group was located in the most meridional area of the system, until Punta Recodo (Figure 4). As this group is influenced by the Caribbean Sea, the highest salinity ($\bar{X} = 28$ psu) and depth ($\bar{X} = 163$ cm) values were recorded here; the dissolved oxygen ($\bar{X} = 8$ mg L⁻¹) and cell counts ($\bar{X} = 85$ cell mL⁻¹) values were the lowest among all the system (Table 2).

During March, the first two axes represented 68% of the total variability. Axis I (42%) separated the Central and Southern groups (Figure 3). The variables that were significant were salinity in the positive part, and the ichthyoplankton density and phytoplankton concentration in the negative part (Table 1). The second component was positively associated with temperature and zooplankton values (Table 1), and the Northern Group was recognized in the negative part of this component (Figure 3). This group occupied a more restricted area than that observed in the previous season (Figure 4), and low salinity ($\bar{X} = 17$ psu) and zooplankton biomass ($\bar{X} = 8$ g 100 m⁻³) values were observed. The Central Group, located mainly in San Miguel and northern La Ría recorded the highest zooplankton biomass ($\bar{X} = 34$ g 100 m⁻³) and ichthyoplankton density values ($\bar{X} = 102$ ind 100 m⁻³). The Southern Group reached three km north of Punta Recodo and had the highest salinities ($\bar{X} = 30$ psu) and the lowest phytoplankton concentration ($\bar{X} = 300$ cell mL⁻¹) in the system, as we observed in the previous season (Table 2).

Relative to species composition of each group (Table 3), it was observed that several species that inhabit the Southern Group, including the herrings *Jenkinsia lamprotaenia*, *Brevoortia* sp, the carangid *Oligoplites saurus*, and *Diplogrammus pauciradiatus*, have a coastal pelagic origin. No exclusive taxa were observed in the Northern and Central groups. The most frequent and abundant taxa in these groups included *Microgobius* spp and *Anchoa mitchilli*. Some species, such as *Membras martinica*, *Hyporhamphus unifasciatus*, *Syngnathus scovelli* and *Lupinoblennius nicholsi* had a wide distribution occurring in salinities of 6.3 to 32.9 psu.

Discussion

Considering previous records together with the results of this study, the presence of at least 35 species can be estimated in this system. These results correspond to 14,110 specimens collected during six sampling periods and 189 paired sampling stations. Only the anchovies *Anchoa mitchilli* and *Anchoviella elongata* represent new records in this estuarine system. Various authors have documented a pattern of decreasing diversity with increasing latitude, in both the benthic (McClatchie et al. 1997; Culver and Buzas 2000) and the pelagic environments (Angel 1993; McGowan and Walker 1993). However, the number of species recorded here seems to disagree with these observations. One of the hypotheses that explain diversity patterns states that low productivity is associated with low diversity (Rosenzweig and Abramsky 1993; Kassen et al. 2000). We believe that the low species richness found here may be partly due to the low phytoplankton production in the area (Table 2). Phytoplankton cell counts registered here are slightly higher than those recorded (33 to 133,000 cell L⁻¹) in some marine areas of the Caribbean Sea (Bhattathiri et al. 1991).

In turn, the low phytoplankton production may respond to the karstic nature of the system (Gischler and Lomando 1999) that limits primary productivity and, in consequence, secondary productivity. In accordance with Wetzel (1981), extremely karstic water bodies represent a type of

maintained oligotrophy, as the conditions generated by the carbonates have an inhibitory action on the flora.

In Bahía Ascensión, the marine area adjacent to this system (Figure 1), Vázquez-Yeomans and Richards (1999) found 57 families, 82 genera and 74 species among 10,198 larvae collected from 269 plankton tows. This indicates that, in spite of the oligotrophic Caribbean Sea, species richness of fish larvae in adjacent marine waters is higher than that recorded in the lagoon. In another study, Vázquez-Yeomans (2000) observed the highest species richness in August, during the rainy season. We believe that a high water exchange rate in this bay could be the reason for the high species richness. Of the 74 species found in Bahía Ascensión, only 16 have been previously recorded in the studied area, particularly to the south of La Ría. Stress originated by the drastic environmental changes in estuaries might be the cause of low species richness. Several authors (Slobodkin and Sanders 1969; Costanza et al. 1993) have discussed that low diversity of estuarine organisms is the result of physiological difficulties in dealing with high-amplitude stress.

The low species richness recorded here might be comparable to that found in high latitude estuaries. Locke and Courtenay (1995) sampled 35 stations from May to September in Miramichi Estuary, Canada (~48 °N) and identified 20 taxa (three identified to genus, and 17 to species, belonging to 14 families). At lower latitudes, Tzeng et al. (2002) analyzed 34 samples and reported 49 families and 94 species for an estuary to the north of Taiwan (~25°N), and Barletta-Bergan et al. (2002) found 63 taxa represented by 28 families in the Caeté Estuary, Brazil, near the Equatorial line (1° S). In spite of differences in sampling effort and difficulties in the identification of fish larvae, these observations indicate that the fish larvae richness found in the Campechén-La Ría system is lower than other records for tropical and subtropical estuaries. These results suggest that the latitudinal gradient in fish larvae diversity in estuaries may be modified by local environmental conditions.

The environmental conditions in this lagoon are highly variable, especially salinity. Three different groups were identified along the vertical axis of the lagoon (15 km) based on the physical and biological characteristics of the system (Figures 2 to 4), with salinity being the main factor in the formation of station groups (Table 1). According to Flores-Coto (1988), composition, abundance and distribution patterns of fish larvae in shallow coastal lagoons are affected by the exchange between oceanic and lagoon waters. Tzeng et al. (1997) argued that highly variable salinities may influence species richness and lead to the dominance of ichthyoplankton communities by a few species only.

The Northern Group was characterized by the highest phytoplankton concentration and oxygen values and the lowest zooplankton biomass records; whereas the Southern Group, with its higher salinities and lower phytoplankton cell count values reflects a major influence of the oligotrophic (Bhattathiri et al. 1991) Caribbean Sea. The Central Group registered the highest zooplankton biomass values during both seasons (Table 2). It is probable that the semi-enclosed characteristic of the basin in this area limits the immigration of planktivore species and determines low predation on the plankton. Nevertheless, for ichthyoplankton density, the Central Group registered the highest values in March and the lowest in October. In San Miguel lagoon, Gómez-Aguirre et al. (1998) observed an inverse relationship between some fish larvae species and dinoflagellates.

Species composition in the three defined groups revealed that the Central and Northern groups

are composed mainly of estuarine resident species like *Microgobius* spp *Anchoa mitchilli* and *A. hepsetus*, while the Southern Group is composed of oceanic species such as *Jenkinsia lamprotaenia*, *Brevoortia* sp, *Oligoplites saurus* and *Diplogrammus pauciradiatus* (Table 3; Davis 1966; Walls 1975; Jones 1978; Flores-Coto 1988; Raynie and Shaw 1994). In another study, biological information (species composition and abundance) treated alone with a Correspondence Analysis revealed two main ichthyoplankton communities, one oceanic and one estuarine, during an annual cycle (Sanvicente-Añorve et al. 2002). Considering the physical and biological information together, a Central Group characterized by intermediate salinity values, high temperatures, and a relatively high zooplankton biomass, was also evident (Figures 2 to 4, Table 2). In Laguna Celestún, in the northern area of the peninsula of Yucatán, Herrera-Silveira (1994) used a multivariate approach and also found three different areas: an area near the sea with high salinity and a low concentration of nutrients, an inner area characterized by low salinity, and a middle transition area.

In conclusion, it can be established that the lagoon system studied here presents oligotrophic conditions, a zonation determined by the marine and freshwater inputs, and an ichthyoplankton fauna that includes both oceanic and estuarine species. Notwithstanding the relatively high diversity of fish from the adjacent oceanic waters, this system has low species richness. These observations suggest that the latitudinal gradient in the diversity within estuaries may be modified by local environmental conditions, such as the low primary and secondary productivities and the high variability in the physical environment that characterize this type of ecosystem. This study supports theories including the relationship between diversity, habitat productivity and environmental variability.

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Appendix

List of species recognized in the Campechén-La Ría estuary in the Sian Ka'an Biosphere Reserve during six sampling periods (October 1996 to March 1998).

Elopidae

Megalops atlanticus Valenciennes, 1946

Clupeidae

Opisthonema oglinum (LeSueur, 1818)

Harengula jaguana (Goode & Bean, 1879)

Jenkinsia lamprotaenia (Gosse, 1851)

Brevoortia Gill, 1861

Engraulidae

Anchoa hepsetus (Linnaeus, 1758)

Anchoa mitchilli (Meek & Hildebrand, 1923)

Anchoviella elongata (Valenciennes, 1848)

Atherinidae

Membras martinica (Cuvier & Valenciennes, 1835)

Hemirhamphidae

Hyporhamphus unifasciatus (Ranzani, 1842)

Syngnathidae

Syngnathus scovelli (Evermann & Kendall, 1895)

Hypocampus erectus Perry, 1810

Carangidae

Oligoplites saurus Bloch & Schneider, 1801

Gerreidae

Eucinostomus Baird & Girard, 1855

Type I

Type II

Blenniidae

Lupinoblennius nicholsi (Tovolga, 1954)

Gobiidae

Bathygobius soporator (Valenciennes, 1837)

Gobionellus boleosoma (Jordan & Gilbert, 1882)

Gobionellus Girard, 1858

Gobiosoma (Girard, 1858)

Microgobius (Poey, 1876)

Callyonimidae

Diplogrammus pauciradiatus (Gill, 1865)

Tetraodontidae

Type I

Sparidae

Archosargus rhomboidalis (Linnaeus, 1758)

Archosargus probatocephalus Jordan & Dickerson, 1908

Eleotridae

Dormitator maculatus (Bloch, 1785)

Cynoglossidae

Symphurus plagiusa (Linnaeus, 1776)

Soleidae

Achirus lineatus Linnaeus, 1758

Trinectes maculatus Bloch & Schneider, 1801

Belonidae

Strongylura marina Walbaum, 1792

Gobiesocidae

Gobiesox strumosus Cope, 1870

Monacanthidae

Type I

Cyprinodontidae

Type I

Distribution of age-1 and age-2 walleye pollock in the Gulf of Alaska and eastern Bering Sea: sources of variation and implications for higher trophic levels

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Key words: *vertical distribution, North Pacific, juvenile fish, walleye pollock*

Abstract

Walleye pollock (*Theragra chalcogramma*) is the predominant groundfish species in the North Pacific Ocean, and it is a focal point in the ecology of the region. However, there is only a limited knowledge of the distribution of the juveniles of this species (age-1 and age-2 individuals). We examine the horizontal and vertical distribution of age-1 and age-2 walleye pollock in the eastern Bering Sea and Gulf of Alaska and relate observed patterns to key physical (temperature, latitude, longitude, bathymetry) and biological (diet, physiology) characteristics. We used data collected from three sources: a field survey conducted in the Gulf of Alaska (2001), field data collected from echo integration trawl surveys in the eastern Bering Sea (1994, 1996, 1997, 1999), and laboratory experiments investigating the behavior and physiology of age-1 and age-2 individuals under various thermal conditions. Results indicate there is the potential for differences in the ecology of walleye pollock between the Bering Sea and the Gulf of Alaska. Data from 1996 and 1997 indicate that age-1 and age-2 walleye pollock in the Bering Sea are vertically separated in the water column

(though they co occur in other years), with age-1 pollock located near bottom and age-2 pollock schooling higher in the water column. However, we did not find evidence of vertical separation among these cohorts in the Gulf of Alaska. Adult pollock (age-4+) appear to be demersal during the day in both systems. Diet analyses of pollock collected in the Bering Sea (1990-1997) indicate a high degree of cannibalism of age-0s by age-1 and age-2 individuals, though there is no evidence of inter-year class cannibalism in samples collected from the Gulf of Alaska (2001). Additionally, laboratory experiments show that the thermal range of pollock decreases with age, suggesting that younger fish may be able to exploit more of the vertical water column than older fish because they have greater thermal tolerances. Further work needs to be done to pursue the study of potential differences in spatial separation among cohorts between the Gulf of Alaska and the Bering Sea. However if the differences that we observed are reproduced, our laboratory results suggest that, in the Bering Sea, spatial separation could be related to a combination of temperature tolerance and intensive intraspecific predation pressure, while the lack of intraspecific predation pressure in the Gulf of Alaska might permit a greater co-mingling of age classes.

Introduction

The North Pacific Ocean is a highly productive ecosystem that supports a vast array of fishes, birds, and mammals. The dominant fish component of this ecosystem is the walleye pollock (*Theragra chalcogramma*), which sustains one of the world's largest commercial fisheries and provides a forage base for higher trophic level animals (Springer 1992). The immature stages of this species transfer energy from zooplankton to higher trophic levels (Brodeur and Wilson 1999), making them an important link in the food chain. Several recent studies have focused on the ecology of age-0 pollock (Brodeur 1998; Swartzman et al. 1999; Wespestad et al. 2000), but distribution patterns of later immature stages (age-1 and age-2s) remain uncertain. Bottom trawl surveys have been used to provide information on the dispersal of age-1 juveniles, but age-2 juveniles are infrequently collected in bottom trawls (Karp et al. 1989). Some information on the distribution of pollock juveniles has also been gathered from existing midwater trawl and hydroacoustic data (McKelvey 1996), but the overall spatial and temporal patterns of distribution between these two cohorts remain unclear. Studies that clarify where and when juvenile pollock are present can help to reduce concerns about bycatch of juveniles, and are important in understanding energy transfer to higher trophic levels.

Data from the National Marine Fisheries Service bottom trawl and echo integration trawl surveys suggests that, in the Eastern Bering Sea (EBS), age-1 and age-2 pollock schools may be vertically separated in the water column. These observations suggest that age-2s may be schooling higher in the water column, while age-1s are primarily located on-bottom. The factors that motivate this presumed stratification are unexplained, and it is also not known whether such a stratification exists in the Gulf of Alaska (GOA). Studies of young-of-the-year pollock suggest vertical distribution is influenced by predation pressure (Bailey 1989), bioenergetic criteria (Sogard and Olla 1994; Ciannelli et al. 1998), and prey availability. These factors and others probably contribute to the patterns of distribution of later immature stages as well, acting synergistically to affect re-

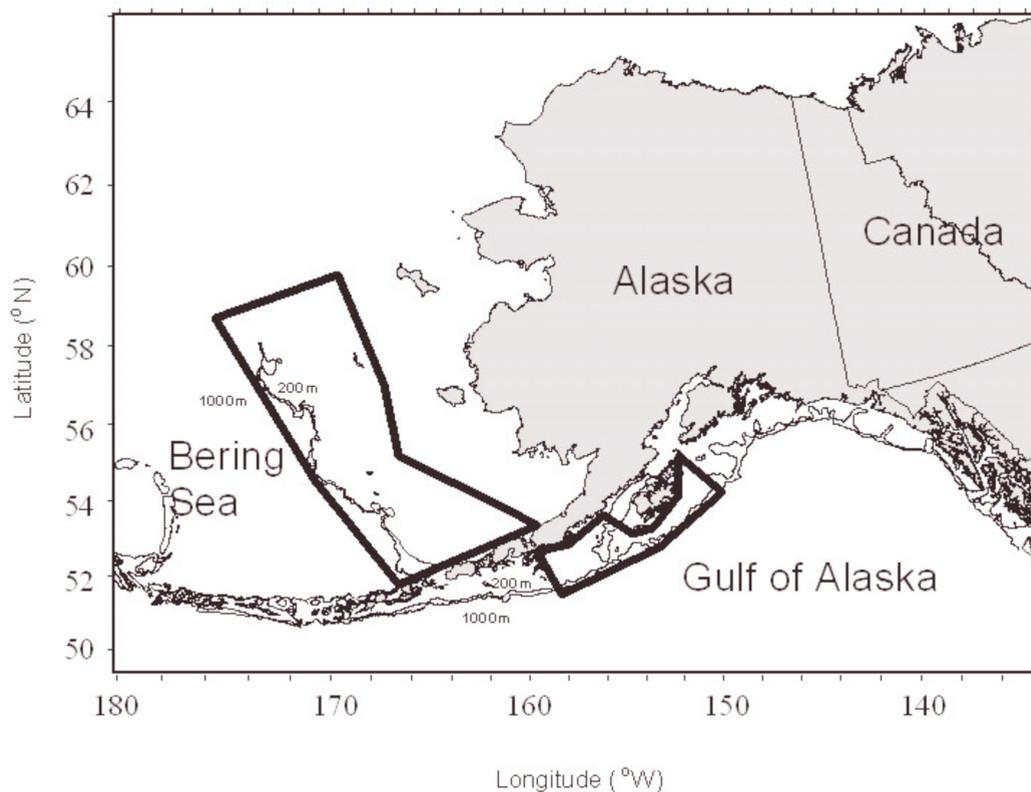


Figure 1. Extent of field surveys (black boxes) in the eastern Bering Sea and Gulf of Alaska in 1994, 1996, 1997, 1999 (EBS) and 2001 (GOA).

sponse. This paper: 1) examines the spatial distribution of age-1 and age-2 walleye pollock in the EBS and GOA, 2) relates observed patterns to predominant physical and biological characteristics to identify potential determinants for segregation, and 3) examines the implications of these results on upper trophic level organisms. Our multifaceted approach provides a preliminary analysis of the distribution of immature walleye pollock in the North Pacific Ocean, and examines some of the underlying physical and biological factors contributing to patterns in distribution and behavior.

Materials and methods

Gulf of Alaska – Field Surveys. Two field surveys were conducted in the GOA in August and September 2001, which assessed the vertical distribution of immature walleye pollock. Cruises were conducted on board the NOAA ship *Miller Freeman* in the vicinity of Kodiak Island, Alaska (Figure 1).

Age-1 and age-2 pollock were collected at discrete depths, either from bottom trawls (Poly Nor'easter bottom trawls (3.5 inch codend) or shrimp trawls (3.2 cm stretched mesh, 3mm mesh codend liner)) or from depth discrete mid-water trawls (anchovy trawls (3.2 cm stretched mesh, 3mm mesh codend liner) or Aleutian wing trawl (3.5 inch codend)). A randomly selected subset of walleye pollock was taken from each trawl, frozen, and returned to the laboratory for stomach content analyses to provide data on diet. An analysis of variance (ANOVA) was used to test the hypothesis that there was no difference in the vertical distributions of age-1 and age-2 walleye pollock.

Bering Sea – Acoustic Surveys. Acoustic data on fish distribution in the EBS were collected between June and September (1994, 1996, 1997, 1999) with a Simrad EK500 scientific split beam echo sounding system. The transducer operated at 38 kHz and was mounted on the bottom of the NOAA ship *Miller Freeman's* retractable centerboard. Echo integration and target strength data were collected simultaneously. Trawling was conducted opportunistically to sample echo sign observed on the echosounder display. Midwater sign was sampled with a large, commercial midwater trawl fitted with a 3.2 mm/1.25 in codend liner. Near bottom sign was sampled with a survey bottom trawl that was fitted with the same sized mesh. Estimates of pollock biomass and numerical abundance by length and age (Traynor 1996) were developed from the acoustic and trawl data.

Stomach Content Analyses. Fish collected from the field (GOA) were measured and weighed, and the stomachs excised. Stomach contents were visually evaluated for fullness (empty, trace prey, 25%, 50%, 75% 100%, distended). Upon dissection, individual taxa were enumerated and identified to the lowest possible taxon. Wet weights for each prey species were measured. Data were grouped into higher taxonomic categories for statistical analyses and an ANOVA (Zar 1984) was used to determine whether there were differences in composition of stomach contents with respect to age (size class) and location (EBS vs. GOA).

Stomach content data for pollock collected from the EBS was compiled from the Alaska Fisheries Science Center's fish food habits database that includes individuals of age-1+ captured throughout the eastern Bering Sea from 1990 to 1997. Methods for stomach content analyses for fishes collected in the EBS were similar to those used in the analyses of GOA-collected samples (but see Livingston and deReynier 1996 for a complete description).

Behavioral Experiments. Age-0 walleye pollock for laboratory experiments were collected near Port Townsend, Washington, in June 1999, and returned to the Center's laboratory in Newport, OR, to be reared for use in behavioral experiments. Pollock were reared until age-2, and behavioral studies were conducted in controlled tanks to examine patterns in vertical migration (similar to methods outlined in Sogard and Olla 1994, 1996). Briefly, behavioral observations were made in two 15,000 l tanks with Plexiglas¹ walls. Stratified temperature conditions were created by slowly adding cold water to the bottom of the tank. Observations under isothermal (iso) and stratified (strat) conditions were made using a video monitoring system, and behavioral responses were scored according to level of activity. In this study we focused on the effects of food availability (fed

and starved fish) on behavioral thermoregulation among age-2 pollock. Each experiment consisted of a set of two individuals (group) for each thermal treatment. A total of 25 groups were partitioned among treatments as follows: 6 iso-starved, 6 iso-fed, 7 strat-fed, 6 strat-starved. Data were analyzed using a nested ANOVA on vertical position (i.e., temperature) in the water column, with group used as the nesting factor. Similar studies using age-1 pollock and mixed schools of age-1 and age-2 pollock were planned, but poor survival of the age-1 cohort made it impossible to conduct these trials.

Energetics Simulations. A bioenergetics model (see Ciannelli 2002) was used to determine juvenile pollock growth response as a function of size and water temperature. In particular, we estimated the 50% thermal range (i.e. the temperature interval within which fish reach 50% of their maximum growth rate) as a function of juvenile pollock size.

Results

Gulf of Alaska – Field Surveys. Over 39,000 walleye pollock were collected from midwater trawls and nearly 2,000 pollock were collected in bottom trawls from the field survey conducted in August 2001. Over 4,000 individuals were collected from midwater trawls and nearly 400 individuals were collected from bottom trawls in the September 2001 survey. There were no statistical differences in vertical distribution of age-1 and age-2 walleye pollock ($p > 0.05$), evidenced by similar catches of these individuals in bottom and mid-water trawls (Figure 2).

However, there was a tendency for adult walleye pollock (age-4+) to occur in greater numbers in bottom trawls, indicating that older walleye pollock are primarily demersal in the Gulf of Alaska.

Bering Sea – Acoustic Surveys. Echo integration trawl data were examined from surveys conducted in summer 1994, 1996, 1997, and 1999. At each trawl site, weighted average distance off bottom was computed for pollock in each of three age groups (age-1, age-2, and age-3+ pollock) and then stratified by known geographic-bathymetric features (east or west of 170 °W, and shallower or deeper than the 100 m isobath). The following observations apply to vertical stratification within the stratum west of 170 °W and deeper than the 100 m isobath, where juveniles and adults were most numerous; horizontal stratification (differences between areas and within and between years) is not treated here. In 1994, both age-1 and age-2 walleye pollock schooled higher in the water column than pollock age-3+. Age-1 vertical distribution was not found to be different from that of age-2s. In 1996, age-1 pollock tended to be found near the bottom, while age-2 individuals were again more often observed higher in the water column. In 1997, age-1 pollock were found in both dense, mid-water schools and in aggregations closer to the bottom, whereas age-2 pollock were found higher in the water column, as in 1994 and 1996. In 1999, all age groups were highly concentrated in one or two locations, and the age-1 and age-2 juveniles were not spatially separated. Thus, for some years, there is evidence for spatial separation between age-1 and age-2 pollock on the EBS shelf.

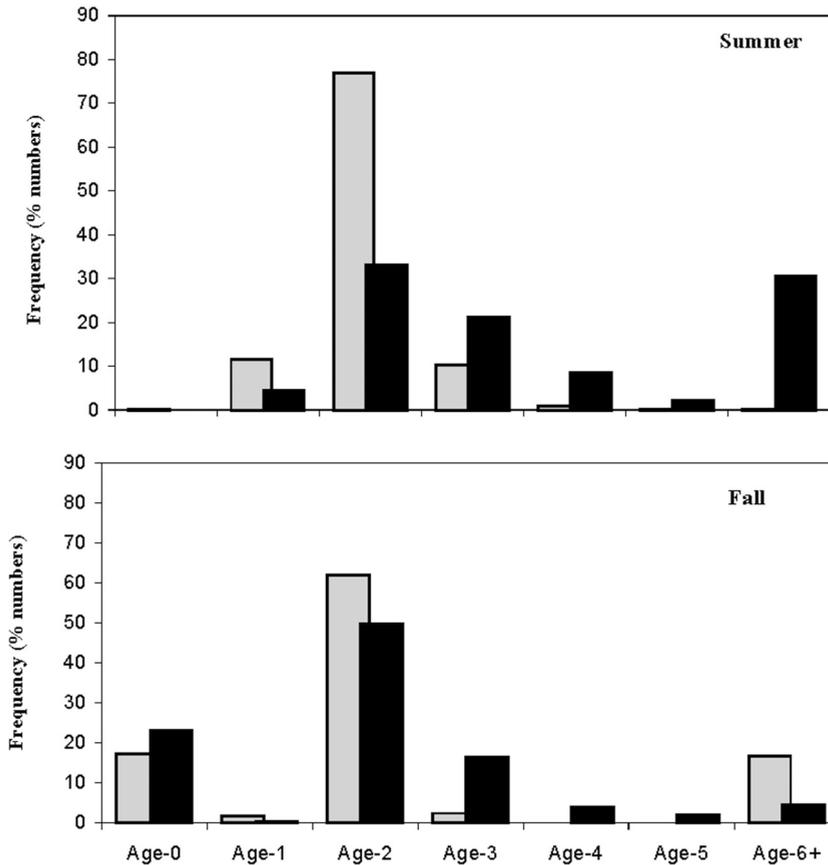


Figure 2. Age-frequency distribution of walleye pollock in the Gulf of Alaska in summer (top) and fall (bottom) 2001. Age-0 < 11 cm, age-1 = 12-18 cm, age-2 = 19-28 cm, age-3 = 29-36 cm, age-4 = 37-41 cm, age-5 = 42-47 cm, and age-6+ > 48 cm. Absence of age-0 individuals in summer collections was due to large mesh size used during towing. Black = bottom trawl, gray = midwater trawl.

Stomach Content Analyses. Over 300 walleye pollock were collected for diet analyses from cruises in the GOA in August and September 2001 (Figure 3), and to date, stomach content analyses have been performed on 61 of the individuals collected in August.

Data from these analyses suggest that the diets of age-1 and age-2 individuals were somewhat dissimilar, with age-1 pollock consuming proportionally more cumaceans than age-2 individuals (51% and 12%, respectively), though both age-classes consumed euphausiids in significant quantities (47% and 85%, respectively; $p < 0.001$). Fish made up a substantial portion (80%) of the diet of adult pollock. There was no evidence of inter-cohort cannibalism among any of the size classes, and all the fishes consumed by adults were identified as sticklebacks or other non-pollock fishes (Figure 4).

Observed differences in diet with age (size class) were not statistically significant because of the large variability in diet composition within size classes.

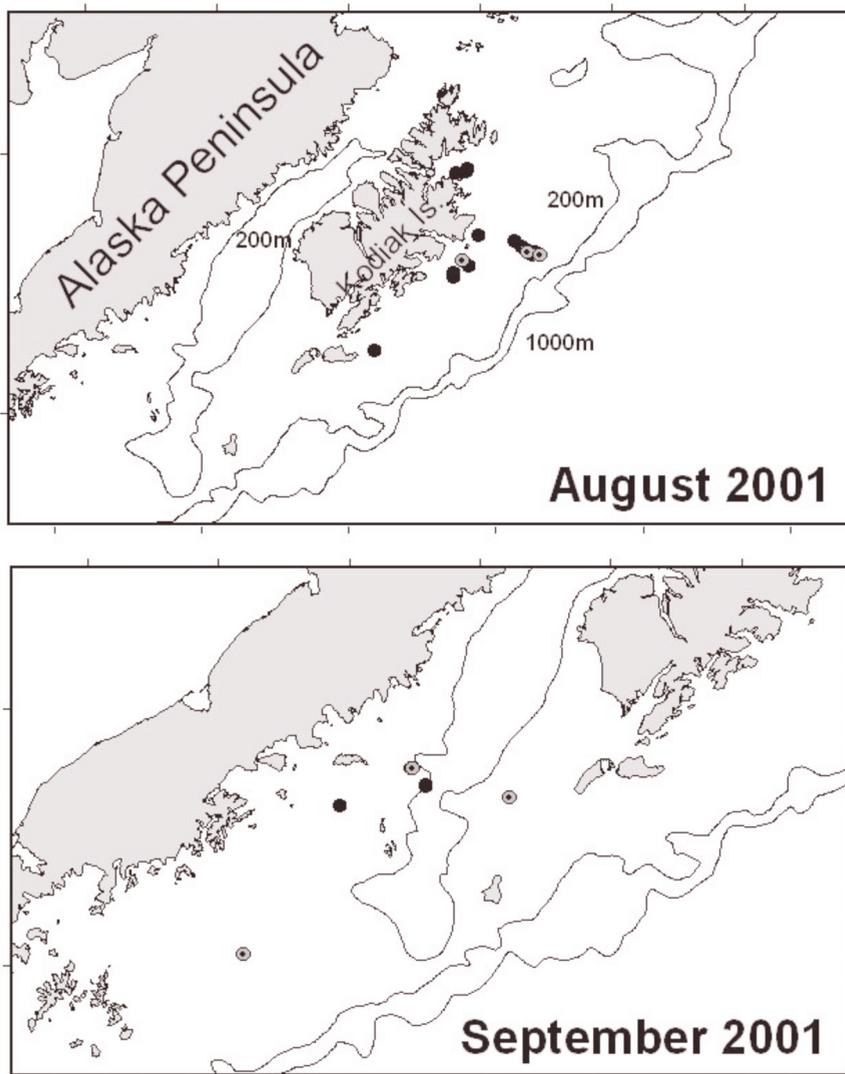


Figure 3. Location of walleye pollock collections for stomach content analyses in summer (top) and fall (bottom). Filled circles = stations where midwater tows were conducted; circles with a dot = stations where bottom trawls were conducted.

Walleye pollock diet data from fishes collected in the EBS indicated that the diet composition of juvenile pollock changed with size, especially with respect to cannibalism and copepod consumption. Importance of cannibalism increased from 5.5% in age-1 pollock to 46.9% in early age-2 (16-20 cm standard length) and then decreased to 28.6% in later age-2 pollock (21-25 cm standard length). Cannibalism ranged between 1.1% and 11.0% in later and larger individuals.

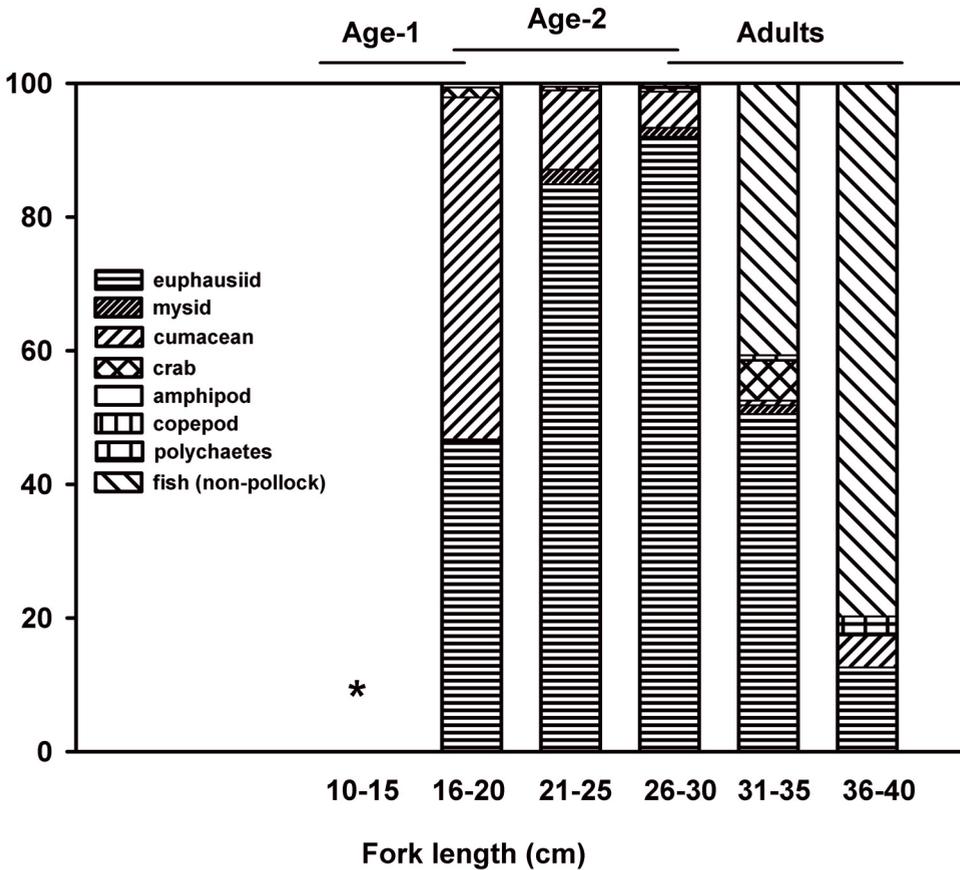


Figure 4. Percent composition by weight of the diet of walleye pollock collected in the Gulf of Alaska in summer 2001. Asterisks = no data available. Dominant prey categories are shown.

The proportion of copepods in the diet changed from 54.0% to 20.8% and 28.0% in age-1, early and late age-2 pollock, respectively. Copepods in the diet ranged between 47.0% and 50.1% in later age classes (Figure 5). All other major prey items included in the diet did not considerably change among age classes. Most of the fish consumed by larger pollock were age-0 pollock (< 100 mm SL).

Behavioral Experiments. We found no difference in behavioral thermoregulation between fed and starved age-2 pollock. In a vertical gradient tank, fed fish experienced an average temperature of 10.8°C (\pm 2.2 SD) while starved fish experienced 10.1°C (\pm 2.7 SD), and the two values were not statistically different ($F_{1,11}$, $p = 0.41$).

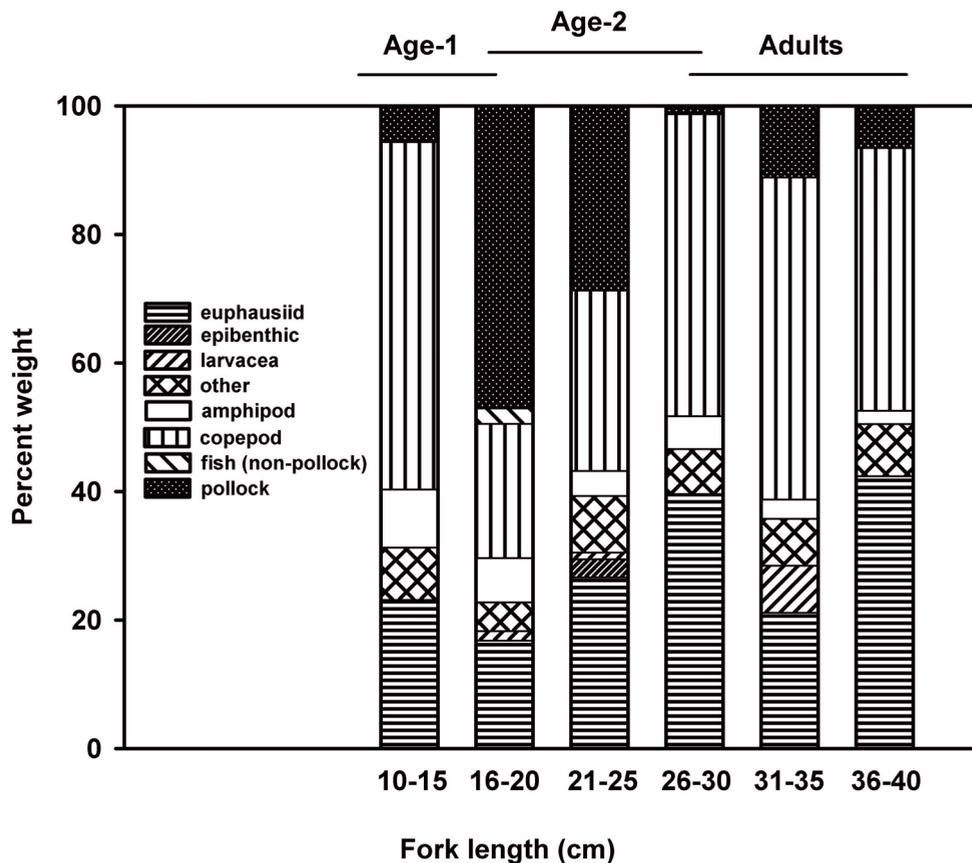


Figure 5. Percent composition by weight of the diet of walleye pollock collected in the eastern Bering Sea in summer (1990-1997). Dominant prey categories are shown.

Energetics Simulations. Estimates of thermal tolerance in juvenile pollock were considerably wider in younger and smaller fish (Figure 6). The bioenergetics simulation found that the 50% thermal range spanned over 7.6°C (from 3.3°C to 10.9°C) for a 0.5 g pollock, while it only spanned 2.7°C (from 2.4°C to 5.1°C) for a 500 g pollock.

Discussion

Based on our sampling, we suggest that the distributional ecology of immature walleye pollock may differ between the Eastern Bering Sea compared to the Gulf of Alaska, at least in some years. In the EBS, age-0 walleye pollock occurred throughout the water column (Tang et al. 1996), age-1 walleye pollock occurred near bottom (in 2 of 4 years examined), and age-2 and -3 individuals

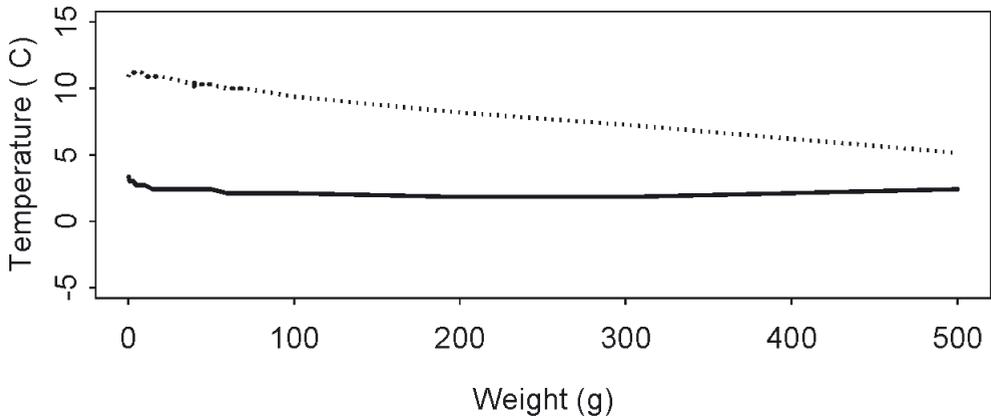


Figure 6. Thermal range of pollock as a function of weight (g). The thermal range includes temperature interval within which fish can reach 50% of its maximum daily growth rate.

schooled higher in the water column. Adults (age-4+) were demersal. In the GOA, age-0 individuals also occurred throughout the water column (Brodeur and Wilson 1996a), though cohorts of age-1 and age-2 individuals in the GOA appeared to co-occur throughout the water column. Adults in the GOA were primarily demersal.

We were able to provide several years of data for vertical distribution of immature walleye pollock in the EBS (1994, 1996, 1997, and 1999), though we were only able to make collections in the GOA in one year (2001). Our data show interannual differences in vertical distribution for the EBS, and it could be that there are interannual differences in vertical distribution of cohorts in the GOA as well. We cannot discount the possibility such occurrences also exist in the GOA, but our preliminary evidences of cohort-specific spatial differences between the two systems from this study warrant further consideration.

Results from our stomach content analyses indicate that diet differences could contribute to potential variations in the vertical distribution of age-1 and age-2 pollock between the EBS and GOA. A high degree of inter-year class cannibalism in the EBS, (Figure 5 in this study, Dwyer et al. 1987; Livingston 1991; Livingston 1993), particularly among age-1 and age-2 pollock on age-0s, may prompt differential vertical positioning in that system relative to the GOA. In the EBS, competition between the age-1 and age-2 cohorts for age-0 pollock prey could precipitate their spatial partitioning, alleviating competition between them. In contrast, the lack of piscivory (or availability of preferred prey) and a reduced degree of diet overlap between the two cohorts in the GOA permits vertical comingling. The finding of limited piscivory and cannibalism from our GOA diet analyses are similar to results from more extensive, multiyear diet analyses of walleye pollock in the GOA (Yang and Nelson 2000).

Further, our physiological analyses indicate that the temperature tolerance range of younger fish is greater than that of older fish, offering one explanation for our observations that adult fish occur almost exclusively on-bottom where temperatures are low and relatively consistent (0-2°C in

the EBS, 4-6 °C in the GOA), while immature walleye pollock (age-0, age-1, age-2) schooled higher in the water column where temperatures are higher and more variable (4-7 °C in the EBS, 7-10 °C in the GOA). Our results indicate that adult pollock may not be able to endure the higher upper water column temperatures (or perhaps cannot move through the thermocline), effectively making them obligate demersals. However, since younger individuals can tolerate higher temperatures, they enjoy greater vertical flexibility, move through the thermocline (Brodeur and Wilson 1996b), and exploit a greater portion of the water column. The thermal range simulation is in part based on bioenergetics parameters derived from juvenile pollock captured in Washington, which are possibly genetically distinct from the North Pacific populations of walleye pollock (Ciannelli 2002). Thus, our results when applied to North Pacific populations are to be taken with caution, particularly because genetically distinct populations could have different thermal adaptations. However, while it is possible that at any given age, southern range populations of pollock can tolerate higher temperatures, the ontogenetic changes of thermal tolerance should remain unaltered across latitudinal gradients.

Of course, other factors are likely to affect vertical distribution as well, and while this study offers some explanations for potential differences, it does not attempt to characterize all the influences on vertical positioning. For example, there is evidence of diel variability which has been linked to ontogeny and feeding (Brodeur and Wilson 1996a, 1996b). Likewise, schooling, temperature and salinity gradients, and threat of predators all influence vertical distribution of walleye pollock (Sogard and Olla 1994). However, it should be noted that possible diel differences should have been mitigated to some extent since pollock from this study were collected day and night from both bottom and midwater trawls. Additionally, patterns of spatial separation of juvenile pollock in the EBS and our ability to detect differences between age groups was probably influenced by year-class strength. For example, in 1997, a year when age-1 and age-2 pollock were spatially separated, age-1 pollock were part of a very large year class (1996). In 1999, when no separation was detected, there were very few age-1 individuals. Spatial distribution is also likely to be influenced by environmental conditions and seasonality. Finally, we should also note that potential differences in catchability of age-1 and age-2 fish could have influenced estimates of their abundance in midwater and bottom trawls, as could differences in mesh size between the gears used.

Our laboratory studies of behavioral thermoregulation in age-2 pollock did not show differences in water column usage under starved and fed conditions. Fish used in this experiment were collected from Port Townsend, Washington, and may have been locally adapted to higher temperatures. As such, it was not altogether unexpected that they would use the majority of the water column under both conditions. Similar experiments with fish collected from the EBS and GOA are necessary to further examine putative differences between these systems.

Topographical differences between the EBS and the GOA may give rise to some of the differences in vertical distribution of immature walleye pollock in these systems. The GOA has a narrow continental shelf (65 – 175 km) which may limit the availability of suitable habitat for walleye pollock. If suitable horizontal space is limiting, immature pollock may be forced to co-occur vertically, piling the size classes on top of one another. In contrast, the EBS has a broad shelf (> 500 km), which may permit greater age class separation over horizontal space, resulting in less vertical stacking. Ultimately, these differences could have prompted diet specializations among fishes in the two areas over long time scales.

Overall in this study we have shown some differences in juvenile pollock vertical distribution between the EBS and the GOA. Such differences could be in part physiologically driven due to ontogenetic shifts in diet and metabolism, and in part ecologically driven, motivated by the partitioning of available resources (i.e., space or food), leading to a reduction of intra-specific competition. In both cases though, fish respond to environmental stimuli. Differences in spatial distribution is of particular interest in the study of energy transfer to upper trophic levels. Subadult pollock in both the EBS and the GOA are a primary prey item for a variety of demersal fishes (Livingston 1993), marine mammals (Sinclair et al. 1994) and seabirds (Hunt et al. 1996). Hence, the relative distribution of juvenile pollock throughout the water column is likely to have repercussions not only on their own survival but also on the feeding success of higher trophic level species. For example, northern fur seals breeding on the Pribilof Islands confine their feeding to mostly above the thermocline, particularly during years with a sharp temperature gradient (Robson 2001). It is speculated that fur seals follow the distributional response of prey fishes which are in turn affected by water column properties (Brodeur et al. 1999), thereby linking hydrography and fish prey distribution with energy transfer to upper trophic level species. It is likely that juvenile pollock distribution varies with changes in environmental conditions (i.e., annually). The inter-annual variability of juvenile pollock distribution and its relation to environmental variability in the GOA was unexplored in this study and, based on the pivotal role of subadult pollock in the local trophic webs, of considerable scientific relevance.

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¹ Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA

Larval abundance, growth, and recruitment of Japanese Spanish mackerel *Scomberomorus niphonius* in the Seto Inland Sea, Japan

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Key words: *Spanish mackerel, larvae, growth, survival, piscivory, Seto Inland Sea*

Abstract

We examined the relationships among larval and juvenile abundance and catch per unit fishing effort (CPUE) of subsequent 1-year-old Japanese Spanish mackerel in the central Seto Inland Sea, Japan, from 1995 to 1999 to test the hypothesis that year-class strength of the mackerel is determined by the juvenile stage. There was no significant correlation between larval and juvenile abundance while juvenile abundance was positively correlated with CPUE of subsequent 1-year-old fish, indicating that year-class strength was determined by early juvenile stage. Mean larval growth rate estimated by otolith microstructure ranged between 0.43 and 0.92 mm day⁻¹. There was a significant positive correlation between prey fish (clupeiform larvae) abundance and mean growth rate of the mackerel larvae. The fish prey abundance during peak occurrence period of the mackerel larvae was higher in 1995 and 1999 than in the other three years. The larval growth rates were significantly higher and strong year-class occurred in 1995 and 1999. The larval abundance was highest, but the juvenile abundance was lowest in 1997, indicating higher mortality during larval period in this year. We concluded that fish prey availability is one of the most important determinants for growth and survival of Japanese Spanish mackerel larvae, which are almost completely piscivorous from their first feeding stage.

Introduction

Japanese Spanish mackerel *Scomberomorus niphonius* (scombridae) is distributed in the southwestern coastal waters of Japan. The species is a particularly important fisheries resource in the Seto Inland Sea. Total catch of the species exceeded 6,000 t in the mid 1980s but has recently decreased to less than 5% of what it was before. The species grows fast with piscivorous and voracious feeding and is a new target for stock enhancement projects in Japan. Approximately 100,000 artificially raised juveniles have been released for the past few years in the Inland Sea with a hope of recovering the stock. However, recruitment mechanism is poorly understood.

Spanish mackerels (*Scomberomorus* fishes) are considered to have specialized survival strategies characterized by piscivory and high growth in their early life stages (Jenkins et al. 1984; Grimes and Isely 1996). Spanish (*S. maculatus*) and king (*S. cavalla*) mackerels larvae feed on larval fish and grow fast at 1.15 and 0.89 mm d⁻¹, respectively, off the southeast United States (Finucane et al. 1990; DeVries et al. 1990). Japanese Spanish mackerel larvae are also piscivorous at their first feeding (Shoji et al. 1997) and grow at a mean growth rate of 1.03 mm d⁻¹ (Shoji et al. 1999). Tanaka et al. (1996) demonstrated a precocious development of adult type digestive system with a functional stomach at the first feeding stage of Japanese Spanish mackerel larvae. In culture, Japanese Spanish mackerel larvae cannibalized when they were fed only invertebrate plankton prey (rotifer and *Artemia*) and preferred fish prey to the invertebrate plankton prey (Fukunaga et al. 1982; Shoji and Tanaka 2001). The larvae all died until 4 days after the first feeding when they were fed only rotifers, while more than 90% of the larvae survived the same period when fed fish prey (Shoji et al. 2002a). These observations indicate that fish larvae, not invertebrate plankton, are essential prey at the first feeding stage of Japanese Spanish mackerel larvae.

Piscivorous feeding during the larval period of fish promotes high growth rates, and high growth rates would be advantageous to survival because of the reduced larval period which is vul-

nerable to predation (Houde 1987). On the other hand, higher swimming performance and metabolic rate required for piscivorous feeding may lead to a high susceptibility to starvation of the predatory larvae (Hunter 1981; Grimes and Isely 1996). Margulies (1993) examined nutritional condition of *S. sierra* larvae in the Panama Bight by histological analysis and showed that 25% of the larvae

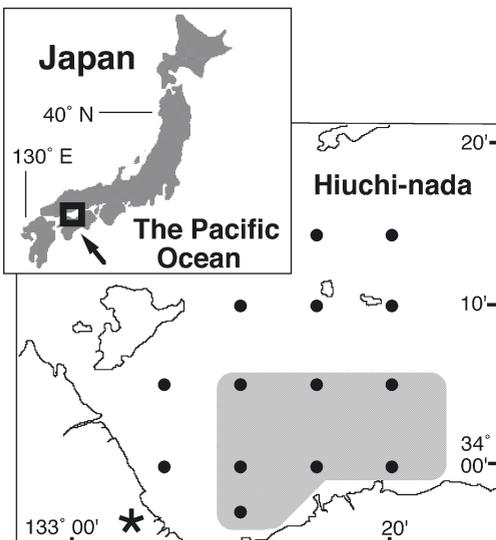


Figure 1. Map showing sampling stations in the Hiuchi-nada, central Seto Inland Sea, where ichthyoplankton were collected from 1995 to 1999. Shaded area shows waters where juvenile mackerel was collected by seine-net and asterisk Kawarazu Fisherman's Association from which catch records of 1-year-old mackerel were collected.

were starving and that the percentage in smaller-size larvae was higher (stage-specific starvation mortality). The nutritional point-of-no-return was estimated to be 0 to 1 day in the first feeding Japanese Spanish mackerel larvae (Shoji et al. 2002a). These observations would lead us to expect that fish prey availability is a key factor for growth and survival during early larval period of the Spanish mackerels. However, no study has been conducted on relationships between food availability and larval growth/survival of Spanish mackerels.

The hypothesis that fish prey availability alters larval growth rate of the Japanese Spanish mackerel was tested in the central Seto Inland Sea, Japan. Relationships among larval abundance, growth rate, prey fish (clupeiform larvae) abundance, and catch per unit fishing effort (CPUE) of subsequent 1-year-old fish were examined from 1995 to 1999.

Materials and Methods

Sampling surveys were conducted in the Hiuchi-nada (Fig. 1), central Seto Inland Sea, four to seven times per year from 1995 to 1999. The Hiuchi-nada is a major spawning ground for Japanese Spanish mackerel stock that distributes in the central and western Seto Inland Sea (Kishida 1991). The mackerel migrates into the Hiuchi-nada to spawn in May (Kishida and Aida 1989), and the mackerel eggs and larvae are abundant in the central Hiuchi-nada in May and June (Kishida 1988, 1991).

Horizontal tows with a conical larva-net (mouth diameter 1.3 m, mesh aperture 0.5 mm) were made to collect the Japanese Spanish mackerel larvae by the R/V Hiuchi of the Ehime Prefecture Chuyo Fisheries Experimental Station (EPCFES). The net was towed for 10 minutes at a ship velocity of 2 knots in the mid-depth (10-15 m) layer considering the larval vertical distribution in this area (Kishida 1988). Vertical hauls of a conical plankton net (mouth diameter 0.6 m, mesh aperture 0.315 mm) were made to examine seasonal changes in abundance of the mackerel larval prey. Post-first-feeding Japanese Spanish mackerel larvae feed mainly on clupeiform larvae at around 3 mm SL (Shoji et al. 1997). Significant loss of clupeiform larvae at this size through the mesh (0.5 mm) of the net used for the mackerel larval sampling is expected. Abundance of clupeiform larvae obtained from the plankton net tows was used as an index of prey abundance. Ichthyoplankton samples were initially preserved in 10% formalin and the mackerel larvae were preserved in 90% ethanol within 24 hours after the formalin fixation. Volumes of seawater filtered by the nets were measured by flowmeters mounted on the mouth of the nets. The number of ichthyoplankton collected by the horizontal and vertical tows were converted to catch per 1000 m³ and 1 m², respectively. The mackerel larvae larger than 12 mm SL were classified as juvenile since their fin formation is completed at this size (Kishida 1991). Water temperature and salinity were measured with a salinity-temperature-depth sensor at each sampling station. The samplings were conducted between 0900 and 1700.

Ages of the mackerel larvae were determined by an analysis of sagittal otolith increments, which are deposited daily in the larval and early juvenile periods (Shoji et al. 1999). There were a few diffuse and poorly defined increments in the core area surrounded by a clearly defined increment, which corresponds to the initiation of feeding (Fig. 2). Right-side sagittal otoliths of 175 fish (40, 25, 40, 30, and 40 in 1995, 1996, 1997, 1998, and 1999, respectively) were removed under a dis-

secting microscope, and otolith increments were counted under a compound microscope with a video monitor. We estimated larval growth rate during post-first-feeding period for inter-year comparison since growth rate during yolk-sac period of the mackerel is negligible (Shoji et al. 1999). The mackerel larvae initiate feeding on day 6 at 18.5°C (day 4-6 depending on temperature: Fukunaga et al. 1982; Shoji et al. 2002a). Mean water temperature at 1 m depth ranged between 17.4-18.2°C during the cruises. The larval growth rate during the post-first feeding period (G , mm d⁻¹) was estimated for each larva following the equation:

$$G = (L_{\text{capture}} - L_1) / (A_{\text{capture}} - A_1),$$

where, L_{capture} = standard length (SL, mm) at capture, L_1 = SL at the first feeding which was fixed at 5.59 mm (Shoji et al. 2002a), A_{capture} = age (day) at capture, and A_1 = age at the first feeding which was fixed at 6 day. Mean growth rate, with at least 25 individual growth rates, was calculated for the larvae collected from the cruises during the peak-occurrence period of each year. An analysis of variance (ANOVA) followed by Turkey's test was applied to compare the larval growth rate among different years.

The Japanese Spanish mackerel spends its first growing season (May to November) in the central Seto Inland Sea (Kishida 1989). The mackerel juveniles were collected once or twice per week from mid June to late July in the southern waters of the Hiuchi-nada from catches by a seine-net fishery, which primarily targets larval, juvenile and adult Japanese anchovy *Engraulis japonica*. The net had a cod end of 2 mm in mesh aperture and was towed by two boats at a ship velocity of 3 to 4 knots. Twenty to fifty kg of the collection was sampled at each sampling, and juvenile mackerel were preserved in 90% ethanol on the boat. Abundance of juvenile mackerel was expressed as total number of fish per tow.

We used catch-per-unit-fishing-effort (CPUE) of 1-year-old Japanese Spanish mackerel fished by the commercial gill net fishery as an index of recruitment of those originating in the Hiuchi-nada (Kishida 1991). The mackerel distributed in the central and western waters of the Seto Inland Sea is regarded as a single stock (Hayashi 1919). Catch records of 1-year-old mackerel and the number of boats operating from the Kawarazu Fisherman's Association were recorded daily during the main fishing season (May) from 1995 to 1999. CPUE was expressed as total catch divided by total number of boats operating.

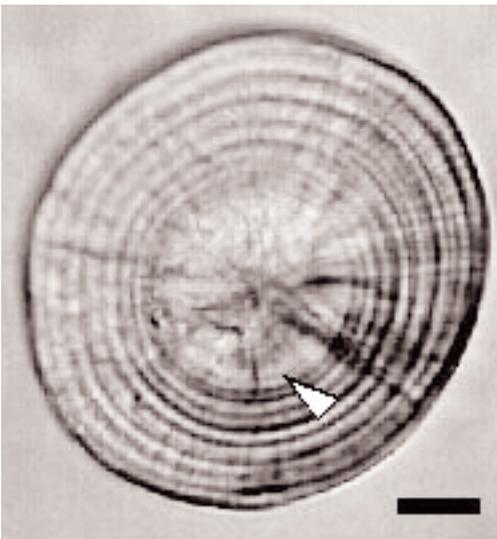


Figure 2. Sagittal otolith of a Japanese Spanish mackerel larvae (8.7 mm SL) collected from the Hiuchi-nada in May 1997. Arrow indicates a clear ring corresponding to first feeding. Bar shows 0.05 mm.

Results

Larval abundance. A total of 1070 mackerel larvae (3.0-9.8 mm SL) were collected from the larva-net tows from May to July (Fig. 3). The larvae first occurred in mid May in 1997, 1998, and 1999 and late May in 1995 and 1996. Larval abundance peaked in early June in 1995 and 1996, late May in 1997 and 1999, and mid May in 1998. Mean water temperature at a depth of 1 m depth during the each peak-occurrence period was 18.2, 17.8, 17.7, 17.5, and 17.6°C in 1995, 1996, 1997, 1998, and 1999, respectively. The larval abundance decreased rapidly following each peak occurrence period. Highest larval abundance (40.2 per 1000m⁻³) was observed in late May in 1997.

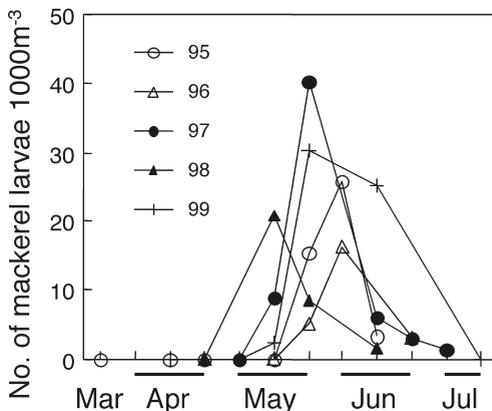


Figure 3. Seasonal changes in mean abundance (number per 1000m⁻³) of Japanese Spanish mackerel larvae collected by horizontal tows with a larva-net in the Hiuchi-nada from 1995 to 1999.

Juvenile abundance. A total of 382 juvenile mackerel (10.8-34.5 mm SL) were collected by seine-net. Most of the juveniles were collected from late June to late July when larval mackerel abundance was low. The juveniles were most abundant in 1999 (412.5/tow) followed by 1995. In the other three years, 1996-1998, juvenile abundance was much lower at less than 50/tow. There was no significant correlation between abundance of larvae and juveniles (n=5, r=0.14, p>0.05). Juvenile abundance was lowest (10.3/tow) in 1997 when larvae were most abundant, indicating higher mortality during the larval period among the five years (Fig. 4A).

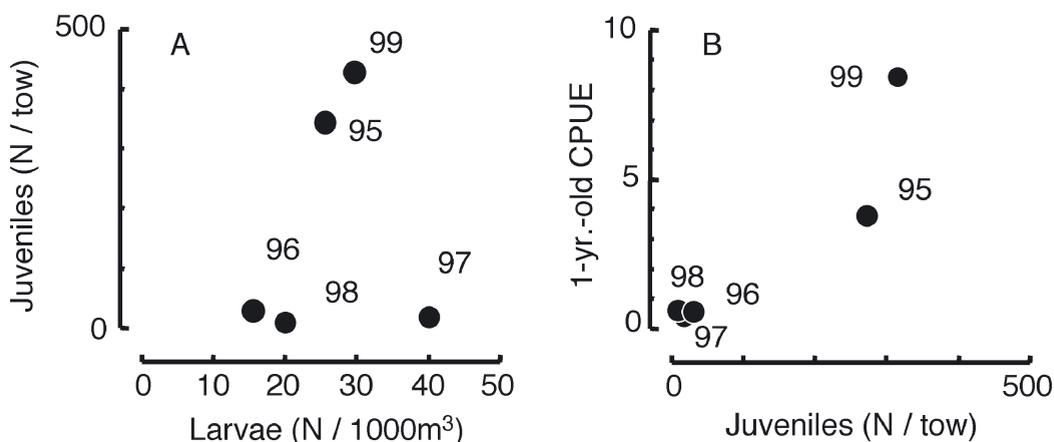


Figure 4. Relationships between abundance of larvae and juveniles (A) and between abundance of juveniles and CPUE of subsequent 1-year-old fish (B). Figures indicate year-classes.

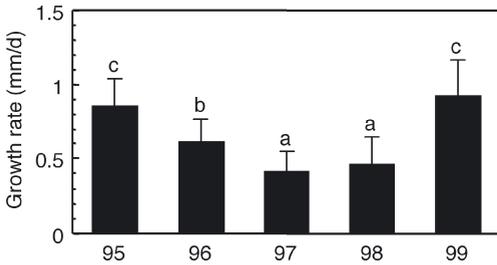


Figure 5. Mean growth rate of Japanese Spanish mackerel larvae collected in peak-occurrence period of the larvae from 1995 to 1999. Vertical bar shows standard deviation of the mean and different alphabetical characters significant differences (all $p < 0.001$).

(Turkey's test, $p < 0.001$). The growth rates in 1997 and 1998 were significantly lower than those in other three years (Turkey's test, $p < 0.001$). There was a significant positive correlation between the mean larval growth rate and juvenile abundance ($n=5$, $r=0.97$, $p < 0.01$) and between the mean larval growth rate and CPUE of subsequent 1-year-old fish ($n=5$, $r=0.89$, $p < 0.05$).

Fish prey abundance. Seasonal changes in abundance of clupeiform larvae peaked in mid May in 1995, 1996, and 1999, while it seemed to have peaked before mid May in 1997 and 1998 (Fig. 6). During the peak-occurrence period of the mackerel larvae (early June in 1995 and 1999, late May in 1997 and 1999, and mid May in 1998; Fig. 3), clupeiform larvae abundance was higher in 1995 and 1999 (252.7 and 180.5 m^{-2} , respectively) than in 1996-1998 (all < 100 m^{-2}). In 1997, the clupeiform larvae abundance during the mackerel larval peak occurrence period was much lower (11.9 m^{-2}) than the abundance in other years. There was a significant ($n=5$, $r=0.89$, $p < 0.05$) positive correlation between clupeiform larvae abundance and the mean larval growth rate.

Discussion

Timing of recruitment establishment. We found a significant positive correlation between abundance of juvenile Japanese Spanish mackerel and CPUE of 1-year-old mackerel, while there was no significant correlation between abundance of larval and juvenile mackerel (Fig. 4). There also was a significant positive correlation between abundance of juvenile mackerel and CPUE of 1-year-old mackerel in the central Seto Inland Sea from 1983 to 1988 (Kishida 1991). Japanese Spanish mackerel initiate feeding at 5.59 mm SL (Shoji et al. 2002a) and the mean larval growth rate ranged between 0.43-0.92 mm day^{-1} in the present study. Size at the transformation to juvenile is considered about 13.2 mm SL assuming shrinkage of 9% body length of the mackerel larvae by ethanol preservation (Shoji unpublished data). Kishida (1991) and the present study indicate that recruitment of the mackerel would have been established by early juvenile stage (9-18 days after the first feeding) and that juvenile fish abundance would be an indicator of recruitment abundance.

1-year-old fish abundance. The CPUE of subsequent 1-year-old mackerel was highest in 1999 (3.86/boat/day) followed by 1995. In the other three years, CPUE was lower than 1/boat/day with the lowest abundance in 1997. There was a significant ($n=5$, $r=0.93$, $p < 0.05$) positive correlation between juvenile abundance and CPUE of 1-year-old fish (Fig. 4B).

Larval growth. Mean larval growth varied between 0.43 and 0.92 mm day^{-1} (Fig. 5) with a significant year effect (ANOVA, $p < 0.001$). The growth rates in 1995 and 1999 were significantly higher than those in 1996 to 1998

Length of the period, during which recruitment abundance is essentially determined, seems to vary among fish species and circumstances. Campana (1996) found a positive correlation between juvenile growth rate and subsequent year-class strength of Atlantic cod *Gadus morhua*. Watanabe et al. (1995) conclude that cumulative mortality throughout first one year after the first feeding stage determined recruitment of Japanese sardine *Sardinops melanostictus*. Butler (1991) found that there was no correlation between larval mortality rates and year-class strength of the California sardine *Sardinops sagax*. Compared to these findings, year-class strength of Japanese Spanish mackerel seems to be established in a shorter period (by early juvenile stage: 9-18 days after the first feeding) in early life. Japanese Spanish mackerel grows at a high rate (3.0 mm day^{-1}) during early life stages, reaching 100 mm in total length in one month (Fukunaga et al. 1982) and 500 mm in fork length five months after hatching (Kishida et al. 1985). Due to the extremely high growth rate, accumulative mortality of the mackerel throughout juvenile stage would be smaller than those of many other fish species with slower growth rates.

Larval growth rate and survival. Generally growth rate is an important factor which can determine mortality during larval period. Fast growth reduces duration of larval period, which is vulnerable to predation (Houde 1987). In the central Seto Inland Sea, there seemed to be a correlation between growth and mortality rates during the larval period of Japanese Spanish mackerel. The mean larval growth rate was significantly correlated with juvenile abundance although the larval growth rates were obtained from only their peak occurrence periods in the present study because of sufficient numbers of fish for the growth analysis from the other sampling periods. Mortality of the mackerel larvae seemed to be highest in 1997 (Fig. 4A), when the larval growth rate was lowest among the five years (Fig. 5).

The mean larval growth rate varied between $0.43\text{--}0.92 \text{ mm day}^{-1}$ among the five years. We speculate that total mortality through the larval period varies by 3800 times depending on duration of the period (9-18 days) by using the daily mortality rate ($60\% \text{ day}^{-1}$) estimated by Margulies (1993) for larval scombrid in the Panama Bight. This calculation suggests that larval growth rate has a substantial effect on early mortality in Japanese Spanish mackerel.

Importance of fish prey. The larval growth rate was significantly higher in 1995 and 1999 when clupeiform larvae abundance was relatively high during the mackerel larval peak occurrence period. The significant positive correlation between clupeiform larvae abundance and the mackerel larval growth rate indicates fish prey-dependent growth of the mackerel larvae. Further, in the

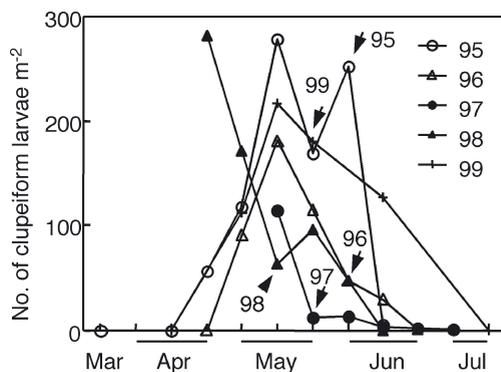


Figure 6. Seasonal changes in mean abundance (number per m^2) of clupeiform fish larvae collected by vertical tows with a plankton net in the Hiuchinada from 1995 to 1999. Arrow shows peak occurrence period of Japanese Spanish mackerel larvae in each year.

central Seto Inland Sea, there was no significant correlation between the larval growth rate and water temperature (Shoji unpublished data). DeVries et al. (1990) compared larval growth rates of king mackerel by sampling site and found higher growth rate (0.95 mm day^{-1}) in areas where ichthyoplankton concentrations were much higher (the Mississippi River plume) than from other locations combined (0.79 mm day^{-1}). Larvae of Spanish mackerels are almost totally piscivorous from their first feeding stage (Jenkins et al. 1984; Finucane et al. 1990; Shoji et al. 1997). We conclude that fish prey availability would be one of the most important determinants for larval growth rate of Spanish mackerels.

Comparative recruitment mechanism: the 1980s and the 1990s. Kishida (1991) focused on relationships between oceanographic condition and recruitment of Japanese Spanish mackerel in the central Seto Inland Sea in the 1980s. Strong year-classes occurred in years when stratification in the main spawning area, the Hiuchi-nada, was obscured in June. There is unfortunately no biological data such as fish prey availability and growth of the mackerel larvae in the 1980s. Based on the results of this study, however, we would suggest that fish prey availability is a key factor in determining recruitment of the mackerel. We did not find such a correlation between oceanographic conditions and the mackerel recruitment in the 1990s. For example, high recruitment was observed in 1999 with high water temperature and stronger surface water stratification, while low recruitment in 1997 coincided with intermediate water temperature and weak stratification. These results may indicate that a single oceanographic condition in the Hiuchi-nada affected recruitment of the mackerel in different ways between the two decades.

In the Seto Inland Sea, change in the pelagic fish community occurred in the late 1980s with a replacement of dominant clupeiform fish species composition (Hashimoto et al. 1995). Japanese sardine *Sardinops melanostictus* and gizzard shad *Konosirus punctatus* (clupeidae) larvae were dominant in the central Seto Inland Sea during the spawning season of Japanese Spanish mackerel (May and June) in the late 1990s (Shoji et al. 2002b) while Japanese anchovy *Engrauris japonica* (engraulidae) larvae was dominant before. Main prey item of Japanese Spanish mackerel larvae also shifted from Japanese anchovy (1981-1983; Shoji et al. 1997) to the two clupeid larvae (1995-1996; Shoji et al. 1999). Japanese anchovy larvae are abundant in the eastern waters of the Hiuchi-nada (Kuroda 1993) while the two clupeid larvae are abundant in the central waters (Shoji 1997). Change in species composition and difference in spatial distribution pattern of larvae of the three clupeiform fish lead us to speculate that there were different spatial patterns in growth and survival of the mackerel larvae in the central Seto Inland Sea between the decades. A single oceanographic condition in the Hiuch-nada might have influenced the mackerel recruitment with different mechanisms between the 1980s and the 1990s because of changes in the dominant prey fish species.

In conclusion, recruitment of Japanese Spanish mackerel in the central Seto Inland Sea seemed to be determined by early juvenile stage for the last two decades. Juvenile fish abundance could be used as an indicator of recruitment abundance. Fish prey availability is important as a potential determinant of growth and survival of the mackerel larvae since they are almost completely piscivorous from their first feeding stage.

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Characterizing natural intervals of development in the early life of fishes: an example using blennies (Teleostei: Blenniidae)

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Abstract

We examined patterns and timing of ontogeny in five species of blenny (Teleostei: Blenniidae) from the northern Gulf of Mexico by assigning a suite of discrete character scores to individual ontogenetic events (10 external characters; 218 specimens). Our approach applies scaling techniques and statistical methods to quantify, differentiate, and select criteria to delimit intervals of development across taxa. We consistently identified three natural intervals of development (labeled 'Larvae', 'Metamorphs', and 'Settlers'). Larvae had total character scores ≤ 13 and ontogenetic index (O_L) values < 80 . Metamorphs had total character scores from 15 to 30, and O_L values from 80 to 90.3. Settlers had total character scores ≥ 25 and O_L values ≥ 83 . State of orbital cirrus and fin development, number of teeth, and body pigmentation patterns provided most of the power to discriminate intervals. We recommend the use of numerous, general (not species-specific), and diverse (i.e., morphological, behavioral, physiological, pigmentation, etc.) characters to delineate intervals of development, but especially fin ontogeny and dentition. Assignment of character scores to discrete ontogenetic events, when combined with a dimensionless index permits quantification of the timing of ontogeny, identification of the events that delimit intervals of development, and interspecific comparison. The diversity of teleosts and their life-history patterns may prohibit development of a standardized staging system across higher taxonomic levels, but standardization at the family level appears possible.

Introduction

The complex life cycle of many marine fishes consists of intervals of development, each interconnected but with different growth and survival requirements and population dynamics (Hempel 1965; Frank and Leggett 1994). Objective characterization of intervals is crucial for examination of factors that influence survival and year-class strength because these processes may be interval-specific (Richards and Lindeman 1987; Kingsford 1988; Noakes and Godin 1988). Insight into developmental processes requires knowledge of the timing and synchronization of ontogenetic change (McCormick 1993; Higgs and Fuiman 1998). Poorly defined characters and lack of precise information on the position of a character within a developmental sequence have weakened attempts to delimit intervals in bony fishes (Ahlstrom 1968; Youson 1988; Fuiman and Higgs 1997). In fact, what appear to be similar characters are often used to describe completely different intervals (see Kingsford 1988) and sometimes multiple intervals (Gorodilov 1996). The wide variety of criteria and characters used to delimit intervals, the common misuse of developmental terminology, and the myriad of descriptive names that depict intervals has restricted our understanding of developmental processes.

Many aspects of development vary with an individual's size and/or age across species. These differences require a dimensionless metric that accounts for non-linear rates of ontogeny in order to make interspecific comparisons (Dettlaff and Dettlaff 1961; Fuiman et al. 1998). Customarily, length or age has been the reference point for interspecific comparisons. While length is an adequate basis for an index that permits intraspecific comparisons, length is not as effective in interspecific comparisons because genetic and environmental factors can induce size differences at comparable states of ontogeny that confound interspecific comparisons (Dettlaff and Dettlaff 1961; Fuiman and Higgs 1997; Fuiman et al. 1998).

Recent advances in developmental scaling methods (Fuiman 1994; Fuiman et al. 1998) permit comparison of larvae of different species and evaluation of differences in the timing and synchrony of developmental events. 'Ontogeny,' as defined here, is the change (either progressive or regressive) in character state (i.e., morphological, behavioral, physiological, pigmentation, etc.), the appearance of new characters, or the loss of existing characters. The ontogenetic index (O_L) of Fuiman (1994) expresses the state of ontogeny of a larva at any point in a developmental sequence ($O_L = \log L / \log L_{juv} \cdot 100$) as a proportion of a logarithmic developmental sequence, where L = standard length (SL) and L_{juv} = SL at the beginning of the juvenile stage for a given species. Using L_{juv} to formulate the ontogenetic index corrects for differences in size at a given comparable state of ontogeny among taxa. We demonstrate a new methodological approach to delimit natural intervals of development in fishes by employing quantitative characters and objective statistical treatment of those characters. This methodology can be used to characterize an individual's state of ontogeny, group individuals into comparable intervals of development, and facilitate interspecific comparisons on a common, dimensionless scale.

Materials and Methods

Light trap collections from oil and gas platforms off Louisiana over a three-year period (1995-1997) contained five species of blenny, including tessellated blenny (*Hypsoblennius invemar*), freckled blenny (*Hypsoblennius ionthas*), *Hypleurochilus multifilis* (no common name), seaweed blenny (*Parablennius marmoreus*), and molly miller (*Scartella cristata*), all in the tribe Parablenniini, according to Bock and Zander (1986). Settlers were hand-netted over oyster shell reefs and along rock jetties; collected with slurp guns along the legs of oil and gas platforms; and gathered following explosive removal of oil and gas platforms. Specimens were fixed in 10% formalin and transferred to 70% ETOH after 24 h. Each data set contained a nearly continuous size series of specimens with only minor gaps in SL.

We examined 55-*Hypsoblennius invemar*, 42-*H. ionthas*, 41-*Hypleurochilus multifilis*, 50-*Parablennius marmoreus*, and 30-*Scartella cristata*. Each specimen was scored for a suite of characters with each score representing a discrete ontogenetic event or character state (Table 1). We use the term 'state' to designate an instantaneous position within an ontogenetic sequence. 'Stage' represents an interval of development as traditionally defined in early life history literature. Dipping specimens into a solution of Cyanine Blue 5R stain, also known as Acid Blue 113 (Saruwatari et al. 1997), improved the contrast of anatomical structures, such as sensory pores, fin rays, and cephalic cirri. Scores for individual characters were summed to produce a 'total character score' for each individual, with total scores ranging from 1 to 42 for the suite of characters used.

Assigning individuals to intervals of development with confidence requires methods that reduce ambiguity. We performed a cluster analysis on the total character scores for the species-pooled data set using complete linkage and Manhattan distance rules to organize and map group structure (James and McCulloch 1990). A minimum linkage distance of 20% separated major clusters. Cluster analysis attempts to find the best solution to classify cases into groups even when data lack clear group structure (DePatta Pillar 1999). Consequently, cluster stability requires a method to test partition strength (James and McCulloch 1990). We used the bootstrap resampling method of DePatta Pillar (1999) with 1000 iterations to test cluster stability, to determine the probability distribution, and to obtain nonparametric estimates of standard error. If clusters are stable, random variability between clusters should exceed variability within clusters. Accordingly, failure to reject the null hypothesis of stable group structure for the proposed number of clusters is consistent with group stability at the suggested confidence level of $\alpha = 0.10$ (DePatta Pillar 1999). We assigned resultant clusters descriptive labels, but do not imply support for any particular hierarchical terminology. After assigning individuals to an interval of development, a Discriminant Function Analysis (DFA) performed on the species-pooled scores for the 10 characters provided the interspecific criteria that discriminated intervals.

Results

Analysis of total character scores revealed three primary clusters or intervals of development (Figs. 1 and 2). Bootstrap resampling of data at the three-cluster level failed to reject the null hypothesis of group stability ($p > 0.15$); therefore, each cluster was assigned a descriptive label. One cluster, termed 'Larvae,' contained specimens with total character scores ≤ 13 and O_L values < 80 . A second

Table 1. Character states used to score the young of five species of blenny from the northern Gulf of Mexico. Each character state represents a separate ontogenetic event.

| | |
|--------------|---|
| Score | Dorsal fin rays |
| 0 | Finfold, fin base thickening, or anlag only |
| 1 | Initial segmentation of earliest developing ray |
| 2 | Terminal ray of fin initially segmented |
| 3 | Pigment along shaft of at least one ray |
| 4 | Pigment along shaft of all rays |
| 5 | Consolidation of pigment into stripes, bars, or bands |
| | Caudal fin |
| 0 | Preflexion |
| 1 | Initial segmentation of earliest developing primary caudal ray |
| 2 | All primary rays initially segmented; secondary rays thickening |
| 3 | All rays formed (primary and secondary) |
| 4 | All primary rays with pigment along shaft |
| 5 | Initial bifurcation of any primary ray |
| | Body pigmentation pattern ¹ |
| 0 | No trunk pigment laterally (excludes visceral mass and pectoral fins) |
| 1 | Proliferation of head pigment, especially above hindbrain and along operculum |
| 2 | Epidermal pigment behind nape (initial formation of first band of trunk pigment) |
| 3 | Two or more bands of pigment forming dorsolaterally along trunk |
| | Pectoral fin |
| 0 | Finfold or incipient rays only |
| 1 | Initial segmentation of earliest developing ray |
| 2 | All rays initially segmented |
| 3 | New epidermal pigment present on pectoral axil, rays, or fin membrane |
| 4 | Reduction in original pectoral fin pigmentation (fin initially pigmented) ² |
| 5 | Complete loss of original pectoral fin pigmentation pattern (All rays pigmented) ² |
| | Pelvic fin pigment |
| 0 | Absent |
| 1 | Usually a single melanophore midway along shaft of ray or on membrane between rays |
| 2 | Loss of aforementioned melanophore; pigment now scattered over pelvic fin base and basal portion of shaft |
| | Orbital cirrus |
| 0 | Absent or thickening nub without free distal margin |
| 1 | Distal margin free, filamentous, and unpigmented; may be furcate |
| 2 | Cirrus lightly pigmented; may be multiply furcate |
| | Nasal cirrus |
| 0 | Absent or thickening nub without free distal margin |
| 1 | Distal margin free, filamentous, and unpigmented; may be furcate |
| 2 | Cirrus lightly pigmented; may be multiply furcate |

(Table 1 continued)

| Score | Number of teeth |
|---|--|
| 0 | 6 or fewer |
| 1 | 8–10 |
| 2 | 12–14 |
| 3 | 16–18 |
| 4 | 20–22 |
| 5 | 24 or more |
| Extent of bony ossicle formation along upper portion of lateral line | |
| 0 | Anterior to 3 rd dorsal spine |
| 1 | Terminates between 3 rd and 6 th dorsal spines |
| 2 | Terminates between 6 th and 9 th dorsal spines |
| 3 | Beyond 9 th dorsal spine |
| Longest preopercular spine relative to orbit diameter | |
| 0 | Increase in spine length |
| 1 | Decrease in spine length |
| 2 | Longest spine nub-like or entirely resorbed |
| Type of teeth ³ | |
| 0 | Villiform teeth only |
| 1 | Mixed villiform and 'spade-shaped' incisiform teeth |
| 2 | Incisiform teeth (except posterior-most), minor changes in tooth shape hereafter |
| Dorsal spines ³ | |
| 0 | Finfold or anlag |
| 1 | Formation of less than 50% of spines |
| 2 | All spines formed and structurally distinct |

¹ See text for explanation of differences in pigmentation patterns between *Parablennius marmoratus* and the other four species

² Opposite fin pigmentation pattern. Young *Parablennius marmoratus* lack pectoral fin pigment until just before settlement

³ Characters included in total character state score but not analyzed further

cluster (termed 'Metamorphs') included specimens with total character scores that generally ranged from 15 to 30. Metamorphs had O_L values between 80 and 90.3. A third cluster, labeled 'Settlers,' contained specimens with total character scores ≥ 25 , and O_L values ≥ 83 . Settlers were collected from a demersal habitat (Table 2). The term larvae as commonly defined includes both our Larvae and Metamorphs categories, which are artificial labels assigned for convenience (Table 3).

Discrimination of interspecific intervals of ontogeny was achieved in the species-pooled data set. Two canonical roots extracted all variability between intervals ($p < 0.0001$; Fig. 1B; Table 3).

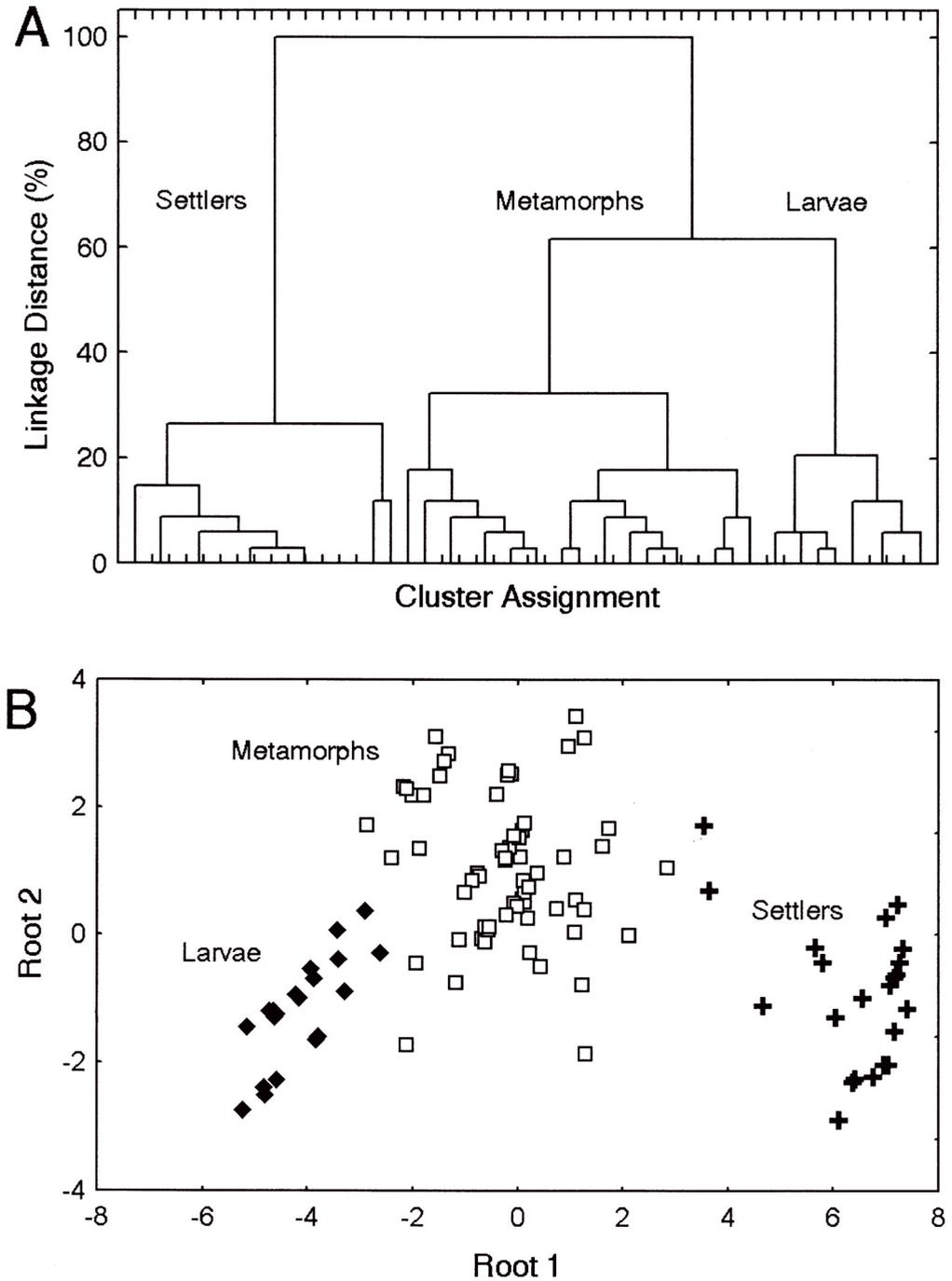


Figure 1. Intervals of development for the pooled data set of five species of blenny from the northern Gulf of Mexico. A. Typical cladogram from cluster analysis. B. Intervals of development as recognized by discriminant function analysis.

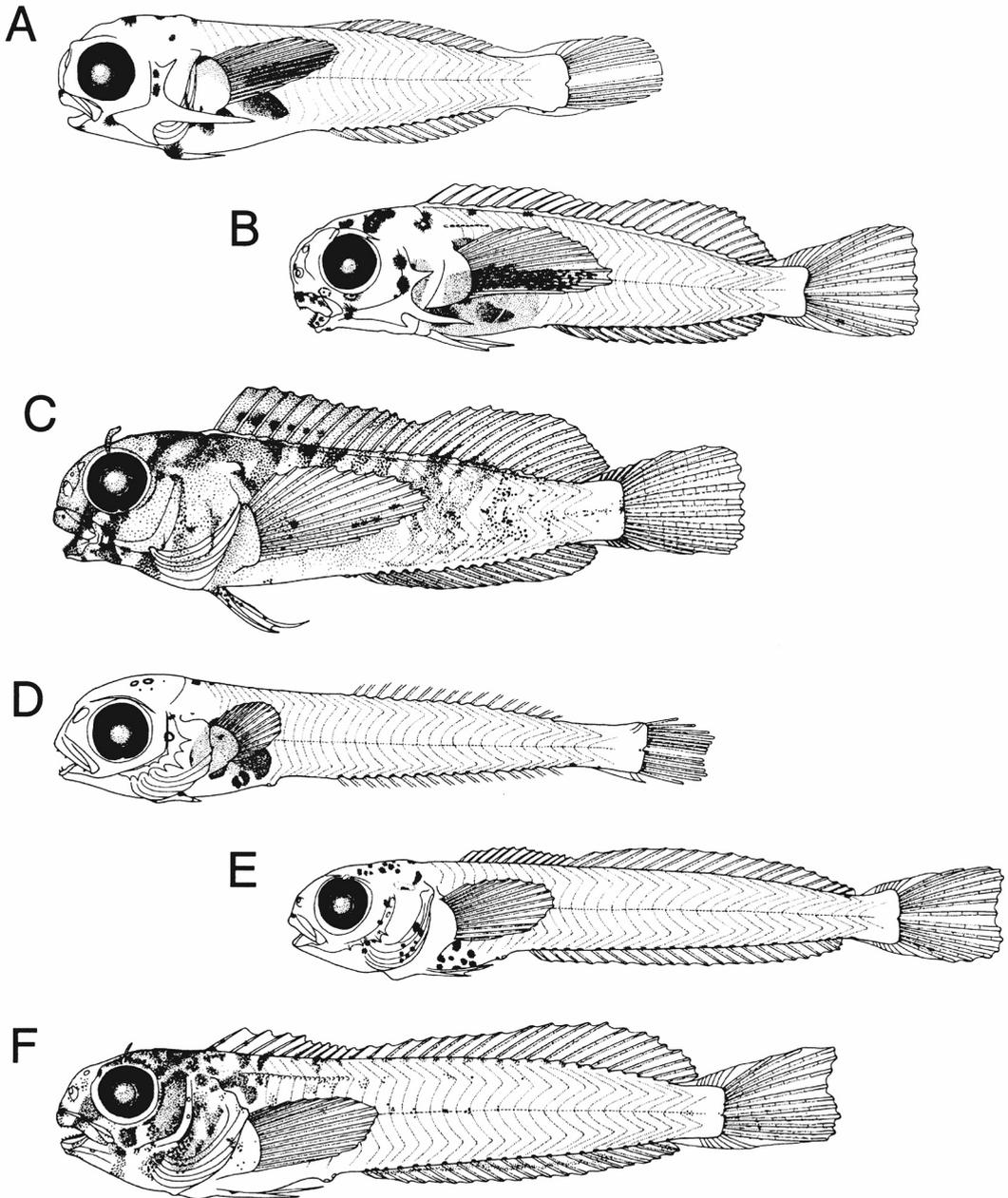


Figure 2. Early life stages of *Hypsoblennius ionthas* (A-C) and *Parablennius marmoreus* (D-F) from the northern Gulf of Mexico. A. 5.4-mm; B. 10.2-mm; C. 11.8-mm; D. 5.8-mm; E. 13.7-mm; F. 17.3-mm (standard length). Larva (A), Metamorph (B), recent Settler (C) of *H. ionthas*; Larva (D), Metamorph (E), late Metamorph (F) of *P. marmoreus*. These two species represent the extremes in development for the five species studied.

Table 2. Summary information for five species of blenny from the northern Gulf of Mexico. Intervals (Larvae, Metamorphs, Settlers) were determined by clustering total character scores for a suite of 10 characters. Ontogenetic index = $\log SL/\log L_{\text{juv}} \times 100$, where *SL* is standard length of an individual and *L_{juv}* is *SL* at the start of the juvenile stage. Total character score is the sum of scores assigned to the individual characters listed in Table 1. All sizes are mm SL. Statistics given are mean and (range).

| Category | <i>Hypsoblennius invemar</i> | <i>Hypsoblennius ionthas</i> | <i>Hyppleurochilus multifilis</i> | <i>Parablennius marmoreus</i> | <i>Scartella cristata</i> |
|--|------------------------------|------------------------------|-----------------------------------|-------------------------------|---------------------------|
| Overall summary | | | | | |
| Sample size | 55 | 41 | 42 | 50 | 30 |
| Size range | 5.4–18.3 | 5.0–17.5 | 5.3–18.3 | 5.8–21.5 | 5.8–18.0 |
| Range of ontogenetic index | 58.5–100.7 | 56.8–101.0 | 57.4–100.0 | 50.7–88.5 ¹ | 60.6–100.0 |
| Range of total character score | 1–41 | 1–41 | 1–41 | 1–37 ¹ | 1–42 ² |
| Larvae | | | | | |
| Sample size | 9 | 8 | 6 | 14 | 5 |
| Size | 9.7 (8.2–11.0) | 8.0 (7.0–9.1) | 8.9 (8.0–11.0) | 14.2 (12.2–16.0) | 7.9 (6.8–9.5) |
| Ontogenetic index | 78.4 (72.9–81.2) | 73.3 (68.7–77.9) | 75.1(71.5–82.5) | 76.4 (72.2–80.0) | 71.3 (66.3–77.9) |
| Total character score | 9.2 (6.0–13.0) | 6.8 (4.0–9.0) | 7.7 (6.0–12.0) | 9.7 (7.0–11.0) | 7.0 (6.0–9.0) |
| Metamorphs | | | | | |
| Sample size | 19 | 18 | 19 | 19 | 6 |
| Size | 12.3 (11.0–13.5) | 10.7 (9.7–11.5) | 12.5 (11.5–13.8) | 19.5 (17.0–21.5) | 10.6 (10.2–11.0) |
| Ontogenetic index | 86.9 (83.1–90.2) | 83.5 (80.2–86.2) | 86.9 (84.0–90.3) | 85.6 (81.8–88.5) | 81.7 (80.3–83.0) |
| Total character score | 19.7 (16–27) | 14.9 (12–18) | 19.5 (15.0–24.0) | 20.8 (15–30) | 21.0 (16–25) |
| Size range ³ | 12.7 (12.0–13.5) | 11.3 (11.0–11.5) | 12.9 (12.2–13.8) | 20.9 (20.5–21.5) | 10.6 (10.2–11.0) |
| Ontogenetic index ³ | 88.1 (86.1–90.2) | 85.6 (84.6–86.2) | 87.8 (86.1–90.3) | 87.7 (85.7–88.5) | 81.5 (80.3–83.0) |
| Total character score ³ | 23.2 (22–27) | 17.2 (16–18) | 26.4 (23–31) | 21.8 (19–24) | 22.0 (19–25) |
| Settlers | | | | | |
| Sample size | 18 | 8 | 14 | 9 | 17 |
| Size ⁴ | 12.2 (11.8–12.8) | 12.1 (11.7–12.7) | 12.1 (11.8–12.3) | 19.3 (19.0–19.5) | 11.3 (11.0–11.5) |
| Ontogenetic index ⁴ | 86.7 (85.6–88.4) | 87.9 (86.8–89.7) | 85.8 (84.9–86.3) | 86.0 (85.5–86.5) | 84.0 (83.0–84.5) |
| Total character score ⁴ | 27.3 (25–32) | 32.7 (30–38) | 34.0 (31–36) | 37.0 (37–37) | 29.3 (27–33) |
| Size at juvenile (<i>L_{juv}</i>) ⁵ | 17.9 | 17.0 | 18.3 | 31.2 ⁶ | 18.0 |

¹ Low upper range of ontogenetic index and total character score is the result of not having collected specimens with bifurcate caudal fin rays (i.e., juveniles)

² Only *Scartella cristata* have nuchal cirri

³ Three largest Metamorphs

⁴ Three smallest Settlers

⁵ Size at initial bifurcation of any primary caudal ray

⁶ Estimated size at initial bifurcation of any primary caudal ray

Table 3. Canonical root means used to discriminate intervals of development for the pooled data set of five species of blenny from the northern Gulf of Mexico. Distance between means is a measure of how clearly discriminant functions separate intervals. Compare sign of each root with characters in Table 4 to determine which characters are associated with each interval. The developmental interval most clearly separated by a given root is in bold.

| Interval of development | Numbers of specimens | Size range (mm SL) | Root 1 | Root 2 |
|-------------------------|----------------------|--------------------|--------------|--------------|
| Larvae | 42 | 6.8–16.0 | –4.283 | –1.154 |
| Metamorphs | 82 | 9.7–21.5 | –0.206 | 1.013 |
| Settlers | 64 | 11.0–20.5 | 6.410 | –0.978 |

Root 1 discriminated Settlers from Metamorphs and Larvae, and root 2 separated Metamorphs from the other two intervals (Fig. 1B). State of orbital cirrus and fin development, number of teeth, and body pigmentation pattern provided most of the power to discriminate intervals in these blennies.

Larvae of all five species had 10 or fewer fang-like or conical teeth along each jaw, lacked pigment laterally on the trunk, and most lacked the ocular cirrus (if developing, then nub-like and unpigmented). Early Metamorphs had 12 to 14 incisiform teeth along each jaw; filamentous, unpigmented orbital cirri; and epidermal pigment on the trunk immediately behind the nape. Pigment behind the nape initiated formation of the first trunk band. *Parablennius marmoratus* had a somewhat different pattern of body and fin pigmentation (Fig. 2). Whereas presettlers of the other four species of blenny had moderately to heavily pigmented pectoral fins, *P. marmoratus* lacked pectoral fin pigment until just before settlement. In addition, late Larvae had a single melanophore along the dorsal midline of the caudal peduncle behind the last pterygiophore at about 14.0-mm SL (Fig. 2E). Melanophores were added along the dorsal midline in an anterior direction and reached the nape in late Metamorphs. Thereafter, bands of pigment formed along the trunk (Fig. 2F). Consolidation of pigment into bands, pigmentation of the orbital cirrus, the presence of 16-18 teeth, and formation of the nasal cirrus signaled approaching settlement in all five species of blenny. Recent Settlers had multiply furcated orbital cirri, a full complement of well-developed elements in all fins, typically 18 or more teeth, and a mottled pigmentation pattern.

Discussion

Assigning discrete character scores to individual ontogenetic events identified three natural intervals of development in these five blennies. Settlers had higher dorsal/anal ray and orbital cirrus scores than Metamorphs, and Metamorphs had higher trunk pigmentation, dorsal/anal ray, orbital cirrus, and dentition scores than Larvae (Tables 3 and 4). While changes in pigmentation may permit rapid and reasonably accurate determination of developmental intervals for large numbers of specimens with little manipulation, the use of general (i.e., non-specific) pigmentation patterns combined with other ontogenetic characters will provide better resolution in interval discrimination than pigment alone. Pigmentation patterns are genetically determined and among the most useful characteristics of fish taxonomy (Kendall et al. 1984), but are subject to preservation problems associated with bleaching and melanophore contraction. Consequently, the use of general but quantifiable patterns would minimize problems associated with the presence/absence of specific melanophores. Although convergent patterns can limit the usefulness of pigment in systematic studies (Kendall et al. 1984), this same commonality in pigmentation is valuable for interval discrimination.

Behavioral changes, such as settlement in blennies (Ditty 2002), and the initiation of schooling in some clupeids (Noakes and Godin 1988) and carangids (Masuda and Tsukamoto 1999) often coincide with changes in relative growth. Quantification of shape change based on body depth to body length ratios (Leis and Carson-Ewart 2000) or allometric methods (van Snik et al. 1997;

Table 4. Standardized canonical coefficients from a Discriminant Function Analysis of the species-pooled data set of 10 ontogenetic characters to identify which characters delimit intervals of development in five species of blenny from the northern Gulf of Mexico. Coefficients identify the interspecific suite of characters that best delimit intervals of development and represent the magnitude of each variable's contribution to that root. Compare sign of each character with roots on Table 3 to determine which characters are associated with each interval. Characters that provide the best discrimination are in bold.

| Character | Root 1 | Root 2 |
|---------------------|--------------|--------------|
| Preopercular spine | -0.047 | 0.042 |
| Orbital cirrus | 0.399 | 0.451 |
| Nasal cirrus | 0.058 | 0.075 |
| Number of teeth | 0.067 | 0.601 |
| Dorsal/anal rays | 0.580 | -0.576 |
| Pectoral fin | -0.025 | -0.040 |
| Pelvic pigmentation | -0.099 | -0.201 |
| Caudal fin | 0.256 | 0.125 |
| Body pigmentation | 0.057 | -0.594 |
| Lateral line | 0.361 | 0.294 |

Gisbert 1999; Neuman and Able 2002; Ditty 2002) may help delimit at least some intervals of development. In fact, changes in shape often occur at discontinuities, such as metamorphosis (Emerson and Bramble 1993) and the onset of the juvenile stage (Copp and Kovac 1996; Vilizzi and Walker 1999).

Lack of guidelines for character selection has made it difficult to choose a uniform suite of characters to develop a universal staging system, a problem magnified for blennies and other fishes that have relatively little spination (other than preopercular spines) and few obvious external characters. Traditionally, SL at complete formation of all median fin rays, at initial squamation, or size at settlement has defined the beginning of the juvenile stage, L_{juv} (Fuiman 1994), although Fuiman (1997) has suggested more recently that squamation should be complete. Reliance on a single character, such as initial or complete squamation, to determine when the juvenile stage begins as has been proposed, creates problems in scaleless fishes, such as blennies, and in fishes (swordfish, squirrelfish, tilefish, and anthiinae serranids) that complete scale development while still considered larvae (Kendall 1979; Potthoff and Kelley 1982; Leis and Carson-Ewart 2000).

Some types of characters are more difficult to evaluate than others are. Incorporation of behavioral characters into a staging system necessitates live material, a requirement not easily met. Similarly, clearing and staining to observe structural development is labor intensive and requires a large number of specimens to establish patterns of ossification (Potthoff 1984). In addition, progressive and continuous changes in ossification are more difficult to quantify than characters that exhibit a distinct ontogenetic change. Monitoring changes in body shape, scoring discrete ontogenetic events, such as segmentation of fin rays, and the use of general pigmentation patterns are more-practical criteria, although quality of pigmentation is subject to various preservation related problems. We encourage the assessment of a variety of characters (i.e., morphological, behavioral, physiological, pigmentation, etc.) to delineate intervals, when possible, because the number of characters can influence the likelihood that intervals coincide with any natural process or impor-

Table 5. A standardized procedure to determine 'natural' intervals of development in the early life of fishes.

Steps:

- 1) Select character set to examine
- 2) Assign scores to individual character states (each change in state represents an ontogenetic event)
- 3) Dip specimen in a solution of Cyanine Blue 5R (Acid Blue 113) to enhance anatomical contrast
- 4) Score individual characters and calculate a total character score for each specimen
- 5) Select linkage and distance rules, then submit total character scores to a clustering program
- 6) Review resultant tree diagram and determine the minimum linkage distance that separates clusters
- 7) Determine the number of clusters and submit data to a bootstrap resampling procedure to test cluster stability¹
- 8) If clusters are stable, assign a descriptive label to each interval
- 9) Assign individuals to their interval of development
- 10) Run a Discriminant Function Analysis on the species-pooled suite of scores for the characters examined to determine the interspecific criteria that best delineates intervals
- 11) Formulate an ontogenetic index to scale data (for interspecific comparisons)²
- 12) Calculate basic statistics to summarize data

¹ DePatta Pillar (1999)

² Fuiman (1994)

tant function (Crowley 2000). A large number of characters will improve the resolution of patterns, while too few characters can impair pattern interpretation (Crowley 2000).

Evaluation of external characters to develop a staging system should provide information equivalent to that of internal characters, at least until all larva-specific characteristics are lost. The fact that all teleosts have fins and that patterns of fin development are generally familial (Kendall et al. 1984) encourages the use of fin ontogeny as a primary source of characters to develop an interspecific staging system. Dentition offers another reliable source of characters because most, but not all, teleosts have teeth. Species-specific characters (e.g., length of preopercular spines and when they are resorbed), however, must be eliminated from the suite of characters used to demarcate intervals, if the objective is interspecific comparison.

Our methodology (Table 5) reduces the subjectivity inherent in traditional staging systems and is robust, if characters typically considered qualitative (e.g., degree of pigmentation, body shape) are quantified carefully. Different patterns of pigmentation, as described here, can be quantified if analogous patterns are established 'a priori' for all species studied. As with any staging system, the researcher must make certain initial decisions about character selection and score assignment. Incorporation of the ontogenetic index into our methodology minimizes interspecific differences in size, given that size is not a reliable indicator of developmental state across taxa. Environmental factors can slow or interfere with developmental processes and induce differences in size at comparable states of development that confound interspecific comparisons (Dettlaff and Dettlaff 1961; Fuiman and Higgs 1997; Fuiman et al. 1998). In these five blennies, the coefficient of variation (CV) for size at settlement based on SL was taxon-dependent and ranged from 4.6% to 7.1%, but was 24.1% when taxa were combined (Ditty 2002). Employing the ontogenetic index to compare interspecific differences in size at settlement resulted in a CV for the index of 1.6%. The fact that the timing and progression of ontogeny is relatively stable within a species (Alberch et al. 1979; Alberch 1985) is an attribute that makes our ontogenetic approach ideal to discriminate intervals of development.

Improved methodologies for characterizing intervals of development have several advantages. For example, our procedure may permit evaluation of habitat quality because ecological disturbances that disrupt physiological processes can affect both the direction and extent of morphological transformations, thereby altering relative growth rates and the timing of ontogeny (Strauss and Fuiman 1985; Jacobsson et al. 1986). The methodology described here may also facilitate examination of differences in life-history strategies and habitat-use patterns that can promote identification of essential fish habitat and important nursery areas for fishery species (Lindeman et al. 1998; Lindeman and Synder 1999). In addition, if the number of late Metamorphs adequately represents the number of recent Settlers in examining abundance patterns (Schmitt and Holbrook 1999), monitoring the supply of the more easily sampled Metamorphs may allow better prediction of year-class strength (Bradford 1992). Although relationships between fisheries recruitment and stock size do not strengthen sufficiently to predict year-class strength until after settlement in demersal fishes (Bradford 1992), improved methods of estimating the number of potential recruits approaching settlement may increase predictive accuracy (Milicich et al. 1992; Thorrold 1992; Meekan et al. 1993). Better characterization of intervals may also permit evaluation of stage-specific mortality rates and provide a meaningful test of the concept of critical periods of development. Similarly, our methodology could be used to investigate ways to adjust the interpretation of shifts in otolith daily ring structure and improve information in that historical record.

In conclusion, scoring individual characters and summing character scores, combined with clustering techniques (either total scores or O_L values can be clustered), DFA, and statistical resampling procedures, as described here, provide a standardized and more objective methodology by which to characterize an individual's state of ontogeny and group individuals into comparable intervals. Categorization of individuals based on quantitative characters and objective treatment of characters facilitates evaluation and comparison of important ecological and early life history questions (Youson 1988). Assignment of character scores to discrete ontogenetic events, when combined with a dimensionless index of ontogeny, such as the O_L of Fuiman (1994), permits quantification of ontogeny, identification of the events that delimit developmental intervals, and interspecific comparisons. Our approach promotes recognition of intervals of development in preserved samples where it may be difficult to rely on pigmentation patterns and impossible to incorporate behavioral criteria. Elimination of species-specific characters and the diversity of teleosts and life-history patterns may prohibit development of a standardized staging system across higher taxonomic levels, although standardization at the family level appears possible.

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The use of kernel density estimators to analyze length-frequency distributions of fish larvae

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Abstract

The examination of size frequency distributions with time helps to analyze some useful parameters in the study of population dynamics. In this paper, we present the use of Kernel Density Estimators (KDE's) as a modern tool to examine length-frequency distributions of fish larvae with the case of *Pholis gunnellus* larvae as an example. Data were taken from a previous published work carried out at two hydrographically different sites in the Gulf of Maine, USA. At each site, sampling was performed from late winter to early summer during 1979 and 1980, at weekly intervals. Fish larvae were measured to the nearest 0.5 mm total length. As we only knew the number of larvae in each millimeter interval (from 9 to 30), we added random numbers around each discrete value in order to obtain a set of continuous data. The size distribution of each sample was analyzed by means of kernel density estimators, using a Gaussian function with the optimal bandwidth. The resulting distributions were decomposed in their Gaussian components with a computerized version of the Bhattacharya's method. Each component's means (corresponding to dominant modes) were plotted against time to follow the average growth of fish larvae. These values were fitted to a logistic function by means of a nonlinear least squares procedure. These analyses showed that mean size of larvae remains relatively constant during late winter samplings, suggesting a continuous larvae hatching and mortality. At spring establishment, when environmental conditions are more favorable for the survival of fish larvae, body size increases following a logistic function. Results indicated a short time lag (16 days) in larvae growth between the sites only in 1980. Hence, with this we illustrate that the use of KDE's, followed by the Bhattacharya's identification of Gaussian distribution components, and the analysis of main modes versus time, provide a more precise way to study length-frequency distributions than length-frequency tables alone.

Introduction

Many fisheries researchers have developed mathematical expressions to describe the growth of fish. Some of these procedures include the reading of growth marks in the hard fish structures, such as otoliths, escales, opercula, rays, spines and vertebrae (direct methods). Other methods are based on the analysis of the length-frequency throughout the time (indirect methods). Length-frequency distributions are commonly analyzed by means of histograms and frequency polygons. However, these procedures present several problems including dependency on grid origin and the interval width, discontinuity, and the use of fixed width intervals. These problems have motivated the interest of statisticians in alternative more efficient, computationally intensive methods. The Kernel Density Estimators (KDE's) do not depend on the origin position and are continuous distribution estimations (Silverman 1986). Besides, there are several methods for choosing the interval width (Härdle 1991; Scott 1992). These nonparametric estimators result in figures which are smoother than histograms, allowing easy recognition of characteristics such as outliers, skewness, and multimodality (Salgado-Ugarte et al. 1993, 1995). Most of these methods have been employed to describe young and adult fish growth; however, they have not been used to describe fish growth during their early life stages. In this study, we present the use of KDE's as a modern tool to examine length-frequency distributions of fish larvae, with the case of *Pholis gunnellus* larvae as an example.

This species, commonly called Rock gunnel or Butterfish, is widely distributed in the northern Atlantic. The Rock gunnel occurs in shallow waters, near the seashore and tidepools, but during winter, it can descend to 100 m or more (Martin and Bridges 1999). It is also found immersed under rocks, debris and seaweed at the low line tide (Kormanik et al. 1998), and its activity patterns are predominantly diurnal (Nickell and Sayer 1998). As an adult, the Rock gunnel feeds on polychaetes, molluscs, small crustaceans and fish eggs (Martin and Bridges 1999). In turn, this species is an important diet item of otters *Lutra lutra* (Mason and MacDonald 1980; Watt 1995), as well as that of some birds, such as the Double-crested Cormorants *Phalacrocorax auritus* (Blackwell et al. 1995) and the Black guillemots *Cephus grylle* (Ewins 1990). As a constituent of the intertidal fauna, the Rock gunnel can breathe air when it is out of water, and it also possesses a particular ability to excrete ammonia (Kormanick et al. 1998; Martin and Bridges 1999). Females lay eggs on the seabed, in empty shells or under stones (Muus and Nielsen 1999), but the knowledge of the biology and ecology of larvae is still limited. Therefore, in this work we try to document the growth of *Pholis gunnellus* larvae, as well to show the use of the modern statistical tool KDE, to examine fish larvae growth.

Materials and Methods

Data were taken from a previous published work (Townsend 1983) carried out at two hydrographically different sites in the Gulf of Maine: the Sullivan Harbor in the eastern Gulf of Maine, and the Damariscotta River estuary to the west (Fig. 1). According to Townsend (1983), hydrographic differences between these two sites influence the timing of the spring phytoplankton and zooplankton blooms, starting earlier in the western Gulf of Maine.

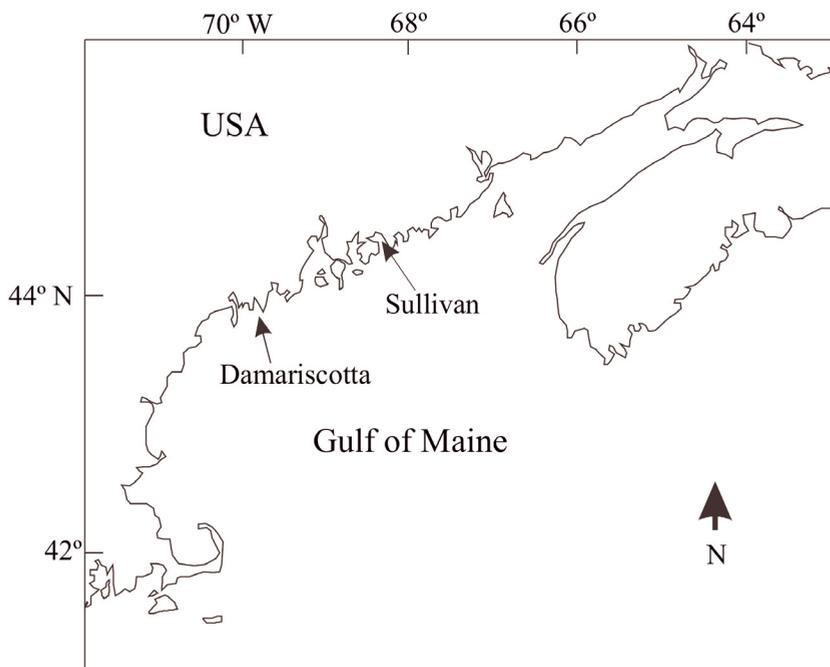


Figure 1. Sites of ichthyoplankton samplings, Damariscotta estuary and Sullivan Harbor in the Gulf of Maine, USA.

At each site, sampling was performed from late January to May in 1979 and 1980, at weekly intervals. Ichthyoplankton samples were collected during the day with a 61-cm mouth diameter Bongo net with 505 μm mesh size. Nets were towed for 10 minutes at ~ 3 knots. Samples were preserved in 5% buffered formalin. The total length of the *Pholis gunnellus* larvae was measured to the nearest 0.5 mm.

As we only knew the number of fish larvae in each millimeter interval (from 9 to 30), we added random numbers (uniform $U(-0.5, 0.5)$) around each discrete value in order to obtain a set of continuous data. Then, size distribution of each sample was analyzed by means of Kernel Density Estimators (KDE), a statistical method first proposed by Rosenblatt (1956) and defined as:

$$\hat{f}(x) = \frac{1}{hn} \sum_{i=1}^n K\left(\frac{x - X_i}{h}\right)$$

where,

$\hat{f}(x)$ = density estimation of the variable x

n = number of observations

h = bandwidth

X_i = length of the i -th fish specimen

$K(\bullet)$ = a smooth, symmetric kernel function integrating to one.

In this case, we use the Gaussian kernel function, *i.e.*:

$$K(z) = \frac{1}{\sqrt{2\pi}} \exp\left(-\frac{z^2}{2}\right)$$

where,

$$z = \frac{(x - X_i)}{h}.$$

One drawback of the KDE's is that they require a large number of calculations. Scott (1985) proposed an alternative more efficient procedure called "averaged shifted histogram" (ASH). Subsequently, Härdle and Scott (1992) developed the more general framework called "weighted averaging of rounded points" (WARP). This approach was used to calculate the KDE's in this work by using the programs presented in Salgado-Ugarte et al. (1997). Additional calculations were carried out with the XploRe software (Härdle et al. 1995).

Next, the optimal bandwidth h was chosen based on the Silverman (1986) rule:

$$h = \frac{0.9A}{n^{1/5}}$$

where,

$$A = \min \left[\left(\frac{\sum (X_i - \bar{x})^2}{n-1} \right)^{1/2}, \frac{\text{Fourth-spread}}{1.349} \right]$$

using the program included in Salgado-Ugarte et al. (1995). Note that A is the smaller of two estimates of the standard deviation: the usual estimate (s) and the so called *F-pseudosigma* a robust alternative for s (Hoaglin 1983). The Fourth-spread is a resistant dispersion measure approximately equivalent to the interquartile range (Tukey 1977).

The Silverman optimal bandwidth is designed for gaussian distributed data. This value is too large when applied with skewed or multimodal distributions. Nevertheless, in these cases the optimal bandwidth may serve as a reference starting point allowing to recover the dominant modes. Of course, the possibility to analyze in detail the distributions remains.

The resulting density distributions were rescaled to a (smoothed) frequency scale and decomposed into their Gaussian components with a computerized version of the method proposed by Bhattacharya (1967) which is included in the Stata software (Salgado-Ugarte et al. 1994; Stata-Corp 2001). Since the slope of a Gaussian curve is positive to the left of the mode, zero at the mode and negative to the right of the mode, the logarithmic derivative decreases linearly. Therefore, each Gaussian component can be determined by identifying negative sloped intervals in the graph of logarithmic frequency differences against length midpoints (Salgado-Ugarte et al. 1994).

Component's means (corresponding to the dominant modes) were plotted against time to follow the average growth of fish larvae. These values were fitted to a four-parameter logistic function by means of a nonlinear least squares procedure. This regression function is described by the following equation:

$$L_t = a + \frac{b}{1 + \exp[-c(t - t_0)]}$$

where,

L_t = average body length at t time

a = initial body length of specimens

b = when added to the a parameter, it indicates the asymptotic body length

c = curvature parameter which determines how fast the asymptotic body length is attained

t_0 = time at which the maximum growth is attained (inflexion point of L_t).

Note that the second part of this expression is similar to three-parameter logistic function.

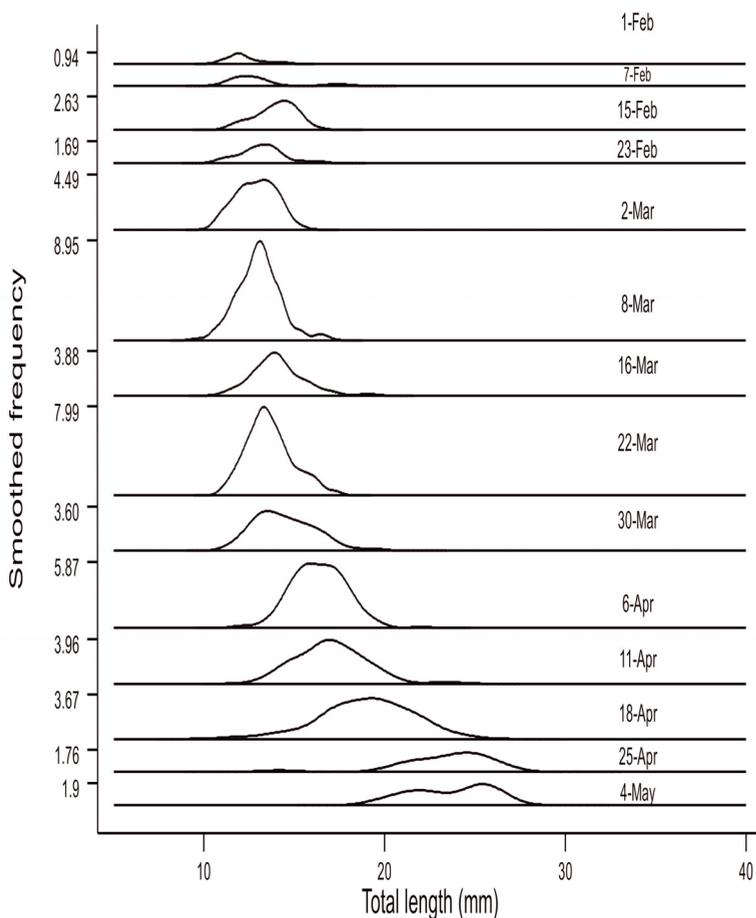


Figure 2. Sequence of smoothed histograms referring body length of Pholis gunnellus larvae from Damariscotta estuary, 1979. Horizontal lines indicate frequency = 0.

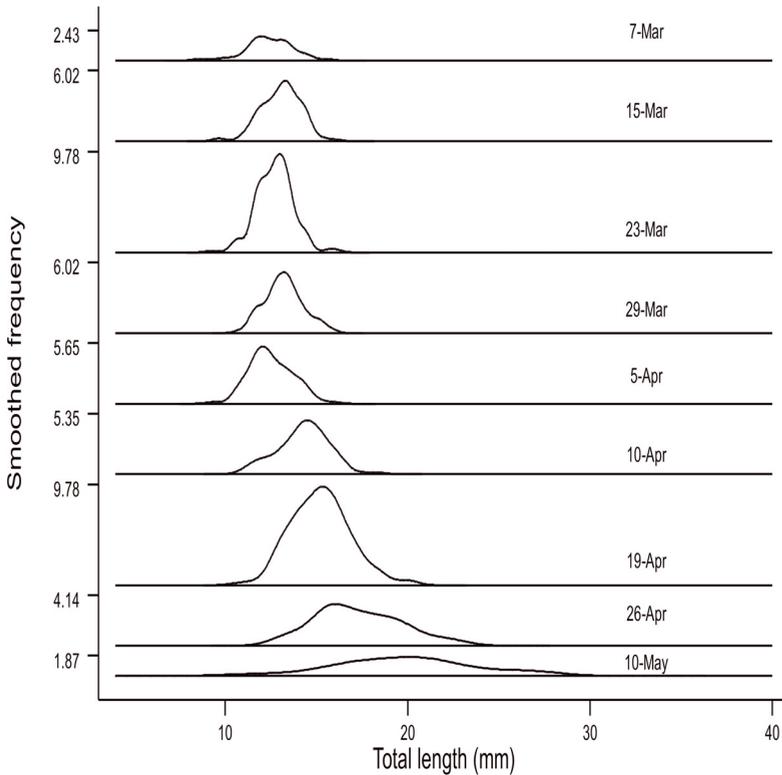


Figure 3. Sequence of smoothed histograms referring body length of *Pholis gunnellus* larvae from Sullivan Harbor, 1979. Horizontal lines indicate frequency = 0.

Results

More than 900 body length observations from Rock gunnel (*Pholis gunnellus*) collected in the Gulf of Maine by Townsend (1983) were analyzed by means of KDE's for each year and site. Figs. 2 to 5 show the weekly sequence of larvae length-frequency distributions. In all cases, at the beginning of sampling period, specimen size was very small with low frequency.

Optimal bandwidths used to construct KDE's varied between 0.22 to 1.8 mm depending on the number of observations and their variation. In most of the smoothed histograms, we observe a dominant mode with several minor modes. Assuming Gaussianity, we estimated the parameters (mean, standard deviation and size) corresponding to each main mode by the Bhattacharya's method (Fig. 6). Dominant modes indicate groups of fish with similar age (cohorts).

The means of the Gaussian components corresponding to the main modes were plotted against dates and fitted according to a logistic function (Figs. 7 to 10). The estimated logistic function parameters by means of a nonlinear least squares procedure, had adjusted $r^2 > 0.88$ and $P < 0.05$ (Table 1). The analysis of the adjusted logistic curves show that the mean size of larvae remains relatively constant during the late winter and early spring samplings (Figs. 7 to 10); at spring establishment, body size increases. Curvature parameters do not show a particular trend between sites

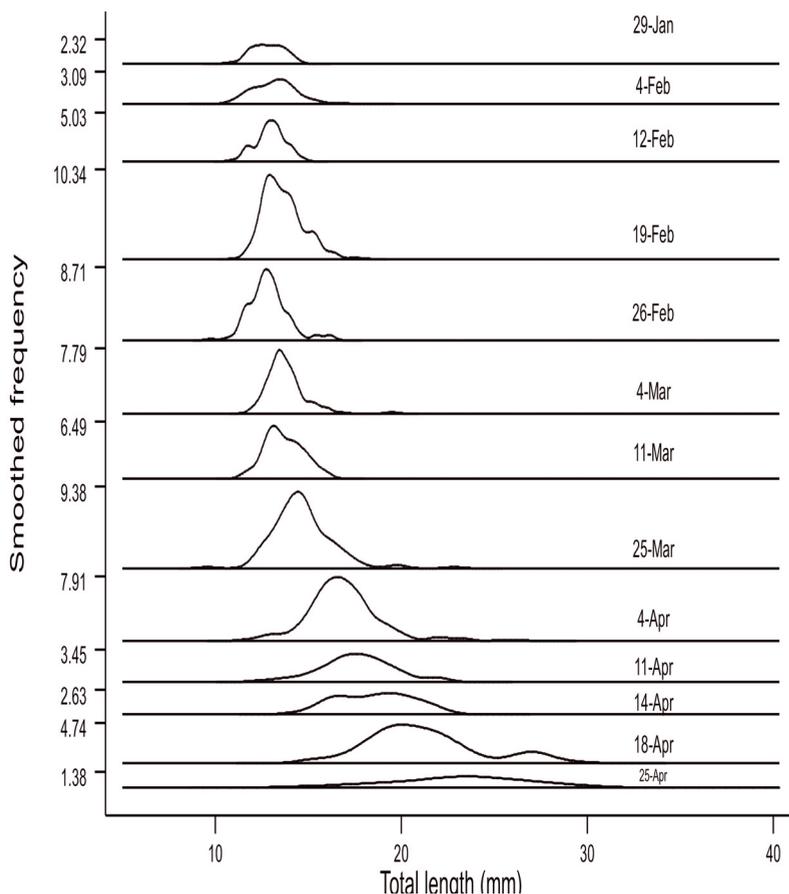


Figure 4. Sequence of smoothed histograms referring body length of *Pholis gunnellus* larvae from Damariscotta estuary, 1980. Horizontal lines indicate frequency = 0.

or years.

Discussion

Most of the studies concerning the analysis of size frequency data use histograms. Nevertheless, according to Fox (1990), the use of this graphical procedure presents some problems. i) Dependency on the origin. Arbitrary position of the histogram origin can result in deceptive estimations of the frequency distribution, since the same data set might show different number of modes depending on the origin position. ii) Dependency on the width and number of intervals (bins). Smoothness of the frequency distributions depends of both these parameters. The use of many bins results in a noisy estimator, and on the other hand, few bins reduce distribution details. iii) Discontinuity. This histogram characteristic depends on the arbitrary bin locations and discreteness of data set (Chambers et al. 1983). iv) Fixed bandwidth. Usually, the data density has a non Gaussian behavior, and it is difficult to choose an optimal bandwidth following simple rules. If a fixed bin is narrow enough to show details where density is high, it cannot avoid noise where density is low.

One of the methods addressed to solve these problems is the Kernel Density Estimator which

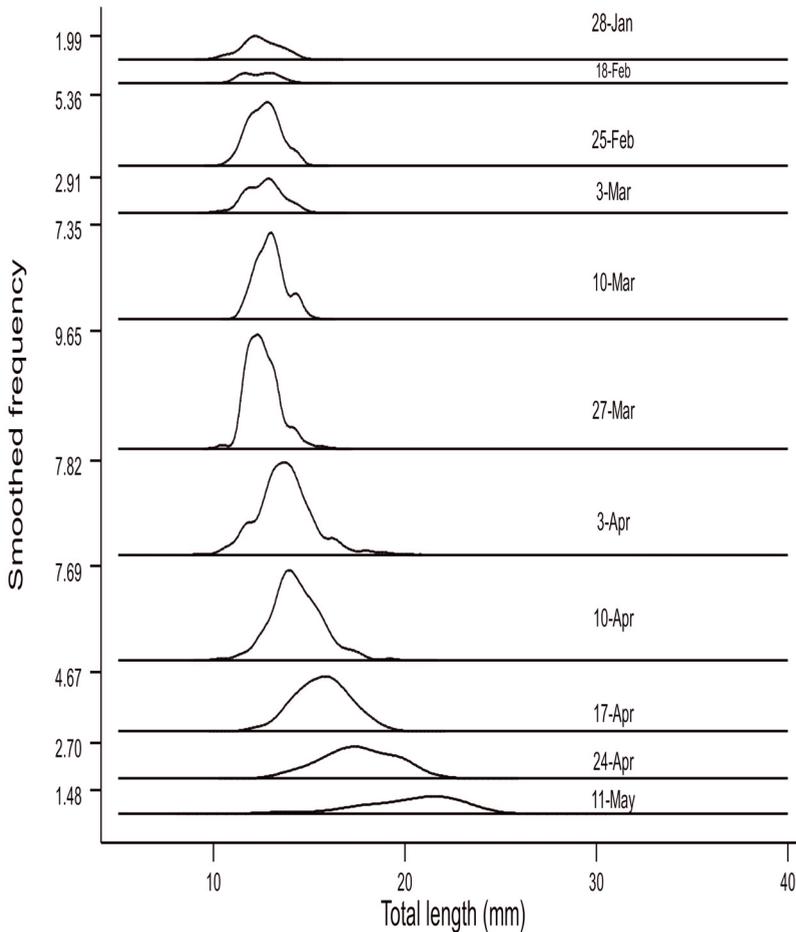


Figure 5. Sequence of smoothed histograms referring body length of Pholis gunnellus larvae from Sullivan Harbor, 1980. Horizontal lines indicate frequency = 0.

does not depend on the origin (the estimation is centered at each data point), is continuous as it uses a smoothly changing kernel function (instead of the rectangular shape), and it can use variable bandwidths. The problem of choosing the bandwidth remains, but several guidelines have been published (Silverman 1986; Härdle 1991; Scott 1992).

In this study, KDE's application and the subsequent fit to a logistic model of the mean values estimated from the main modes, shows that during the first weeks, larvae body length remains nearly constant (around 12 mm). The nearly constant body size observed during the first weeks (Figs. 7 to 10) may be caused by the continuous mortality of larvae, which are replaced by new small plankton recruits, as Townsend (1983) also argued.

Analyzing length-frequency tables alone, Townsend (1983) indicated that periods of an evident increment of fish larvae lengths were: from 8 to 30 March, and 11 to 25 March in Damariscotta during 1979 and 1980 respectively; and from 5 to 10 April, and 27 March to 10 April in Sullivan, during 1979 and 1980. According to the author, these results show the existence of a timing delay

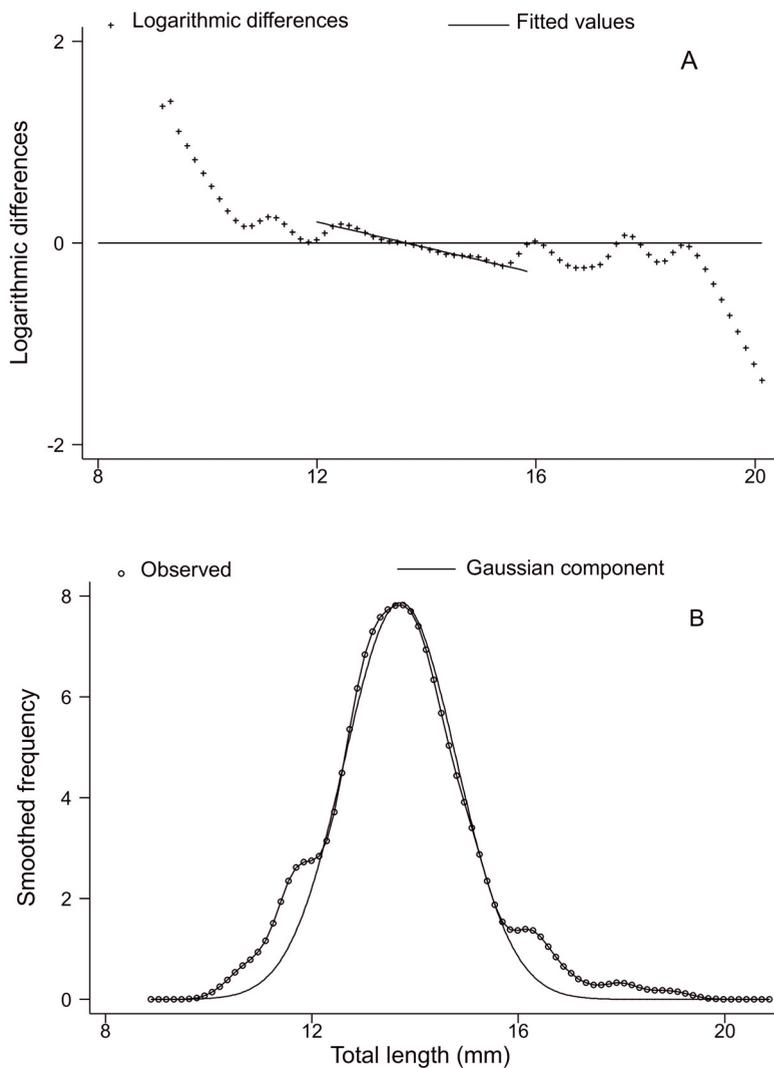


Figure 6. Bhat-tacharya's method for decomposition and characterization of Gaussian components; A) graph of the logarithmic differences against length; B) smoothed frequency and determined Gaussian component.

in the establishment of the size increase period of *Pholis gunnellus* larvae between Damariscotta estuary and Sullivan Harbor. Townsend (1983) argued that difference in temperatures and timing of phytoplankton blooms between both sites are the main causes of differences in larvae growth. If we consider that an evident growth means 0.1 mm/day, the times at which Rock gunnel larvae begin to increase in length were 6 April and 20 March for Damariscotta in 1979 and 1980 respectively; and 9 April and 5 April for Sullivan during 1979 and 1980. These results indicated a short time lag (16 days) between the sites only in 1980; a delay was also observed for the time at which the maximum growth was attained (Table 1).

After those starting growth dates, when favorable conditions are attained, the survival improves

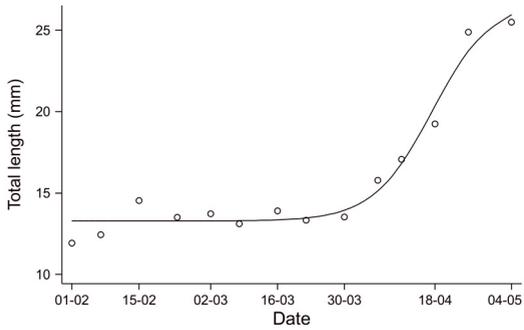


Figure 7. Logistic function adjustment to the growth of *Pholis gunnellus* larvae collected in Damariscotta estuary, 1979.

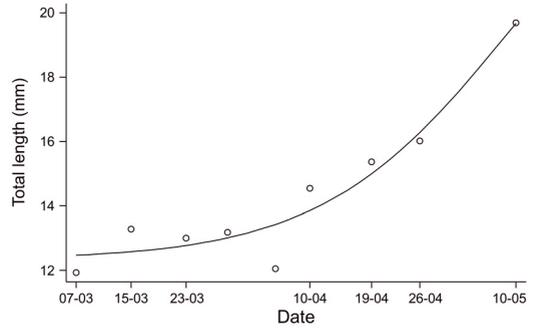


Figure 8. Logistic function adjustment to the growth of *Pholis gunnellus* larvae collected in Sullivan Harbor, 1979.

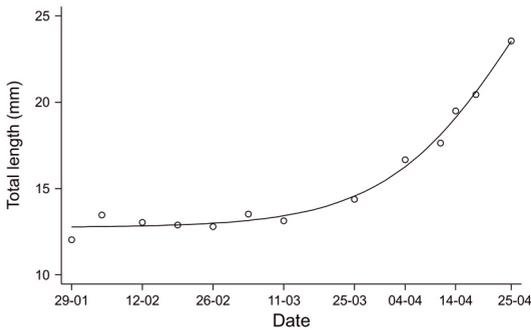


Figure 9. Logistic function adjustment to the growth of *Pholis gunnellus* larvae collected in Damariscotta estuary, 1980.

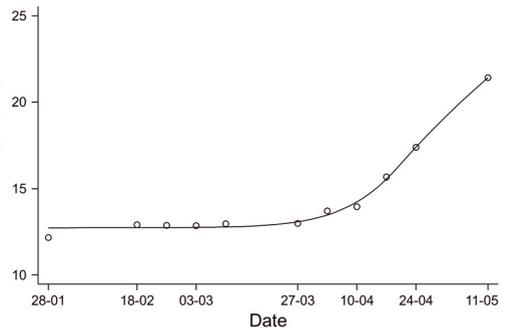


Figure 10. Logistic function adjustment to the growth of *Pholis gunnellus* larvae collected in Sullivan Harbor, 1980.

and the dominant group (successful cohort) begins to grow, this is easy to follow in the smoothed histograms (Figs. 2 to 5). According to Warlen (1988) larvae growth is controlled by two main external factors: temperature and food availability. Townsend (1983) mentioned that the small copepod nauplii (*Acartia* sp and *Eurytemora herdmani*), invertebrate eggs and barnacle casts are important food items in the diet of *Pholis gunnellus* larvae. As well, in 1979, water temperatures increased from about 3 °C at end March, to 8 °C at end April in Damariscotta, and to 6 °C in Sullivan. A similar trend was observed during 1980.

Several studies, based on otolith readings, have demonstrated that fish larvae growth follows a linear trend (Sánchez-Ramírez and Flores-Coto 1998), at least for several days after the yolk absorption. In this study, at which weekly length-frequency distributions were analyzed during a ~3 months period, we found a logistic trend, 30 mm being the biggest body size. We thought that these two procedures could be complementary methods. The logistic trend found here suggests

Table 1. Parameters of the nonlinear regression for the growth logistic function fitted for *Pholis gunnellus* larvae data. n = number of original observations in the length distribution from which dominant mode was followed. N = number of weeks.

| | n | N | r^2 | a | b | c | t_0 (days) |
|--------------------|------|-----|-------|-------|-------|------|--------------------------|
| Damariscotta, 1979 | 924 | 14 | 0.95 | 13.27 | 13.55 | 0.16 | 75.44 \approx 25 April |
| Sullivan, 1979 | 968 | 9 | 0.88 | 12.32 | 14.44 | 0.07 | 63.52 \approx 30 April |
| Damariscotta, 1980 | 1405 | 13 | 0.98 | 12.75 | 24.01 | 0.07 | 89.78 \approx 13 April |
| Sullivan, 1980 | 980 | 11 | 0.98 | 12.73 | 10.17 | 0.11 | 88.45 \approx 25 April |

that the growth of this species, until its adult stage (~ 25 cm), could be described by a series of steps. Then, a seasonal expression may be used for its description (fast growth during spring, slow during winter). Wyatt (1980) has well documented the differences between tropical continuous growth and higher latitudes discontinuous growth, as a consequence of time lag between phytoplankton and zooplankton production.

Despite the fact that frequency tables provide useful information on the growth of larvae cohorts, we have shown in this study an improved and more precise method to analyze length-frequency information. We have illustrated that it is clearer to see features in the smoothed histograms (KDE's), such as the modes of the length distributions, and it is easier to follow the progression of the dominant modes and, once characterized as Gaussian components, to use the estimated means for fitting a mathematical model describing the observed growth trend.

We leave for future research the possibility of using the bivariate kernel density method to estimate the joint distribution of length and time in one step. More advanced smoothing techniques allow for the use of different smoothing parameters in different regions of the data.

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New techniques for sampling larval and juvenile fish otoliths for trace-element analysis with laser-ablation sector-field inductively-coupled-plasma mass spectrometry (SF-ICP-MS)

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Key words: *ICP-MS, otolith, trace-elements, preservation*

Abstract

Trace-element signatures in otolith-growth bands provide a powerful tool for fish biologists to track fish movements, discriminate fish populations, and monitor aquatic environments. The ability to read these trace-element signatures has rested on the development of sophisticated instruments used by analytic chemists. Most recently, the preferred instrument has been inductively-coupled-plasma mass spectrometer (ICP-MS) for analysis of trace elements in biogenic carbonates, such as corals and otoliths. To preserve chronological information in otoliths, scientist use lasers to introduce samples into the ICP-MS. Moreover, the operational-sampling properties of lasers on corals have been well studied, but few studies have addressed laser-sampling performance in otoliths and none in otoliths of larval or juvenile fish. We show that laser-operating properties can be constrained to manipulate crater dimensions in otoliths as desired, and in so doing can be used to determine trace-elements reliably in larval and juvenile otoliths that are less than 1000 μm in size. Crater depths were greatest when the beam was held in one spot at high power and with the largest beam diameter. Crater depths varied from 10 to 500 μm , depending on the combination of beam diameter, laser-pulse frequency, and power. Crater-maximum diameters were consistently smaller than the beam diameters and depended on beam diameter. Importantly, otoliths are three-dimensional structures and to report only the crater width presents an incomplete picture of the life-history period that has been sampled. For example, a beam diameter that covers a week of life may excavate material from one month. Precise-depth laser sampling, as demonstrated here, provides significant improvements to our knowledge of life history and constrains sampling to the period of interest.

Introduction

Development of in-situ microanalytical techniques at trace levels had long been a dream of research scientists. For example, trace-element signatures in otolith-growth bands provide a powerful tool for fish biologists to track fish movements, discriminate fish populations, and monitor aquatic environments (Campana 1999; Smedbold et al. 2001; Thorrold et al. 2001). Several techniques have been applied to otoliths and other biogenic carbonates to obtain these data, such as electron microprobe (Gunn et al. 1992; Secor 1992; Bettencourt and Guerra 2000), proton-induced X-ray emission (PIXE; Sie and Thresher 1992; Arai et al. 1995; Procter et al. 1995; Allison et al. 2001; Weber et al. 2002), and laser-ablation inductively-coupled plasma mass spectrometry (LA-ICP-MS; Campana et al. 1994; Fowler et al. 1995; Chen and Jones 2002). The electron probe has a small beam diameter (5 μm), but is restricted by its high detection limits; 1000 ppm is typical (Cabri et al. 1985). Micro proton-induced X-ray emission (PIXE) which uses a proton probe (beam size 5-20 μm), yields a sensitivity only 3 to 30 times better than the electron probe (Remond et al. 1987). Secondary ion mass spectrometry (SIMS), an ion microprobe, also has been used with reported detection limits near the ppm level (Chrysoulis et al. 1989). Accelerator mass spectrometry (AMS) has also been used to analyze trace elements (Wilson et al. 1991; Rucklidge et al. 1992). Typically, however, the beam diameter is 0.5 mm, which is normally far too large to be focused on small growth bands (Rucklidge et al. 1992). Because of the expense in running an AMS machine, and the difficulties of reproducible preparation of samples, Rucklidge et al. (1992) did not consider this a viable method of analyzing sulphide concentrates for trace elements (Rucklidge et al. 1992). Also, the ion probe, PIXE and AMS are too costly for most scientists to use in routine analyses.

Most recently, the preferred instrument has been ICP-MS for analysis of trace elements in biogenic carbonates (Campana et al. 1997; Campana 1999; Chen and Jones 2002). The first ICP-MS instruments became commercially available in early 1984 following prototype development in the 1970's (Hieftje 1998). These instruments were readily accepted because they were able to detect isotopes in parts-per-billion concentrations, depending on the isotope, and were able to measure as many as 60 isotopes simultaneously (Ireland 1999). Since their introduction in the early 1990's, magnetic-sector ICP-MS is rapidly replacing the older quadropole instruments due to significantly improved mass resolution and sensitivity. The high mass resolution capability allows analysts to resolve polyatomic ion interferences for problematic analytes, e.g. $^{40}\text{Ar}^{16}\text{O}$ on ^{56}Fe (Thomas 2001).

The first use of ICP-MS for otolith trace-element chemistry was dependent on solution-based sample introduction. As sophistication with solution-based methods grew and revealed the value of using trace-element signatures, scientists sought to conduct surface sampling using instruments such as lasers. The desire to conduct surface sampling was mediated by an important consideration. Although detection is greater using solution-based introduction, dissolution of otoliths required by this method results in the loss of information encoded in otolith microstructure (Campana 1999; Kingsford and Gillanders 2000). Microstructural information is preserved when sample introduction is done by surface-based introduction using lasers. The first use of lasers for sample introduction to ICP-MS (LA-ICP-MS) was done in 1985 (Gray 1985) and its use in otoliths followed within a decade (see for example Fowler et al. 1995). Sample introduction using lasers is

not, however, without problems. Limitations of LA-ICP-MS are in its higher detection limits because less material is transported to the plasma, particle size is not optimal for ionization in plasma (Guillong and Guenther 2002), and scientists lack of a matrix-matched calibration standard. Moreover, lasers provide a transient signal that is not stable. Because of this instability, relative standard deviation (RSD) can be as high as 50% depending on the structure of the sample surface with up to 25% RSD using calibration standards (See table 5.3 in Taylor 2001). These RSD are higher than for solution-based introduction, which typically have RSD <1%.

The effects of laser radiation on solids have been reviewed thoroughly by Darke and Tyson (1993; also see Sinclair 1999 for use on corals), but no study has been conducted to determine the operational effects on otoliths. The operational properties of laser ablation depend upon both the characteristics of the laser beam and the physical properties of the solid. The history and recent developments of laser-ablation introduction systems have been extensively reviewed by Moenke-Blankenburg (1989). Otolith-sample introduction by laser ablation offers several advantages over conventional methods such as pneumatic nebulization of a solution. These include: reduced reagent and labor costs; elimination of dilution errors; minimization of reagent contamination; avoiding some interferences; and providing spatial information by allowing analysis of small selected areas.

In this study, we show that laser-operating properties can be constrained to manipulate crater dimensions in otoliths as desired, and in so doing can be used to determine trace-elements reliably in larval and juvenile otoliths that are less than 1000 μm in size. In working with small otoliths, we were forced to confront the lack of information available on the three-dimensional spatial resolution of laser craters. The purpose of this paper is to show the results of experiments to control laser-crater geometry and quantify crater depth, especially in small otoliths, as a function of beam factors such as beam diameter, laser power and frequency.

Materials and Methods

Instrument. The ICP-MS used in this study was a Finnigan ELEMENT 2 double focusing sector field (SF)-ICP-MS (Bremen, Germany). The software has been upgraded to the Windows-NT version 2.2. The magnetic and electric sector mass analyzers were in reversed Nier-Johnson geometry. The instrument can be operated in three resolution modes (M/ Δ M, 10% valley definition, low>300, medium>4000, and high>10,000) by changing manufacturer-predefined slits. Sample introduction was achieved using a combination of a PFA microflow nebulizer and a laser ablation (LA) system. The sensitivity for in low resolution mode is greater than 1.5 million counts per second per ppb in solution. Element concentrations down to sub ppt in solution can be measured.

The LA software was run in remote mode controlled by the ICP-MS software during LA-ICP-MS analysis. A beamsplitter directs approximately 2% of the laser beam to a built-in energy meter and the other approximately 98% of the beam to the objective lens. This arrangement allows an operator to continuously monitor the laser power before the final objective lens during ablation. Operation conditions are summarized in Table 1.

Table 1 Instrument operating conditions.

(a) SF-ICP-MS

| | |
|-----------------------------|--|
| Inductively coupled plasma- | |
| Plasma gas | Argon |
| Forward power | 1200 W |
| Reflected power | <6 W |
| Gas flow- | |
| plasma gas flow rate | 14.0 (L min ⁻¹) |
| Auxiliary gas flow rate | 0.8 (L min ⁻¹) |
| Inner gas flow rate | ~0.60 (L min ⁻¹) from nebulizer and ~0.25 (L min ⁻¹) from laser cell. |
| Interface- | |
| Torch | Fassel-type with guard electrode |
| Sampling aperature | Nickel, 1.1 mm diameter |
| Skimmer aperature | Nickel, 0.8 mm diameter |
| Ion lens settings- | |
| Extraction lens | -2000 V |
| Focus lens | -879 V |
| X-Deflection | -1.00 V |
| Shape Quad | -118 V |
| Y-Deflection | 3.08 V |
| Data acquisition parameters | |
| Resolution | 300 (low), 4000(medium) |
| Scan mode | E-Scan |
| Mass windows | 5% |
| Sample per peak | 200 |
| Dwell time | 15.0 ms |
| Sample introduction | |
| Nebulizer | PFA micro-flow nebulizer (50 μ L min ⁻¹) |
| Spray Chamber | PFA spray chamber |

(b) Laser ablation system

| | |
|------------------------------------|------------------------------|
| System: | New Wave Research EO LUV 266 |
| Laser type | Nd:YAG |
| Laser mode | Q-Switch |
| Wave length | 266 nm |
| Laser output frequency | 5-10 Hz |
| Laser energy before objective lens | <2 mJ |
| Spot size | 5-50(μ m) |

(c) Experimental operating conditions

| | |
|-----------------------|--------------------------------------|
| Raster type | Spot, line, raster |
| Beam diameter | 10, 20, 50 μ m |
| Laser pulse frequency | 5, 10 Hz |
| Laser power | 25, 50, 75, 100% |
| Travel Speed | 10, 20 (μ m sec ⁻¹) |
| Sampling time | 110 sec |

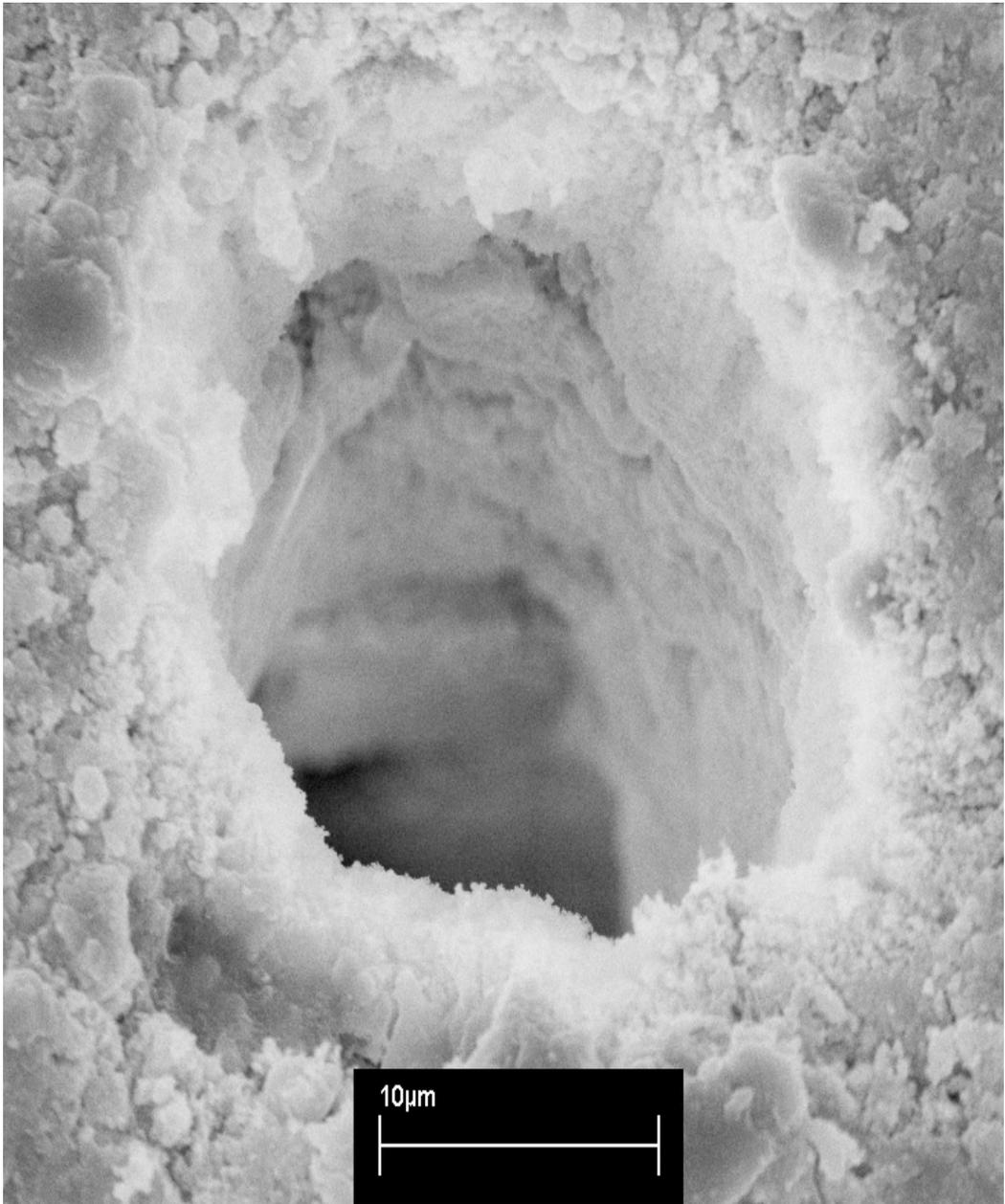


Figure 1. SEM of otolith showing a laser-ablation crater in spot configuration. Crater was made by holding the laser in one position at 50% power, 20 μm diameter beam, and at a pulse frequency of 10 Hz.

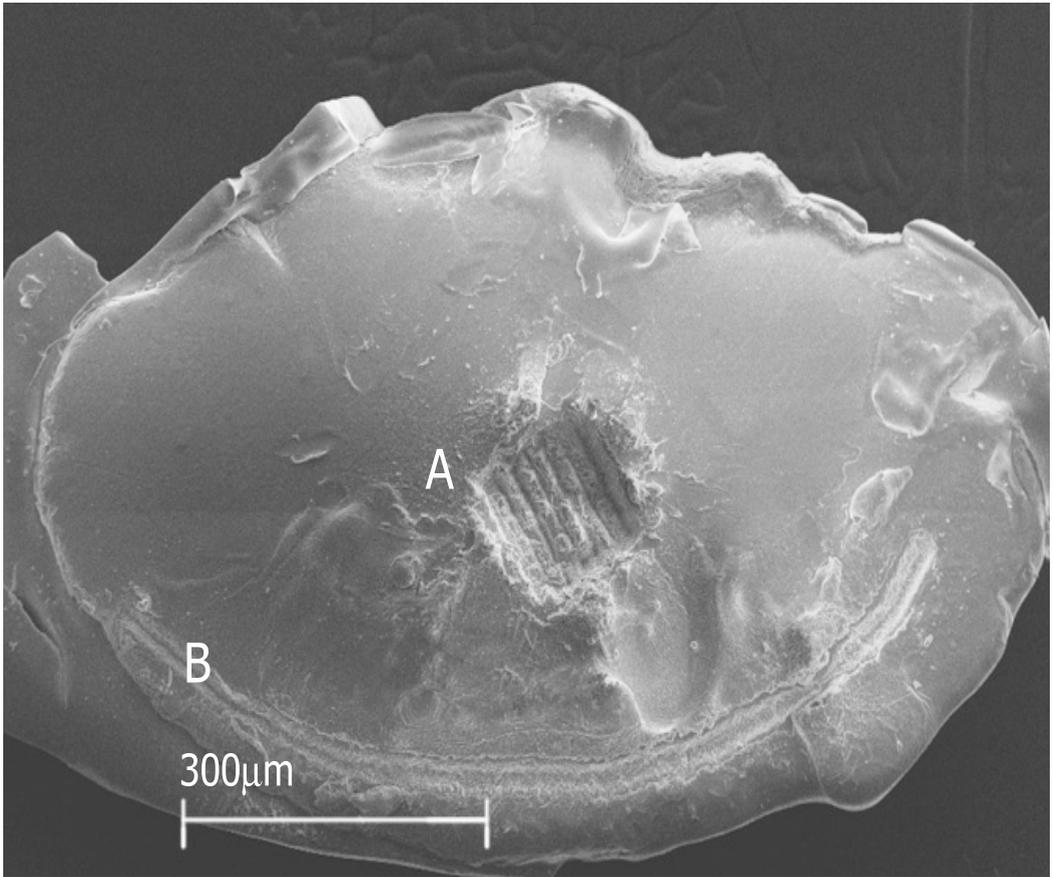


Figure 2. SEM of otolith showing two laser-ablation craters in A) rectangular-raster configuration and B) line configuration. The rectangular-raster crater was made by moving the laser in abutting lines at 50% power, 20 μm diameter beam, 10 $\mu\text{m sec}^{-1}$ travel speed, and at a pulse frequency of 10 Hz. The line-configuration was made using the same settings as the raster, but following a single non-abutting line.

Preparation of fish otoliths. We used otoliths that were previously extracted from late larval and early juvenile Atlantic croaker (*Micropogonias undulatus*, $n = 24$) collected in nearshore waters of Chesapeake Bay, USA, for experimental analysis of two raster types (spot and rectangles) and juvenile red drum (*Sciaenops ocellatus*, $n=7$) collected in Pamlico Sound, NC, USA for the predictive experiment on line raster types. All processing was done using trace-element clean procedures in a class-100 clean room. Upon extraction, we removed all remaining tissues or other particles from the otoliths using acid-washed glass probes. Otoliths were rinsed at least three times with milli-Q water. Otoliths were then soaked for 3-5 minutes in ultra-pure hydrogen peroxide (H_2O_2) and subsequently rinsed with Milli-Q water at least three times. Otoliths were mounted individually onto an acid-cleaned glass slides. Otoliths were ground until the nucleus was clearly exposed with Beuhler 30 μm lapping paper. Ground-otolith sections were then transferred to an acid-cleaned

Table 2. Properties of otolith-crater depth for a beam held in a constant position (raster type = spot) using a combination of beam diameter, laser pulse frequency and power.

| Beam Diameter (µm) | Laser Pulse Frequency (Hz) | Laser Power (%) | Maximum Crater Depth (µm) | |
|--------------------|----------------------------|-----------------|---------------------------|-----|
| 10 | 5 | 25 | 80 | |
| | | 50 | 90 | |
| | | 75 | 100 | |
| | | 100 | 120 | |
| | 10 | 5 | 25 | 50 |
| | | | 50 | 80 |
| | | | 75 | 120 |
| | | | 100 | 140 |
| | 20 | 5 | 25 | 100 |
| | | | 50 | 140 |
| | | | 75 | 160 |
| | | | 100 | 280 |
| 10 | | 5 | 25 | 60 |
| | | | 50 | 80 |
| | | | 75 | 200 |
| | | | 100 | 360 |
| 50 | | 5 | 25 | 200 |
| | | | 50 | 280 |
| | | | 75 | 500 |
| | | | 100 | 500 |
| | 10 | 5 | 25 | 200 |
| | | | 50 | 280 |
| | | | 75 | 440 |
| | | | 100 | 480 |

petrographic slide in groups of five in preparation for laser analysis. Sections were affixed to the slide with silicon glue that had been previously tested for the absence of trace-element analytes. Finally slides were sonicated for 5 minutes in Milli-Q water and then rinsed three times before being dried under a laminar-flow hood for 24 hours.

Data analysis. Predetermined, full-factorial combinations of beam diameter, laser pulse frequency and power, and raster type were randomly assigned to prepared otoliths (Table 1c). Raster types were: 1) spot – laser beam held at one position (Figure 1), 2) raster - laser beam moved along abutting lines to form a rectangular ablation crater (Figure 2), and 3) line – laser beam moved along a pre-determined path at a specified speed (Figure 2). Crater dimensions and depths were measured with an Olympus BH2 compound microscope that was calibrated in the x, y, and z planes. Three

readings were taken and the average of these reported. Readings differed by less than 5%. We applied multiple regression analysis (Hintzie 2001), with crater depth, maximum, and average diameter as the response variables and beam diameter, power, and pulse frequency as the independent factors. Residuals were tested for normality to insure proper application of these tests. We present the data as a response surface in figures for graphical interpretation of laser performance.

Results

Crater depths were greatest when the beam was held in one spot at high power and with the largest beam diameter. Crater depths varied from 10 to 500 μm , depending on the combination of beam diameter, laser-pulse frequency, and power. Crater depths were shallowest, averaging 80 μm or less, at 25% power with beam diameters of 10 and 20 μm , regardless of pulse frequency (Table 2; Figure 3). At 50 μm beam diameter, crater depths averaged 480-500 μm at powers of 75 and 100%, regardless of pulse frequency. Because laser-pulse frequency did not affect crater depth, it was dropped from further analysis. Beam diameter and power were both significant terms in predicting crater depth ($F_{\text{beam dia}} = 49.19$, $df = 3,18$; $F_{\text{power}} = 29.53$, $df = 3,18$; $P < 0.001$). Beam diameter had the greatest influence on crater depth. It accounted for 60% of the variance in crater depth and while laser power accounted for 30%.

Crater-maximum diameters were consistently smaller than the beam diameters and depended on beam diameter ($|t| = 6.10$, $P < 0.001$, $n = 24$) and laser power ($|t| = 7.24$, $P < 0.001$, $n = 24$), but not on the laser pulse frequency ($|t| = 0.40$, $P = 0.69$, $n = 24$). This was also true for crater-average diameters; crater-average diameters were consistently smaller than the beam diameters and depended on beam diameter ($|t| = 7.20$, $P < 0.001$, $n = 24$) and laser power ($|t| = 6.39$, $P < 0.001$, $n = 24$), but not on the laser pulse frequency ($|t| = 0.35$, $P = 0.73$, $n = 24$). The predictive equations for these regressions are:

$$\text{Max. diameter} = 0.26 (0.47) \text{ beam diameter } (\mu\text{m}) + 0.19 (0.03) \text{ laser power } (\%) \text{ (eq. 1)}$$

$$\text{Ave. diameter} = 0.30 (0.47) \text{ beam diameter } (\mu\text{m}) + 0.16 (0.03) \text{ laser power } (\%) \text{ (eq. 2),}$$

where the numbers in parentheses are the standard error of the parameter estimates.

Using a beam diameter of 20 μm with rectangularly configured rasters, crater depths varied from 80 μm at 50% power and travel speed of 20 $\mu\text{m sec}^{-1}$ to 225 μm at travel speed of 10 $\mu\text{m sec}^{-1}$ (Table 3). Because laser operation at 25% power resulted in transient signals and high relative standard deviations ($\text{RSD} > 10\%$), we discontinued further testing at this power. The statistical model fit by multiple regression was significant ($F = 4.89$, $df = 11$, $P < 0.05$) and can be written as:

$$\text{Crater depth} = 19 + 1.4 * \text{Power } (\%) - 0.4 * \text{Travel speed } (\mu\text{m sec}^{-1}) \text{ (eq. 3).}$$

The response surface for these data is shown in Figure 4. Bear in mind that these predictive equations (eq. 1-3) are specific to the configuration of our laser and ICP-MS and may differ for other systems.

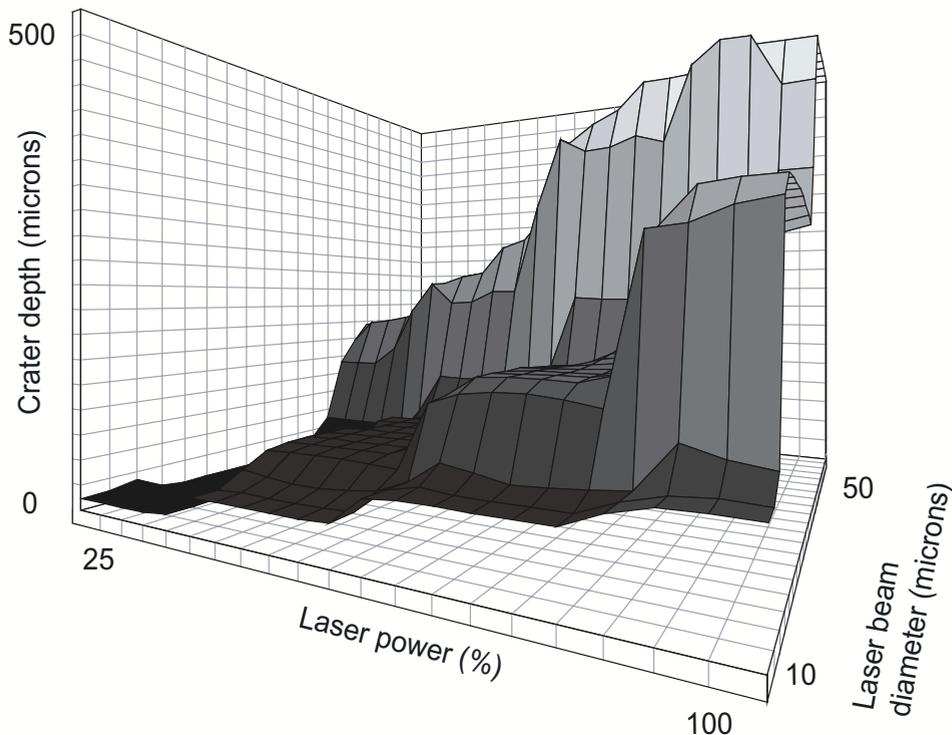


Figure 3. Response surface showing crater depth in spot configuration as a function of laser power and beam diameter when laser is applied to an otolith.

Using equation (3) we predicted that we would produce a crater depth of 85 μm by moving the laser along a predetermined line with a beam diameter of 20 μm , 50% power, and travel speed of 10 $\mu\text{m sec}^{-1}$. The average depth of seven-measured lines (from each of seven individual red drum otolith sections) was 95 μm with a standard deviation of 18 μm ; not significantly different from the crater depth that we predicted.

Discussion

Our results show that biogenic carbonates, such as otoliths can be excavated to predicted crater depths by controlling the operating characteristics of the laser used for sampling solid surfaces with SF-ICP-MS. This was true even though 1) otolith material is heterogeneous in composition and 2) we sampled two different fish species. For materials other than otoliths, scientists have previously shown that depth and width of laser craters depend on the energy of the beam, pulse frequency, pulse duration, power applied, and volatility of sample material (Sinclair 1999; Taylor 2001). However, this is the first time that the three-dimensional characteristics of ablation craters have been demonstrated in otoliths of larval and juvenile fish.

Table 3. Otolith-crater depths as a function of travel speed, power, and raster size. Beam diameter was set at 20 μ m and pulse frequency at 10 Hz.

| Travel Speed ($\mu\text{m sec}^{-1}$) | Power (%) | Raster Size ($\mu\text{m} \times \mu\text{m}$) | Maximum Crater Depth (μm) |
|--|-----------|---|---|
| 10 | 50 | 20 x 20 | 90 |
| | 75 | | 130 |
| | 100 | | 200 |
| 20 | 50 | 40 x 20 | 80 |
| | 75 | | 100 |
| | 100 | | 120 |

There are tradeoffs between crater configuration and the amount of material ablated for vaporization, and thus detection by the ICP-MS. Sinclair (1999) has shown for corals that the smaller the crater, the higher the detection limits, and the lower the precision of measured analytes. Laser ablation must produce enough material to provide sufficient analyte to detect differences in trace-element composition above detection limits. The amount of material necessary depends both on the elemental concentration in the material, and on the size and amount of vaporized material produced by ablation. In otoliths, we are able to sample Mg, Mn, Sr, and Ba routinely in both solution- and laser-introduction, but cannot measure other elements, such as the rare earths, using the laser even though we can measure these elements in solution at concentration of ppt. Laser-based sampling, with its higher detection limits, restricts one's ability to discriminate habitat use based on the trace-element signature compared to solution-based introduction, which can detect more elements. Hence, one limitation of laser-based sample introduction is the less complex trace-element signature that results from its use.

Complexity of the trace-element signature is only one consideration in determining which sample-introduction method to use for otoliths. Even though solution-based sample introduction results in lower detection limits and more trace-element signature complexity, all microstructural (chronological) detail is lost upon dissolution. Regardless of its greater sensitivity, whole-otolith dissolution cannot be used when we require chronological information on habitat use. This is especially true in the small otoliths of larval and juvenile fish. Whereas large otoliths can be cored to produce sections that can then be dissolved to provide sequential life-history information, larval and juvenile otoliths are typically too small for this approach. Hence, we are left to use laser-based sample introduction.

Otoliths are three-dimensional structures and to report only the crater width presents an incomplete picture of the life-history period that has been sampled. Precise-depth laser sampling, as demonstrated here, provides significant improvements to our knowledge of life history. Important life-history events can occur over temporal ranges of a week or two that are contained in growth bands that span regions as small as 10 to 20 μm . Sinclair et al. (1998) and Fallon et al. (1998) have sampled seasonal profiles and shorter time spans in corals with laser-ablation ICP-MS. Their sampling resolved temporal sequences of 20 μm . Similarly, Putten et al. (2000) were able to resolve in-

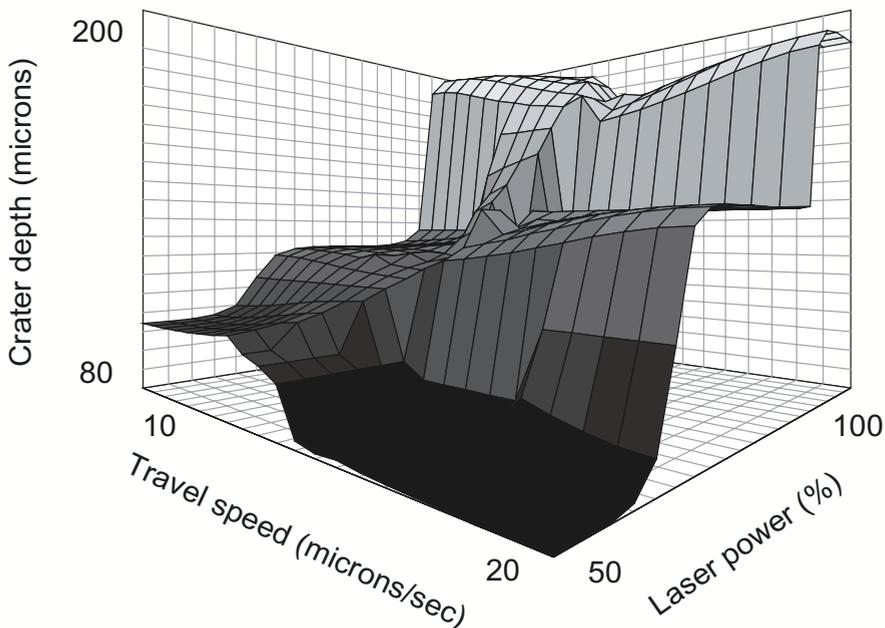


Figure 4. Response surface showing crater depth in line configuration as a function of laser power and travel speed when laser is applied to an otolith using a 20 mm diameter beam.

formation with spatial resolution of $\leq 60 \mu\text{m}$ in bivalve shells to show inter-annual variation in relation to food and environmental exposure. In fish, Fowler et al. (1995) and Thorrold et al. (1997) were able to obtain spatial resolutions of $100 \mu\text{m} \times 100 \mu\text{m}$ in juvenile Atlantic croaker. This spatial resolution represents 20-40 days or more in a fast-growing fish if we assume similar depth. We show with our results that otoliths can now be sampled with greater spatial resolution, thus enabling us to follow specified growth bands. The otoliths of young fish tend to be spherically symmetric and can be ground to predetermined tolerance above the primordium. When otoliths are ground to tolerance, and laser-ablation depths are controlled, spatial resolutions of $\pm 40 \mu\text{m}$ can be obtained around the primordium and this represents a period of 10 days in the fish we used for this study. Thus events in fish-life history can now be sampled with weekly spacing with full three-dimensional control.

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Suitability of a semipermeable membrane to cap storage vials for rainbow trout (*Oncorhynchus mykiss*) eggs

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Key words: rainbow trout eggs, storage, semipermeable membrane.

Abstract

Successful storage of rainbow trout (*Oncorhynchus mykiss*) eggs is dependant upon several factors including the oxygen pressure of the atmosphere under which they are stored. On the other hand, transportation of eggs in uncovered containers is impractical. The present experiment was designed to test if closure of storage vials with a semipermeable membrane ("BioFolie 25", Sartorius, Goettingen, Germany) will allow the eggs to survive. Freshly stripped unfertilized rainbow trout eggs from 3 to 5 year old females were stored 2 or 4 layers deep in vials either uncapped or capped with BioFolie at a temperature + 2°C under a moisture-saturated oxygen atmosphere for up to 30 days. To control bacterial growth, antibiotics at a concentration of 125 IU penicillin and 125 µg streptomycin per g of eggs and coelomic fluid were added. Batches of eggs were fertilized with uniform frozen-thawed semen after either 0, 15, 20, 25 or 30 days of storage. Freshly collected eggs served as controls. The fertilization and hatching rates were expressed as percent of controls. The percentage of eyed eggs was taken as indicator of fertility. The experiment was conducted with five replications. Fertility of the eggs declined significantly during storage ($P < 0.05$). However, fertilization rates around 50% were achieved up to 20 days of storage. Thereafter fertilization rates dropped sharply, reaching levels below 10% by day 30. No significant difference in fertility of eggs was observed between treatments and number of layers over the storage period with the exception of eggs stored for 30 days where there was a slight, though significant reduction in fertility of eggs stored in uncapped vials vs capped vials ($P < 0.05$). Either dehydration, despite the humidified gas atmosphere supplied, or an enhanced metabolic rate due to excessive exposure to oxygen may serve as explanation. Thus, the results show that the capping of egg-containing vials with BioFolie is possible without impairment of fertility in eggs stored for extended periods of time.

Introduction

Rainbow trout (*Oncorhynchus mykiss*) eggs can be maintained in coelomic fluid under various conditions for several days (Withler and Humphreys 1967; Jensen and Alderdice 1984; Lahnsteiner and Weismann 1999). The importance of the gas atmosphere, the number of egg layers and the addition of antibiotics for the survival of stored eggs has been demonstrated by Pueschel (1979) and Stoss et al. (1980). In closed vessels eggs will deteriorate rapidly. Uncapped vessels, however, are hard to ship without spillage. The present investigation was undertaken to determine if closure of storage vials with a newly developed semipermeable membrane ("BioFolie 25", Sartorius, Goettingen, Germany) will ensure sufficient oxygen to pass through to warrant respiration of eggs during storage. Furthermore, the membrane may help to reduce the risk of physical damage to the eggs during handling.

Material and Methods

Eggs were stripped from 3 to 5 year old rainbow trout spawners from our own breeding unit at the University Experimental Farm Reliehausen at the peak of the spawning season (December to February). Good quality pooled semen from 4 to 5 males at a time, cryopreserved in pelleted form (Holtz 1993) was used for fertilization of eggs with density of 8×10^6 frozen-thawed spermatozoa/egg (Stoss and Holtz 1981). Unfertilized eggs were pooled from several females. In order to test the quality of the eggs, five control groups of 200 eggs each were fertilized immediately to provide an estimate of pre-storage egg quality. To control bacterial growth, antibiotics dissolved in coelomic fluid were added to the eggs at concentrations of 125 IU penicillin and 125 μg streptomycin per g of eggs and coelomic fluid. These eggs were then randomly distributed to 80 vials (50 mm height and 30 mm diameter). In 40 vials, eggs were stored 2 layers deep (about 60 eggs), in the other 40 vials, four layers deep (about 120 eggs). In each group half of the vials were uncapped, the other half were capped with a newly developed gas permeable membrane ("BioFolie 25"). The technical data for the 0.025 mm thick "BioFolie 25" at 25°C are as follows: permeability ($\text{cm}^3 \text{m}^{-2} \cdot 24\text{h} \cdot \text{atm}$) for oxygen: 1.6×10^3 , for carbon dioxide: 25.9×10^3 , for hydrogen 34.1×10^3 , for nitrogen: 5.0×10^3 , and permeability ($\text{g m}^{-2} \cdot 24\text{h}$) for water vapour: 7.0.

Vials were placed into a 10-l desiccator kept in a refrigerator at a constant temperature of 2°C. The gas content of the desiccator was exchanged daily by a 10 min steady flow of moisture-saturated pure oxygen (technical oxygen 99,5%) as described in Stoss and Holtz (1983). At 15, 20, 25 and 30 d after collection, the eggs of five vials of each of the four treatment groups were fertilized with frozen-thawed semen. Thus, 5 replications per treatment were performed. Eggs were incubated in a 10-tray vertical incubator system (Veco AG, Horgen, Switzerland) with a constant flow of 1 l min^{-1} of water at 8 to 9°C. Eggs that turned opaque were removed at 3 day intervals. To check for fertilization, these eggs were placed into a solution consisting of 40 ml glacial acetic acid made up to 1000 ml with an aqueous 0.7% NaCl – solution (Romeis 1968) to restore translucence and visualize embryos, if present. After 3 weeks of incubation, number of eyed eggs, and after 4 weeks hatched larvae were recorded. The percentage of eyed and hatched eggs was calculated based on

the initial number of eggs in a replicate. The fertilization and hatching rates were expressed as percentage of what was observed in freshly collected eggs from the same stripping. The percentage of eyed eggs was taken as index of fertility.

Statistical analyses were conducted using the program "SAS version 6.1". Correlation and regression analyses between percentage of eyed and hatched eggs were performed. Changes in fertility of eggs during storage were analyzed using one-way ANOVA with time of storage as the main variable. Statistical differences among treatments were tested using two-way analysis of variance (ANOVA) with treatments (capped vs uncapped vials) and number of layers (2 vs 4) as the main factors. Each of the time periods were analyzed independently. Differences between means were evaluated using F-test ($P < 0.05$). Differences between individual means were estimated using Student's t-Test ($P < 0.05$).

Results and Discussion

It was found that across treatments the percentage of eyed eggs correlated closely with the percentage of hatched eggs ($r = 0.98$, $P < 0.01$). The linear regression coefficient of percent hatched eggs (dependent variable) on percent eyed eggs (independent variable) was 1.009, its standard error was 0.0265. The linear regression was statistically highly significant ($F_{1,65}$, $P < 0.01$) and the linear regression curve was as follows: Percent hatched eggs = $-5.439 + 1.009 \times$ percent eyed eggs. It was decided to use percent eyed eggs as an index for fertilization capacity. Hatching rate may be calculated from it using the above regression equation.

In 5 control fertilizations of freshly collected eggs, fertility averaged 78.4 (SEM 3.3%). Fertility of the eggs declined significantly during storage ($P < 0.05$). However, reasonable fertilization rates of more than 50% were achieved until 20 days of storage irrespective of vials being capped or uncapped (Table 1). In both experimental groups fertility of eggs dropped sharply thereafter, reaching levels of less than 10% by day 30. This is in conformity with Stoss et al. (1980) who found that storage of eggs not more than 4 layers deep in coelomic fluid with antibiotics added kept under a moisture-saturated oxygenated atmosphere at 0°C, provided 50% fertilization after 20 days of storage.

Table 1. Fertilization rate (percent eyed eggs) of eggs stored at 2 °C under a moisture-saturated oxygen atmosphere either 2 or 4 layers deep in uncapped or BioFolie-capped vials expressed as percent of freshly collected eggs (78.4% fertilized) (5 replications/treatment).

| Days of storage | 2 Layers | | | | 4 Layers | | | |
|-----------------|-------------------|-----|------------------|-----|------------------|-----|-------------------|-----|
| | Capped | | Uncapped | | Capped | | Uncapped | |
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 15 | 78 ^a | 5 | 77 ^a | 3 | 69 ^a | 1 | 67 ^a | 3 |
| 20 | 50 ^b | 3 | 50 ^b | 5 | 51 ^b | 4 | 50 ^b | 4 |
| 25 | 28 ^c | 4 | 15 ^c | 3 | 28 ^c | 5 | 29 ^c | 6 |
| 30 | 10 ^{A,d} | 4 | 1 ^{B,d} | 1 | 9 ^{A,d} | 2 | 6 ^{AB,d} | 1 |

A,B: within rows, means with different superscripts differ (F-test, $F_{3,16}$, $P = 0.02$)

a,b,c,d: within columns, means with different superscripts differ (F-test, $F_{3,16}$, $P < 0.01$)

In the present study, no significant changes in fertility of eggs occurred between treatments and number of layers over the storage period with the exception of eggs stored for 30 days (Table 1). In these, there was a slight, though significant difference between treatments, i.e. fertilization rate of the eggs stored in uncapped vials was significantly reduced compared to the eggs in capped vials ($P < 0.05$). Either dehydration, despite the humidified gas atmosphere supplied, or an enhanced metabolic rate due to excessive exposure to oxygen may serve as explanation.

The importance of the availability of oxygen to respiratory exchange has been demonstrated for trout eggs by Czihak et al. (1979) who found the oxygen consumption of unfertilized and non-activated eggs of 1.29 ng min^{-1} per egg. Withler and Humphreys (1967) observed that 1000 stored pink or sockeye salmon eggs in closed 3.8 l plastic vessels at a temperature of 8 to 9°C lost their viability rapidly. The fertility was 50% after 2.5 days of storage and lower than 1% on Day 7.

The encouraging result of the present experiment is that the viability of the eggs in capped vials did not decline more than in uncapped vials. Therefore, the gas-permeable membrane is suitable to cap vials containing rainbow trout eggs, ensuring sufficient oxygen available for respiration during storage.

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Methods for high density batch culture of *Nitokra lacustris*, a marine harpacticoid copepod

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Key words: *harpacticoid copepods*, *live feed*, *batch culture methods*

Abstract

Marine copepods are a potential source of live feed for marine fish larviculture. However, the technology to culture marine copepods economically and at high enough yields is still being developed. This research investigated the effects of container size and configuration, as well as the effects of live versus formulated feed on the overall population yield and population growth rate of short-term high density batch cultures of a marine harpacticoid copepod.

A survey of wild copepods along the Atlantic and Gulf Coast of the U.S.A. identified a harpacticoid copepod *Nitokra lacustris* with exceptional promise for high density culture. *Nitokra lacustris* is a common euryhaline coastal species which is highly adaptable to a variety of culture conditions. This species has been used previously in a toxicological study (Lotufo and Fleeger 1997) and a study on the effects of a muddy substrate on high density culture of meiobenthic copepods (Chandler 1986). Since the survey, this species of copepod has been maintained in continuous culture in the laboratory and has been able to reach population densities of 100,000 individuals l⁻¹ in small culture containers (2 l) consistently. The species has a short generation time (10 to 12 days) at 20° C, produces high numbers of nauplii (7 to 18 nauplii female⁻¹ day⁻¹) at an average ambient temperature of 20° C, and ranges in size from 40 µ to 620 µ, which is ideal for many early marine fish larvae (Walford et al. 1991). This project analyzed the optimum food type and container configuration at low (10 l) and high volumes (100 l to 266 l).

Statistical analysis of replicate treatments showed that supplying an inert formulated feed to the copepod populations did not result in significantly lower population growth in comparison to live or preserved algae ($p > 0.05$) when all treatments were included. If treatments that had gone extinct were excluded, the copepod populations fed formulated feed had significantly higher densities and intrinsic growth rates (r) than the populations fed live algae ($p < 0.01$). In small containers,

copepod populations raised in carboys grew significantly slower than copepod populations in trays for all feed types ($p < 0.05$). No interaction effects were detected between feed type and container type in the 10 l containers. In large containers (> 100 l), the treatments showed no effect from the shape of the container or the surface area to volume ratio of the water after Day 15 of the trial. Based on these results, this paper discusses methods to optimize the high density batch culture methods for *Nitokra lacustris*. Hatchery level production (8.6 million day⁻¹) of a marine harpacticoid copepod could be achieved using these methods.

Introduction

Copepod diets have been shown to increase larval marine fish growth and development better than a diet of rotifers *Brachionus plicatilis* (Kuhlmann et al. 1981) and *Artemia* (Kuhlmann et al. 1981). Copepods can have other beneficial effects. Feeding copepods to halibut (*Hippoglossus hippoglossus*) has resulted in better pigmentation than enriched *Artemia* (McEvoy et al. 1998), which may be due to the higher level of polar lipids in the copepods. Despite these findings, enriched rotifers and *Artemia* will probably continue to be the live feeds of choice in commercial hatcheries, because copepods are difficult to culture at sufficient densities to be economically efficient on a commercial scale. This project attempted to overcome some of the previous obstacles to harpacticoid copepod culture by testing various container sizes and shapes and by developing a formulated feed for the copepods that produces reliable population growth rates.

Only a few species of copepods have been successfully reared at near commercial scale in extensive systems. An extensive analysis of current copepod rearing technologies has been provided by Støttrup (2000). Most copepod rearing trials have been small scale, and have lasted only a few weeks or months (Sun and Fleeger 1995). The most successful methods to date have involved extensive pond or bag culture using large inputs of natural seawater or the placement of bags of various mesh sizes in open sea water (van der Meeren and Naas 1997). According to the review by Støttrup (2000), a reliable system for the continuous large-scale indoors intensive culture of calanoid or harpacticoid copepods has not yet been developed. In more recent efforts, Payne and Ripplingale (2001) were able to produce 878 nauplii l⁻¹ day⁻¹ of the calanoid species *Gladioferens imparipes* in 500 l automated batch cultures for 420 days.

This research focused on the harpacticoid copepods, which seem to offer the best combination of size and adaptability to live feed culture technologies. Harpacticoid copepods, which are usually detritivores, can adapt to both formulated artificial feeds and to algal monocultures (Norsker and Støttrup 1994). Harpacticoids can be grown in densities up to 115,000 individuals l⁻¹ (Kahan et al. 1982). By comparison, most calanoid copepods are small particle feeders (raptorial predators or omnivores), and can be grown at densities of only 100 to 200 adults l⁻¹ (Ogle 1979; Støttrup et al. 1986). The ability to convert shorter chain *n*-3 polyunsaturated fatty acids to the essential fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) has been observed in the harpacticoid copepods *Tisbe* (Norsker and Støttrup 1994; Nanton and Castell 1998) and *Tigriopus* (Watanabe et al. 1978). Harpacticoid copepods do not exhibit reduced reproduction as a result of being fed diets deficient in EPA and DHA (Norsker and Støttrup 1994). Calanoid copepods, which

may have a limited lipid bioconversion capability from 18:3 n -3 to the longer chain n -3 HUFA (Moreno et al. 1979), require dietary sources of EFA's for normal reproduction and growth (Støttrup and Jensen 1990; Jonasdottir et al. 1995).

For this study, wild copepods were collected along the Atlantic and Gulf coast of the U.S., grown in the lab, and compared against the key criteria presented by Uhlig et al. (1984) for potential new live feed species: (1) tolerance of a wide range of environmental conditions; (2) ability to utilize different food sources; (3) short life cycle; (4) high reproductive capacity; and (5) tolerance of high densities. As a result of this survey, *Nitokra lacustris* was selected as the best candidate species. This species has a short generation times (10–12 days) at 20° C, and produces high numbers of nauplii (7 to 18 female⁻¹ day⁻¹) in water temperatures from 7° C to 33° C and salinities from 10 to 40 ppt.

Nitokra lacustris, which have nauplii that are comparable in size to large rotifers (about 100 µm in length), may be an ideal alternative feed for early fish larvae that cannot ingest larger feeds (Walford et al. 1991). In stock cultures, the maximum linear dimension of the nauplii averages 40 µm (width); the largest life stage of *N. lacustris* are the females with embryo sacs, which average 620 µm in total length. The nauplii and adults fall into a range that is smaller than many commercially-reared calanoid and harpacticoid copepods. The first naupliar stage of the calanoid copepods *Euterpina acutifrons* and *Acartia tonsa* have average widths of 70 µm (Kraul 1990) and 145 µm (Støttrup 2000) respectively. The length of the average adult in these two species is 700 µm (Kraul 1990) and 1000 µm (Støttrup 2000). The commercially grown harpacticoid copepod *Tisbe holothuriae* has nauplii that are 100 µm in width and adults that are approximately 750 µm in length. Because of its small size, *N. lacustris* may be an acceptable food for fish larvae that cannot ingest larger copepods or *Artemia*. In preliminary trials to assess the potential of *N. lacustris* for larviculture, speckled sea trout (*Cynoscion nebulosus*), black sea bass (*Centropristis striata*), red drum (*Sciaenops ocellata*) and Southern flounder (*Pleuronechthys lethostigma*) larvae were able to locate, ingest and survive on *Nitokra lacustris* nauplii and adults.

Nitokra lacustris has been cultured in the laboratory since February 2000 and has reached a density of 100,000 individuals l⁻¹ in small (2 l) containers kept at room temperature (20° C). This species of copepod is tolerant of a wide range of conditions, and has been raised in water temperatures from 7 °C to 33 °C and salinities from 10 to 40 ppt on a large variety of algal monocultures and inert feeds. Based on these production characteristics and consistency in maintaining these high-density cultures, it may be possible to successfully mass culture this copepod in sufficient quantities for commercial hatchery operations, either through batch culture or a semi-continuous culture system.

This paper discusses methods to optimise the mass culture methods of *Nitokra lacustris* in batch cultures. First, live algae, frozen algae paste and a formulated feed were analysed for their effect on the overall yield and population growth rates of *N. lacustris* in small containers of two types, trays and carboys. Secondly, six copepod populations maintained on formulated feed in large containers were evaluated for differences in growth due to the two different shapes of the containers used: round and square. Finally, copepod populations grown in large containers of the two different shapes were analysed for their growth in relation to the ratio of the surface area to volume. Surface area in this experiment refers to the air-water interface. Based on the results of these experiments, recommendations are given to optimise copepod population growth in batch cultures.

Materials and Methods

Culture Conditions. Stock cultures of *N. lacustris* were maintained in 2 l of culture media. The culture media was synthetic seawater composed of a Crystal Sea® or Reef Crystal® mixture added to chlorinated/dechlorinated water until the salinity reached 30 ppt (+/- 2 ppt), as measured by a hand-held refractometer. Food was added at a concentration of 50,000 to 100,000 cells (or particles) ml⁻¹ every 9 days to 3 weeks depending on the size of the container, the number of copepods, and whether the food was live algae or formulated feed. Care was taken to avoid overfeeding, which was indicated by a cloudy media or excess precipitation of food. No aeration was provided for the 2 l stock cultures. The culture water was renewed once a month or every other month, by passing the cultures through a series of mesh sizes to collect the different ages of the animals:

| Mesh size (µm) | Stages Retained |
|----------------|---|
| 35 µm | All stages |
| 68 µm | All except smallest nauplii |
| 105 µm | Late stage copepodites, adults and females with embryo sacs |
| 125 µm | Adults and females with embryo sacs |
| 150 µm | Females with embryo sacs and mating adults. |

To restart a fresh culture, females with embryo sacs (50 l⁻¹) were redistributed to new containers. Experimental cultures were maintained at ambient temperatures, varying from 17.4 °C to 33.3°C. Salinities ranged from 26 to 33.4 ppt. The cultures were kept in artificial lighting or natural sunlight, depending on the experiment.

Aeration was provided for small and large experimental treatments. For 10 l containers, the aeration was provided centrally, near the bottom of the tray or carboy, using a glass pipette with an aperture of 1.5 mm controlled to give a bubble of air one to five times per second. For larger treatments (100 l to 260 l), aeration was provided by an airstone. The intensity of the aeration in the larger treatments was strong enough to circulate the water in the container without causing excessive turbulence.

Feed. The copepods in the stock cultures were given either live algae (mainly *Tetraselmis suecica*), a combination of live algae and frozen algae pastes, or a formulated feed from a recipe adapted from a guide for aquarium hobbyists (Moe 1997). The cost of producing the formulated feed was much lower than the labour costs of algal production or the cost of commercially available feeds used for rotifer or *Artemia* enrichment. The recipe was modified to replace the omega-3 fatty acids (FA) provided from fish oil in the original recipe with polyunsaturated omega-6 and omega-3 fatty acids from flax seed oil. This formulated feed recipe is referred to as “Modified Moe’s Media (MMM)” in this paper.

- Tomato or vegetable juice (240 ml)
- enriched brewer’s yeast (10 grams)
- liquid vitamin C (1 ml)
- liquid vitamin B complex (2 ml), and
- flax seed oil (rich in omega-6 and omega-3 FA) (5 ml)

To feed the copepods, these ingredients were combined and blended for two minutes. Then, artificial sea water (30 ppt) was added until the total volume of the mixture was one litre. This larger volume of feed was blended for two more minutes. The MMM was added to treatments at 1 ml l⁻¹ every nine days. This volume of MMM provided approximately 50,000 particles ml⁻¹ of particles in the size range of 10 to 12 µm, assuming that the majority of the particles did not break down or coagulate. Only copepods from stock cultures given formulated feed were used to start experimental cultures, in order to prevent the unwanted appearance of live algae.

Sampling Procedure. Treatments were stirred for two minutes immediately before sampling to attempt to equalize the copepod distribution. Then, the population of copepods was estimated from counting the total number of copepods in multiple (3 or 4) samples. After counting, the copepods were returned to the treatments.

Harvesting Procedure. When the copepod populations were sampled at the end of the experiment, the treatments were drained and the copepods were grouped by size using two sizes of mesh (35 µm and 105 µm). When debris was present, culture media was added to allow filtration of the debris. After collection, clove oil was used to immobilise the copepods before they were counted using a stereo microscope. Total counts of representative samples from the condensed populations were repeated four times for each treatment and size group, recording the proportion of the population in each life stage of interest (nauplii, copepodite, adult or female with an embryo sac).

Total population counts were log normalized. The intrinsic growth rate, r , was calculated by dividing the difference between the log normal populations at the beginning (N_0) and the end (N_t) by the duration of the experiment (t):

$$(1) \quad r = [\ln(N_0) - \ln(N_t)] * t^{-1}.$$

The r values from separate tanks under the same conditions were considered replicates. The r values of treatments run concurrently were compared using an analysis of variance test for three or more replicates and a t-test for two replicates, assuming treatments had the same underlying variance. The r values calculated in the experiments assumed that the population is growing in a constant environment without any density dependence effects. Table 1 summarises the container size, feeding schedule and sampling schedule for the four main experiments.

Feeding Trials (Experiments One and Two). In Experiments One and Two, feed type and container type were compared simultaneously. In Experiment One, 1900 copepods were placed in each of six 10 l carboys and six 10 l trays. The white plastic trays measured 31.5 cm by 58 cm, which provided a surface area to volume ratio of 182.7 cm² l⁻¹. The lids to the trays were clear. The clear plastic Nalgene carboys had a diameter of 24.8 cm with a surface area to volume ratio of 48.3 cm² l⁻¹. Three of the six containers in each treatment were given live *Tetraselmis suecica*. The remainder were given Modified Moe's Media (MMM). *Tetraselmis suecica* was added to the randomly assigned live treatments at 50,000 cells ml⁻¹ at the beginning of the experiment. The algal concentration was reduced to 100,000 cells ml⁻¹ by water exchange on Day 13. MMM was added to the formulated feed treatments at 50,000 food particles ml⁻¹ on Day 1 and increased to 100,000 food par-

Table 1. Experiments and treatments.

| | Size and Type of Container | Number | Type of Feed | Feeding Frequency | Sampling Days | Location |
|----|----------------------------|--------|----------------------------|----------------------|------------------------------------|-------------------------------|
| #1 | Trays 10 l | 6 | Live | 2 | Days 13 and 19 | Indoors, 16 h:8 h light/dark |
| | Carboys 10 l | 6 | <i>Tetraselmis</i> and MMM | (Days 0 and 13) | | |
| #2 | Trays 10 l | 6 | <i>Tetraselmis</i> | 2 | Day 31 | Indoors, 16 h:8 h light/dark |
| | Carboys 10 l | 6 | Paste and MMM | (Days 0 and 15) | | |
| #3 | Round Tank 266 l | 3 | MMM | 4 | Day 54 | Outdoor greenhouse |
| | Square Tank 266 l | 3 | | (Days 0, 16, 32, 48) | | |
| #4 | Round Tank 100 l | 2 | MMM | 3 | Days 9, 12, 15, 18, 21, 24, and 27 | Indoors, 12 h:12 h light/dark |
| | Round Tank 200 l | 3 | | (Days 0,9, 18) | | |
| | Square Tank 200 l | 3 | | | | |

ticles ml⁻¹ on Day 13. The number of food particles in the case of the formulated feed refers to the number of particles that were equal in size to the diameter of the live *Tetraselmis* (10 to 12 µm). The same volume of water was exchanged in the formulated feed treatments as in the live feed treatments on Day 13. It was assumed that the food level was in excess in all treatments for the duration of the experiment, because the algae and formulated feed remained suspended in the water column and some excess precipitation was observed on the bottom of the containers.

In Experiment Two, 6325 copepods were placed in each of six 10 l carboys and six 10 l trays. MMM was randomly added to three trays and three carboys and frozen preserved *Tetraselmis suecica* was given to the remaining containers. This time, trays and carboys were given feed biweekly (100,000 particles or cells ml⁻¹). Copepods from all treatments in Experiment Two were harvested after 31 days, and total numbers were assessed.

Harvest Techniques and Distribution (Preliminary Trial to Experiment Three). Before Experiment Three, a preliminary trial to test harvesting techniques was conducted on a copepod population that had been growing for 26 days in a large square tank (266 l). The culture was started with 1800 copepods and was given 100 ml (0.38 ml l⁻¹) of MMM on Days 0, 14 and 24. The four procedures were:

Procedure 1: The top 20% of the water volume was removed by siphon, with the inlet close to the water surface.

Procedure 2: The valve at the bottom of the tank was opened to release 10% of the total volume close to the bottom.

Procedure 3: After stirring the culture, 60% of the original volume was released through valves at the bottom of the tank.

Procedure 4: The final 10% of the original volume was reduced by reverse filtration, leaving debris and copepods. These were collected with four rinses of 1 to 2 l of seawater.

Large Container Shape and Batch Cultures (Experiment Three). For Experiment Three, which took place in a greenhouse during the summer, three large round tanks and three large square tanks were filled with 266 l of culture media. The square tanks used in all large scale experiments (>100 l) were polyethylene and the round tanks were moulded fibreglass. These tanks had a 400 l total capacity. The tanks were covered with clear plastic and shaded with 80% shade cloth. MMM was added to the randomly assigned formulated feed treatments every two weeks in increasing increments. On Days 0 and 14, 100 ml of MMM (0.38 ml l⁻¹) was added. On Day 28, 150 ml of MMM (0.56 ml l⁻¹) was added and on Day 42, 200 ml (0.75 ml l⁻¹) was added. It was assumed that the feed was provided in excess of grazing rates, because the feed remained suspended in culture and/or precipitated to the bottom. The average temperature of the treatments was 32 °C (+/- 0.7) and the average salinity was 31 ppt (+/- 2.4). The copepods from all treatments in Experiment Three were harvested after 54 days and the total numbers evaluated.

Surface Area to Volume Ratio and Large Containers (Experiment Four). Experiment Four was conducted indoors under artificial lighting (12h light:12 h dark). Three large square tanks and three large round tanks were filled to 200 l. To compare surface area to volume differences, another three large round tanks were filled to 100 l. The tanks were stocked at equal densities (30 copepods l⁻¹). The salinity of the water varied between treatments from 28.6 to 30.5 ppt, the temperature ranged between 17.4 °C and 23.2 °C, and the dissolved oxygen profile remained between 6.0 and 7.3 mg l⁻¹.

MMM was added to the tanks at concentrations of 1 ml l⁻¹ on days 0 and 9. On day 18, MMM was added at 2 ml l⁻¹. From days 9 to 27, the copepod populations were sampled in all tanks at intervals of three days. After counting, the copepods were returned to the tanks. On day 27, all treatments were terminated and counted.

Results

The results for all trials are given in Table 2 and Figure 1. The *r* value given for the population was measured on the final day of the trial. A positive value for *r* indicates that the population is growing. Standard error is calculated when three or more replicates are available.

Table 2. Summary of r values for all treatment.

| | Size and Type of Container | Number | Day of Harvest | <i>r</i> | Standard Error |
|----|----------------------------|--------|----------------|----------|----------------|
| #1 | Trays10 l | 6 | 19 | 0.3468 | 0.0278 |
| | Carboys 10 l | 6 | 19 | 0.1188 | 0.0594 |
| #2 | Trays 10 l | 6 | 31 | 0.0842 | 0.0149 |
| | Carboys 10 l | 6 | 31 | 0.0291 | 0.0242 |
| #3 | Round Tank 266 l | 3 | 54 | 0.0940 | 0.0092 |
| | Square Tank 266 l | 3 | 54 | 0.0947 | 0.0325 |
| #4 | Round Tank 100 l | 2 | 27 | 0.1587 | 0.0023 |
| | Round Tank 200 l | 3 | 27 | 0.1587 | 0.0094 |
| | Square Tank 200 l | 3 | 27 | 0.1688 | 0.0069 |

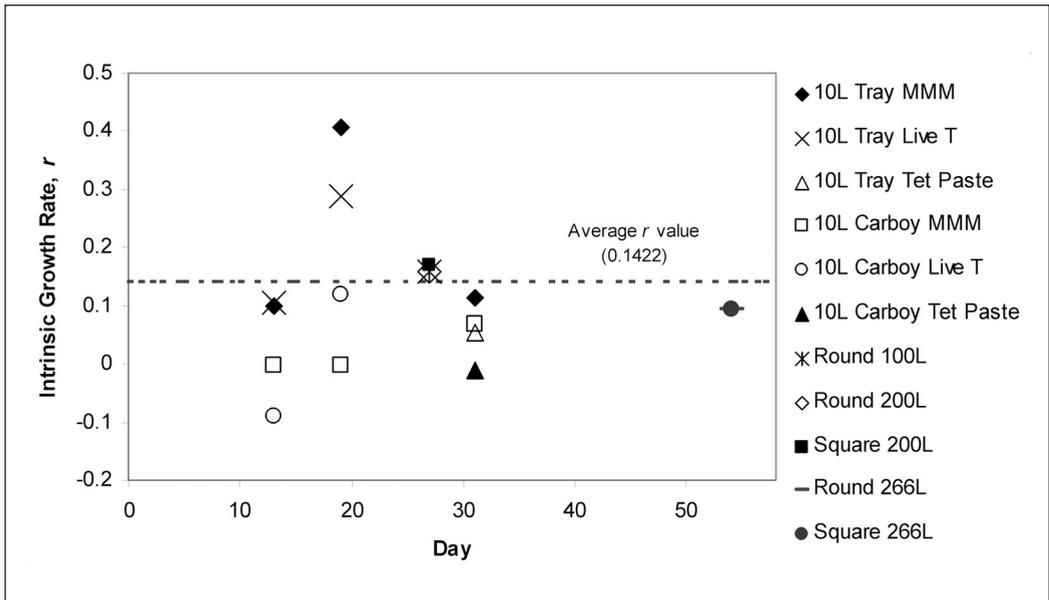


Figure 1. Intrinsic growth rates (r) for all experimental treatments. The r values for every sampling day for the experiments using 10 l volumes are given: 10 l trays fed Moe's Modified Media (MMM) (\blacklozenge), 10 l trays fed live *Tetraselmis* (\times), 10 l trays fed *Tetraselmis* paste (\triangle), 10 l carboys fed MMM (\square), 10 l carboys fed live *Tetraselmis* (\circ), and 10 l carboys fed *Tetraselmis* paste (\blacktriangle). Only the final r values for treatments using volumes greater than 10 l are plotted: round 100 l ($*$), round 200 l (\diamond), square 200 l (\blacksquare), round 266 l ($-$), and square 266 l (\bullet). All treatments greater than 10 l were fed MMM. The dashed line represents the average r value for all sampling days for all treatments in all experiments.

Experiments One and Two. For Experiment One, the natural logs of the populations were compared on days 13 and 19 (Figure 2). Single factor and two factor analysis of variance (ANOVA) on intrinsic population growth of the copepod populations were conducted using container type and feed type as the factors. Single factor ANOVA showed that trays had significantly higher population densities and growth rates ($p < 0.05$) on days 13 and 19. No significant difference was detected between feed types when all treatments were included. When the treatments which died off were excluded, the copepod populations fed formulated feed grew significantly faster and reached significantly higher population densities ($p < 0.01$). The two factor ANOVA with replication showed no significant interaction between food type and container type. The copepods given MMM in trays provided the highest population densities on day 19 (28 to 43 copepods ml^{-1}) and the highest intrinsic growth rates ($r = 0.39$ to 0.42).

For Experiment Two, trays had slightly higher population densities at day 31 than carboys ($p = 0.07$). Copepod populations given MMM had significantly higher r values than the populations given frozen algae ($p = 0.04$). Copepods in trays given MMM had the highest population r values (0.10 to 0.12). Copepods in carboys given *Tetraselmis* paste had the lowest population r values (-0.03). No interaction effect was detected between container type and feed type ($p = 0.28$). Two of the carboys given *Tetraselmis* paste had populations that died off completely.

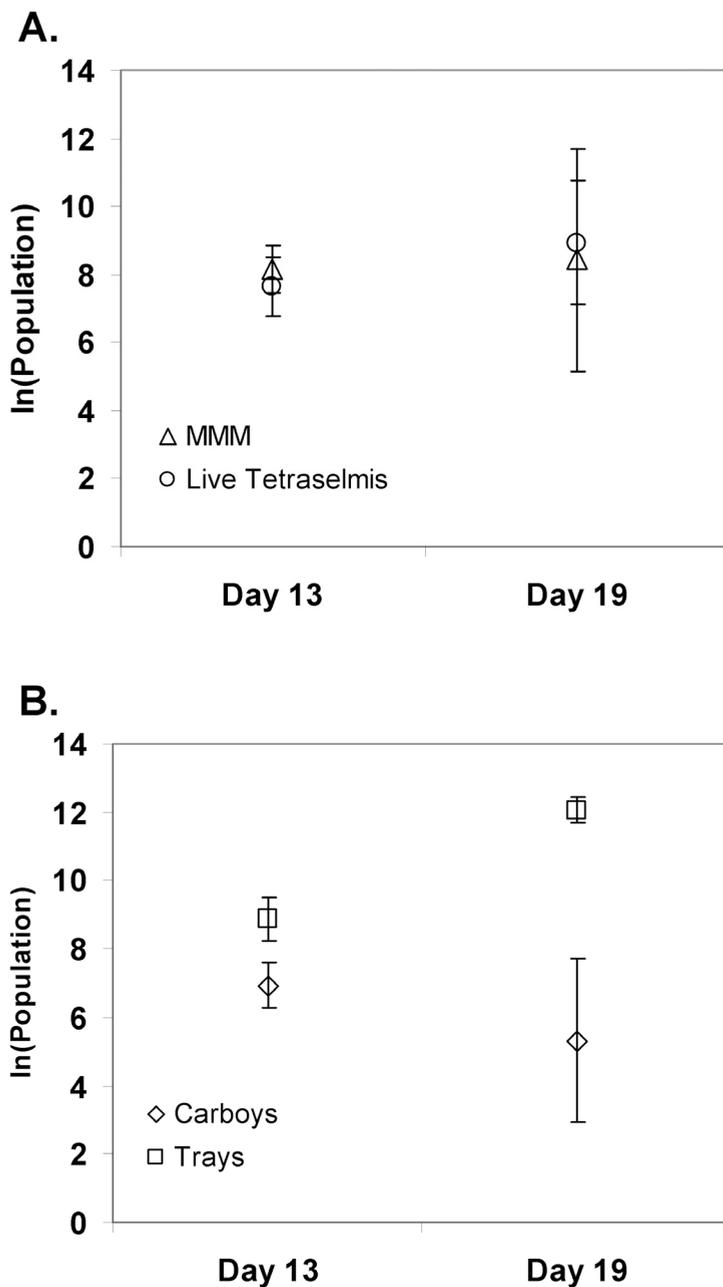


Figure 2. Results from Experiment One, a two factor analysis of feed type and container type on copepod populations. The error bars represent standard error for the three replicates. The copepod populations were measured on days 13 and 19. (A.) Comparison of the natural log of the copepod populations fed the formulated feed, Modified Moe's Media (MMM) (Δ) versus live *Tetraselmis* (\circ). (B.) Comparison of the natural log of copepod populations cultured in 10 l carboys (\diamond) and in 10 l trays (\square).

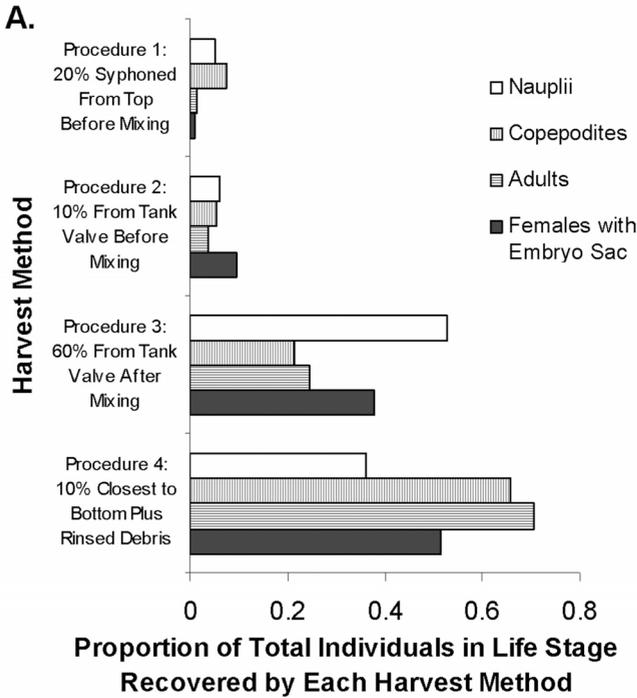
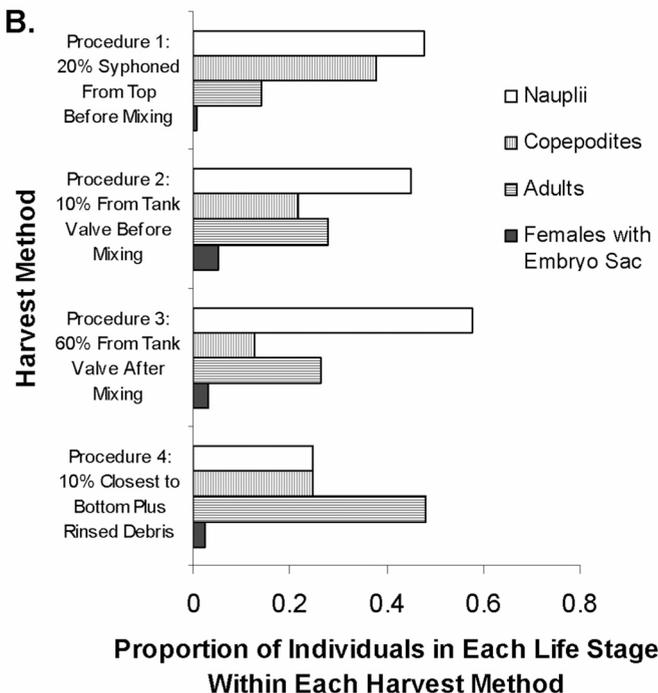


Figure 3. Results of the harvesting trial (preliminary trial to experiment three). (A.) The efficiency of each harvest method to collect each life stage (nauplii, copepodite, adult and females with embryo sac) is calculated by dividing the number of individuals recovered by a particular harvest method by the total number of individuals in that life stage collected by all harvest methods. (B.) The distribution of life stages within each harvest method is calculated by dividing the number of individuals in each life stage recovered by a particular harvest method by all individuals recovered by a particular harvest method.



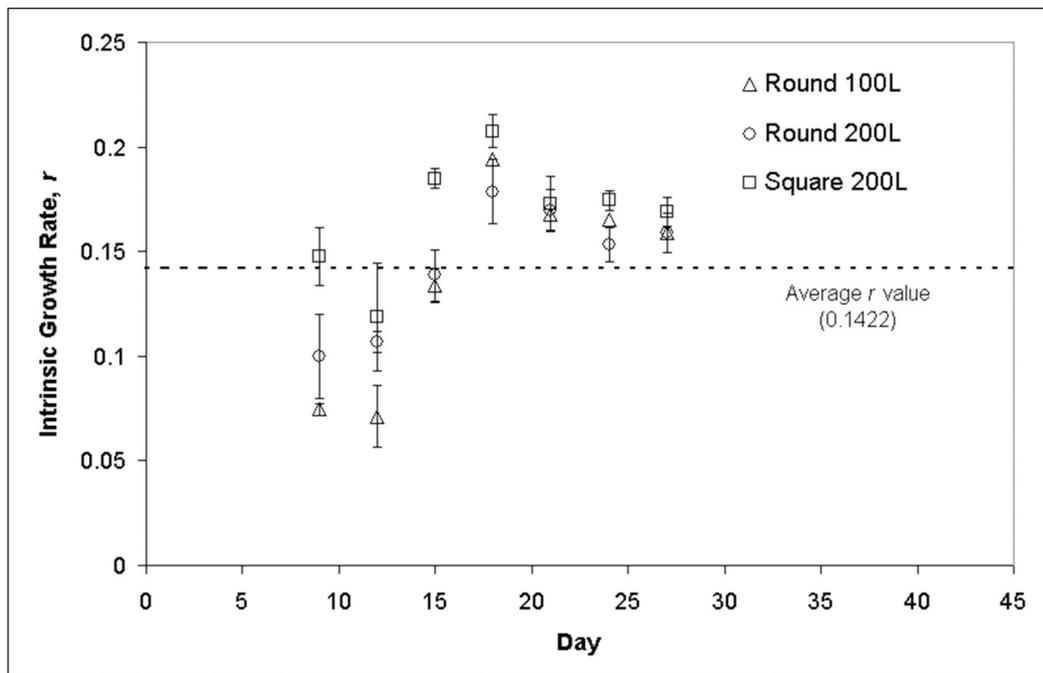


Figure 4. The intrinsic growth rates (r) are calculated for each sampling day for Experiment Four: round 100 l (Δ), round 200 l (\circ), and square 200 l (\square). The beginning density for each treatment was 30 copepods per liter. No evidence for serial autocorrelation was found to affect r values (Durbin-Watson, $p < 0.001$). The dashed line represents the average r value for all sampling days for all treatments in all experiments.

Preliminary Trial to Experiment Three. The proportions of the population recovered by each harvest method are presented in Figure 3a. The distribution of reproductive females, adults, copepodites and nauplii for each harvest method are given in Figure 3b. The 10% volume of water and debris collected at the bottom of the tank using procedure 4 contained the highest proportion of all life stages except nauplii. The general distribution of copepods among life stages varied depending on the harvest method, except for the females with embryo sacs, which composed a consistently low proportion (1 to 5 %) of the total population for all harvest methods.

Experiment Three. A single factor ANOVA between round tanks and square tanks showed no significant ($p < 0.05$) difference between final population sizes, the natural log of the final population sizes, or the intrinsic growth rate, r , at 54 days.

Experiment Four. Because one of the 100 l treatments was terminated early, single factor ANOVA could only be conducted on data collected on days 9, 12 and 15. For other days, t-tests assuming equal variances were used to compare the 100 l treatments to the other treatments. Significant differences ($p < 0.05$) in r values were detected only on days 9 and 15 between containers of different

Table 3. Culture methods for copepods used as live feed for marine fish (Støttrup 2000; Payne and Rippingale 2001).

| Species | Culture Size | Densities | Productivity | Food Conditions | Reference |
|---|---|--|--|--|-----------------------------|
| <i>Acartia tonsa</i> (Calanoid) | 1890 l | 232 l ⁻¹ | 2-75 nauplii/adult 16 days | Natural phytoplankton, extensive | Ogle(1979) |
| <i>Acartia tonsa</i> (Calanoid) | 200 - 450 l | 50 – 100 l ⁻¹ | 200-220 eggs l ⁻¹ ~28 days | <i>Rhodomonas baltica</i> , <i>Isochrysis galbana</i> , intensive | Støttrup et al. (1986) |
| <i>Eurytemora affinis</i> (Calanoid) | 30 m ³ | 300 – 500 l ⁻¹ copepodites and adults, >1000 nauplii l ⁻¹ | 7-10% | Algae and detritus, extensive | Nellen et al. (1981) |
| <i>Gladioferens imparipes</i> (Calanoid) | 2X 1000 l semi- continuous cultures | Stocked at 1000 nauplii l ⁻¹ | 520 nauplii l ⁻¹ day ⁻¹ 242 days | <i>Isochrysis galbana</i> , sometimes with <i>Rhodomonas baltica</i> | Payne and Rippingale (2001) |
| <i>Gladioferens imparipes</i> (Calanoid) | 500 l automated batch cultures | Stocked at 1000 nauplii l ⁻¹ | 878 nauplii l ⁻¹ day ⁻¹ 420 days | <i>Isochrysis galbana</i> , sometimes with <i>Rhodomonas baltica</i> | Payne and Rippingale (2001) |
| <i>Tisbe</i> spp. (Harpacticoid) | 1.5 l floating baskets in 200 l tanks | 92 – 115 ml ⁻¹ | Not estimated | <i>Mytilus</i> powder, lettuce pieces | Kahan, et al. (1982) |
| <i>Tigriopus japonicus</i> (Harpacticoid) | 210 m ³ | 10-22 ml ⁻¹ | 4 – 5 kg at regular intervals | <i>Chlorella minutissima</i> , ω-yeast, baker's yeast, with rotifers; outdoor tanks, semi-extensive | Fukusho (1980) |
| <i>Tisbe</i> spp. (Harpacticoid) | 32 l | 1 ml ⁻¹ adults 31 ml ⁻¹ < 200µm | 1.4 nauplii/ind day ⁻¹ | Microfeast l-10 larval diet or <i>Isochrysis galbana</i> | Nanton & Castell (1997) |
| <i>Tisbe holothuriae</i> (Harpacticoid) | 5-l trays | 8 ml ⁻¹ | 300,000 (20 mg) nauplii/tray day ⁻¹ | <i>Rhodomonas baltica</i> , batch intensive | Støttrup & Norsker (1997) |
| <i>Tisbe holothuriae</i> (Harpacticoid) | 150-l closed tank | Not registered | 500,000 ind day ⁻¹ mixed nauplii and copepodites in food- limited cultures | <i>Rhodomonas baltica</i> , continuous intensive | Støttrup & Norsker (1997) |
| <i>Amphiascoides atopus</i> (Harpacticoid) | Three 40 l aquaria and five 28 l plastic boxes | Not measured | .15 million to 2.8 million individuals day ⁻¹ for 17 weeks | <i>Chaetoceros muelleri</i> or TetraMarin fish flakes | Sun and Fleeger (1995) |
| <i>Euterpina acutifrons</i> (Harpacticoid) | 450 l square tanks, outdoor extensive | 20-50 adults ml ⁻¹ | 10% - 15% day ⁻¹ | <i>Chaetoceros</i> , <i>Tetraselmis</i> , and <i>Nannochloropsis</i> | Kraul (1990) |

shapes and containers with different SAV ratios. A comparison of the change in r values for each sampling period ($N_0 = \text{day } 0$) shows a an increase in r for days 9 through 18, a slight decrease between day 18 and day 21, and level r value from days 21 to day 27 (Figure 4).

Discussion

Table 3 reviews some of the more recent attempts to produce high numbers of copepods. A variety of system configurations and production levels have been tried. One of the most successful recent trials lasted more than one year and produced 440,000 nauplii day^{-1} of the calanoid species *Gladioferens imparipes* (Payne and Rippingale 2001). While an automated continuous system would be ideal for a year-round fish production system, some hatcheries may require high numbers of copepods and copepod nauplii sporadically. In this case, a batch culture system that can reliably produce high numbers of copepods in a short amount of time may be a more appropriate solution.

In both Experiments One and Two, more of the copepod populations fed with MMM survived to the end than those fed with the alternate feed. However, population growth varied between the experiments. Experiment One, which lasted 19 days and was fed twice with MMM had a higher intrinsic growth rate (r) than Experiment Two, which lasted 31 days and was fed four times with MMM.

Some explanations for this population growth difference relate to the purity of the feed and tank hygiene. In Experiment One, live *Tetraselmis suecica* invaded the MMM treatments after day 13. This may have increased the growth rates of the copepod populations fed the formulated feed between days 13 and 19. In Experiment Two, overfeeding could have caused a ciliate bloom or a build up of bacteria as the copepods were unable to utilize all the food. This may have depressed growth rates due to poor water quality. All of the treatments in Experiment Two had thick slimy residue at the bottom of the containers. Unlike Experiment One, no water exchange was conducted throughout the 31 days. The lack of water exchange may have been favourable to the copepod populations in the trays, because the debris was spread over a wider area.

The methods outlined in this paper may be applicable in less than ideal culture conditions, where invasive organisms are present. In Experiments Three and Four, ciliates were present in all of the treatments in concentrations greater than 200 ml^{-1} throughout the experiment. Rotifers were present in Experiment Three and nematode worms were present in Experiments Three and Four. In Experiment Three, the total number of copepods was highly correlated with the total number of invading nematode worms and rotifers (Pearson's R value = 0.98, $p < 0.05$). In Experiment Four, the presence of worms was highly correlated with copepod population numbers (Pearson's correlation, $R = 0.88$, $p < 0.001$). The correlation of invasive organism populations with copepod populations indicates that the conditions in the tanks are favourable for growth. Whether the presence of the invaders inhibits copepod growth is not clear. Understanding the relationship of invasive species, such as rotifers and nematodes, to the population growth of the copepods will help in estimating yields when contamination cannot be avoided. It is interesting to note from Experiment Three, that the copepod population growth rates continued to be positive even after 54 days in the presence of rotifers, ciliates and nematode worms (Table 2 and Figure 1).

When all experiments are compared, the average r value is 0.1422 ± 0.01 . The variability in r can be explained by a combination of factors. Univariate and all multivariate combinations of the following independent variables were tested for explanatory value: surface area to volume (SAV) ratio, length of trial (days), average temperature, average salinity, and the beginning density of copepods per litre. The strongest regression equation is found when the r value is regressed against the SAV ratio, length of the trial in days and average salinity:

$$(2) \quad r = -0.7134 + 0.0006 *(\text{SAV}) - 0.0057 *(\text{days}) + 0.0338 *(\text{salinity})$$

$$(0.012) \quad (0.021) \quad (<0.001) \quad (<0.001)$$

$$R^2=0.56, \text{Adj. } R^2=0.52$$

Surface area in this study refers to the air-water interface instead of the total surface of the container available to the copepods. *Nitokra lacustris* does not stick to the sides of the container, but tends to congregate along the bottom surface, which correlates with the water surface. In other studies, it has been shown that the total container surface area to volume ratio may be more relevant to population growth (Støttrup 2000). This may depend on the behaviour of the copepod

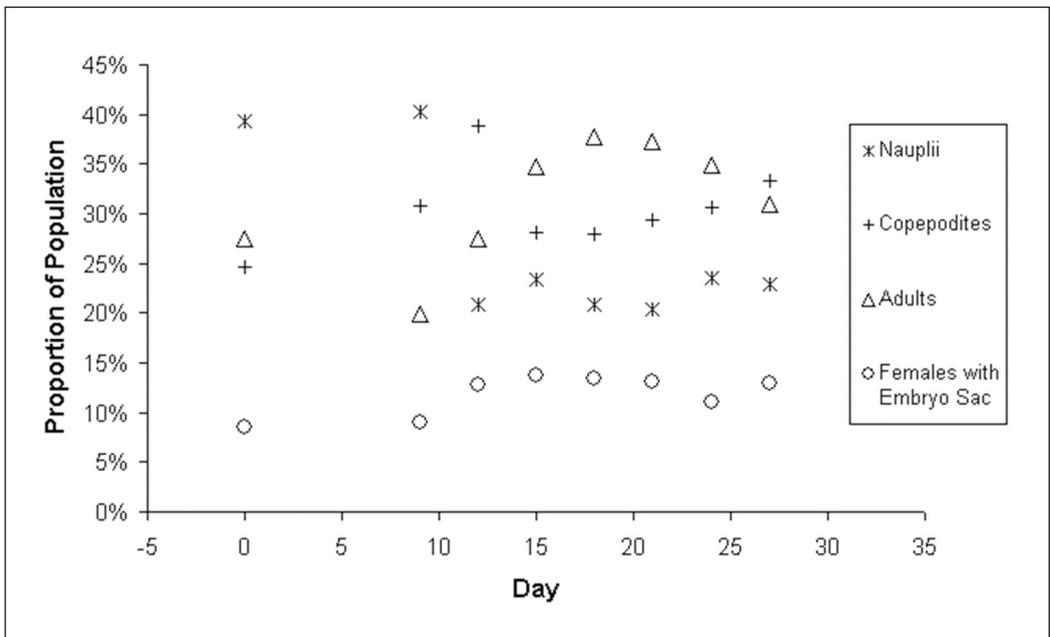


Figure 5. The relative proportions of the life stage changes with time. The proportion of each life stage (nauplii (*), copepodites (+), adults (Δ) and females with embryo sac (○)) is given for each sampling day in Experiment Four. The proportions were calculated by dividing the number of individuals in each life stage by all individuals counted on that day. No significant differences were detected between the arcsin transformed proportions for the three treatments (round 100 l, round 200 l and square 200 l). The data represents the average proportions of all replicates in all treatments.

species. Copepods grown in poor tank hygiene may benefit from a larger surface area to volume ratio, which has been shown to be important in the culture of other harpacticoid copepods (Heath 1994).

From the stepwise multivariate regression analysis, salinity and the length of the trial in combination with the SAV were found to be good predictors of the intrinsic rate of population growth. In Experiment Four, the containers with lower salinity values had slightly higher population densities on day 27, but not significantly higher densities. A small difference in salinity may have a cumulative effect on the daily growth rates, but this remains to be investigated.

The shorter trials, Experiments One and Four, had higher r values than Experiments Two and Three. A comparison of the change in r values for each sampling period ($N_0 = \text{day } 0$) shows an increase in r for days 9 through 18, a slight decrease between day 18 and day 21, and level r value from days 21 to day 27 (Figure 4). This indicates that after day 21, the proportions of the various life stages in the population become more uniform. This suggests that the effect of the length of the treatment on r may diminish after the stable stage population has been reached and if conditions remain favourable for growth (Figure 5).

The reduction in r values in the longer experiments is probably due more to tank hygiene issues as opposed to changes in population composition or the existence of density dependence. For example, copepod cultures in the lab which receive frequent water changes consistently reach much higher densities ($>100 \text{ ml}^{-1}$) than the experimental densities and the presence of density dependent effects on the copepod populations was not detected. In Experiment Four, no significant difference in the ratio of reproductive females to nauplii was detected for days 12 to 27. Data before day 12 is significantly different ($p < 0.05$), but this is to be expected in an exponentially growing population before it has reached a stable stage composition. In Figure 5, it can be seen how the percent composition of nauplii, copepodites and adults on day 9 is quite different from later days.

The development of economical methods to culture copepods would be a significant step toward the successful culture of many species of marine ornamentals and marine food fish. *Nitokra lacustris* is smaller than the harpacticoid copepods *Tisbe sp.* and *Tigriopus sp.*, which are currently used in marine fish hatcheries in Europe and Japan. *Nitokra lacustris* also has other advantages over calanoid species. It can be raised on an artificial feed that is much less expensive than a continuous live culture of algae or commercially available preserved algae. This artificial feed does not rely on marine fish oil, which reduces marine aquaculture's reliance on wild fish stocks. This copepod does not require frequent water changes to grow successfully. It can survive in a wide range of temperatures and salinities. It is very tolerant to invasive species such as ciliates, nematode worms and rotifers.

The methods presented in this paper may provide the framework for further investigations into other potential harpacticoid copepod species. In order to adapt any copepod species to aquaculture, it will be necessary to understand which conditions will allow the copepod population to reach high densities quickly. In this case, short term cultures in 10 l trays fed live algae or formulated feed resulted in the best population growth ($r = 0.34$). These trays have reached densities of 43 copepods ml^{-1} after 19 days when started with less than 0.2 copepods ml^{-1} . Longer term cultures in large containers ($>100 \text{ l}$) were also able to produce high numbers of copepods (4,000 l^{-1}) in less than 30 days when started with 0.03 copepods ml^{-1} . In the larger cultures, starting with a

higher density of copepods could shorten the time to the target density. Transferring a 10 l culture with 43 copepods ml⁻¹ into a 200 l container could result in 43 copepods ml⁻¹ within 3 weeks.

High density batch copepod cultures might be appropriate for hatchery-produced fish that can benefit from being fed copepods for a few days during larval growth. For example, typical defects in Atlantic halibut can be avoided by feeding copepods during a critical window of time from two to three weeks after first feeding (Næss and Lie 1998). Three 200 l containers with a copepod density of 43 ml⁻¹ harvested without replenishment would suffice to meet one week's feeding requirements for 4000 halibut in a 1500 l tank fed 2 copepods ml⁻¹ day⁻¹ (Næss and Lie 1998).

With nine 200 l containers, a rotating harvesting schedule would allow the continuous production of copepods. On average, the copepods increase by 15.3% day⁻¹ (based on the average *r* value of 0.1422). Theoretically, a 200 l tank with 43 copepods ml⁻¹ harvested by one third would replenish in three days. If nine 200 l tanks were used for the system, and were harvested by one third each day, a total sustainable production of 8.6 million copepods day⁻¹ could be achieved. This is enough for 4.3 m³ of fish production using 2 copepods ml⁻¹ (Næss and Lie 1998). If this partial harvest approach were used, this copepod production level could be maintained for longer than 7 days with careful management.

Acknowledgements

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Appendix

Colour plates

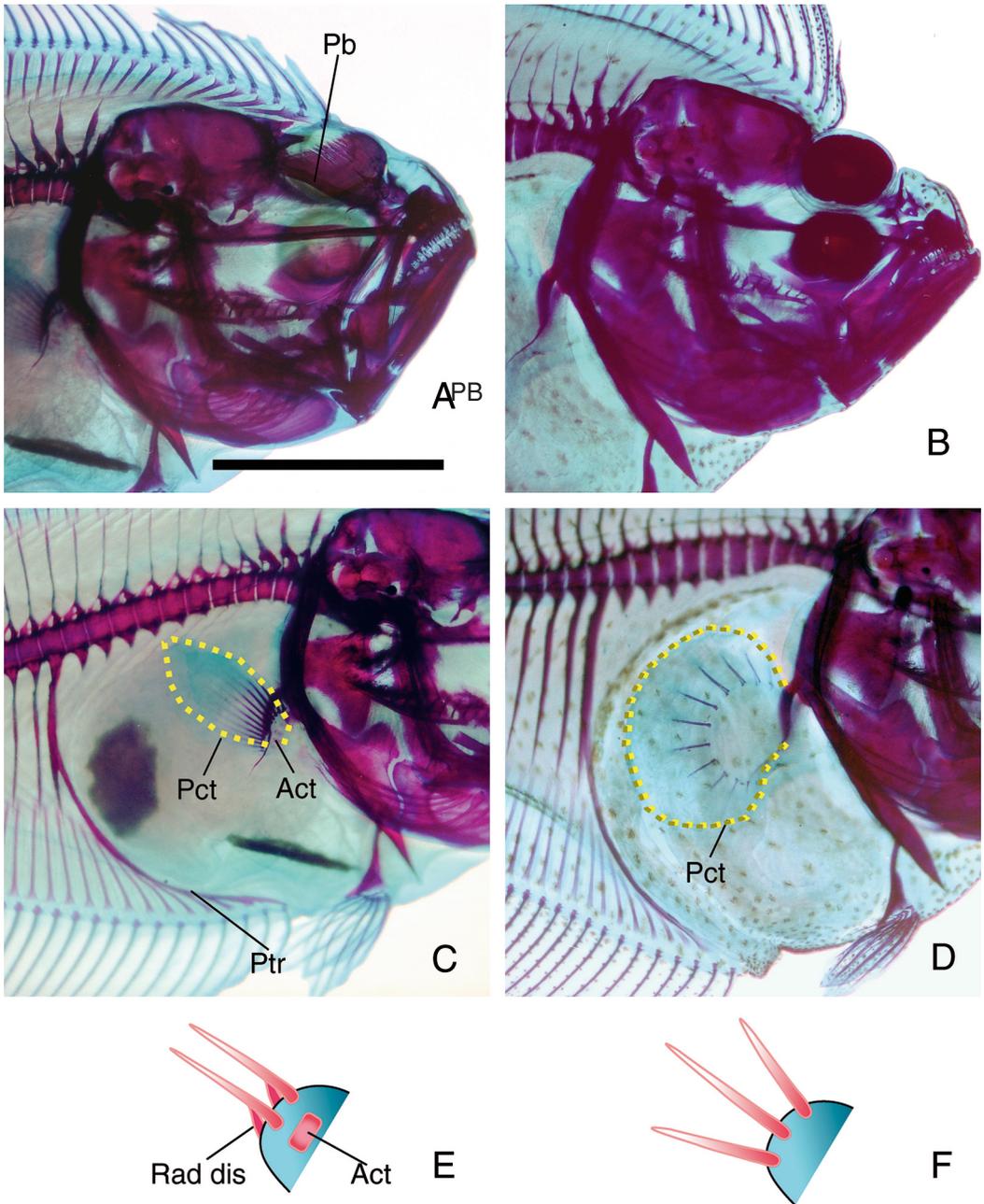


Figure 4 from Okada et al. (page 182). Anterior part of *Paralichthys olivaceus* at 35 dph reared 20 days; (A), (C) in ordinary seawater Control, and (B), (D) in 30 ppm TU. Schematic drawings of the pectoral fin of Control fish (E) and TU-treated fish (F). Photographs were taken on cleared and double stained samples from the blind side. The outline of the pectoral fin is indicated by a yellow broken line. Act, actinost; Pb, pseudomesial bar; Pct, pectoral fin; Ptr, pterygiophore of anal fin; Rad dis, distal radial. Bar 3 mm.

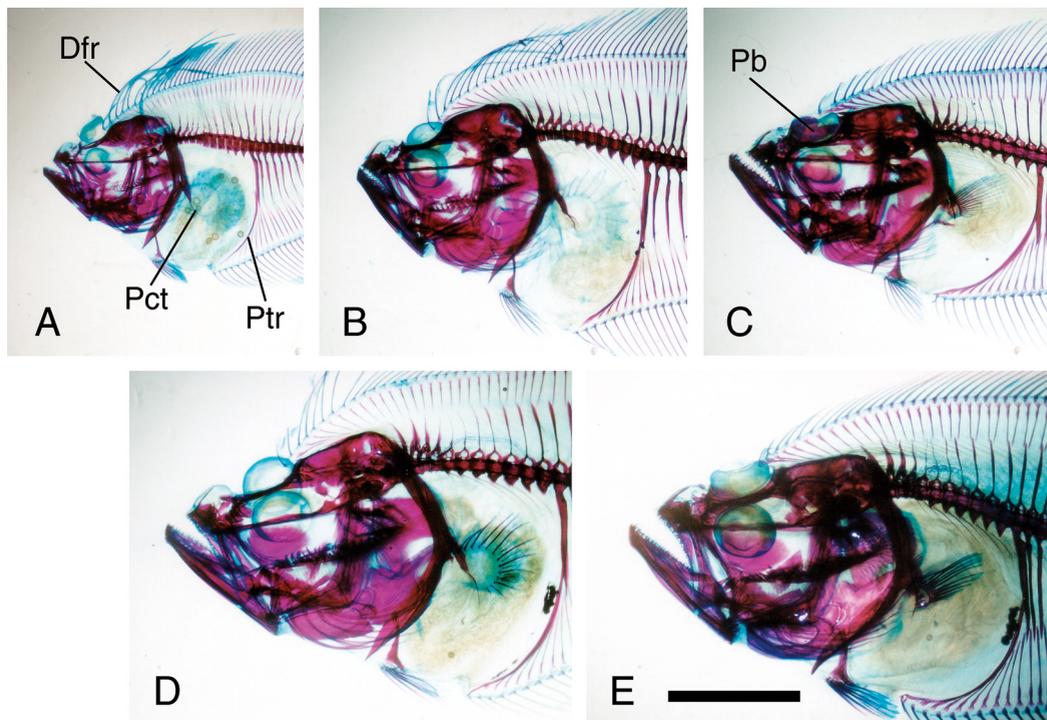


Figure 6 from Okada et al. (page 183). Differential responsiveness of internal morphology of TU-treated fish to exogenous T4 provided at different timings. All the fish including controls were reared under the presence of TU (30 ppm) from 22 dph. Photographs were taken on cleared and double stained samples. (A) 38 dph, initial control; (B) 51 dph, control; (C) 51 dph, T4 (100ppb, 38 to 51dph); (D) 81 dph, control; (E) 81 dph, T4 (100 ppb, 67 to 81 dph). Dfr, dorsal fin ray; Pb, pseudomesial bar; Pct, pectoral fin; Ptr, pterigiophore of anal fin. Bars = 1 cm.

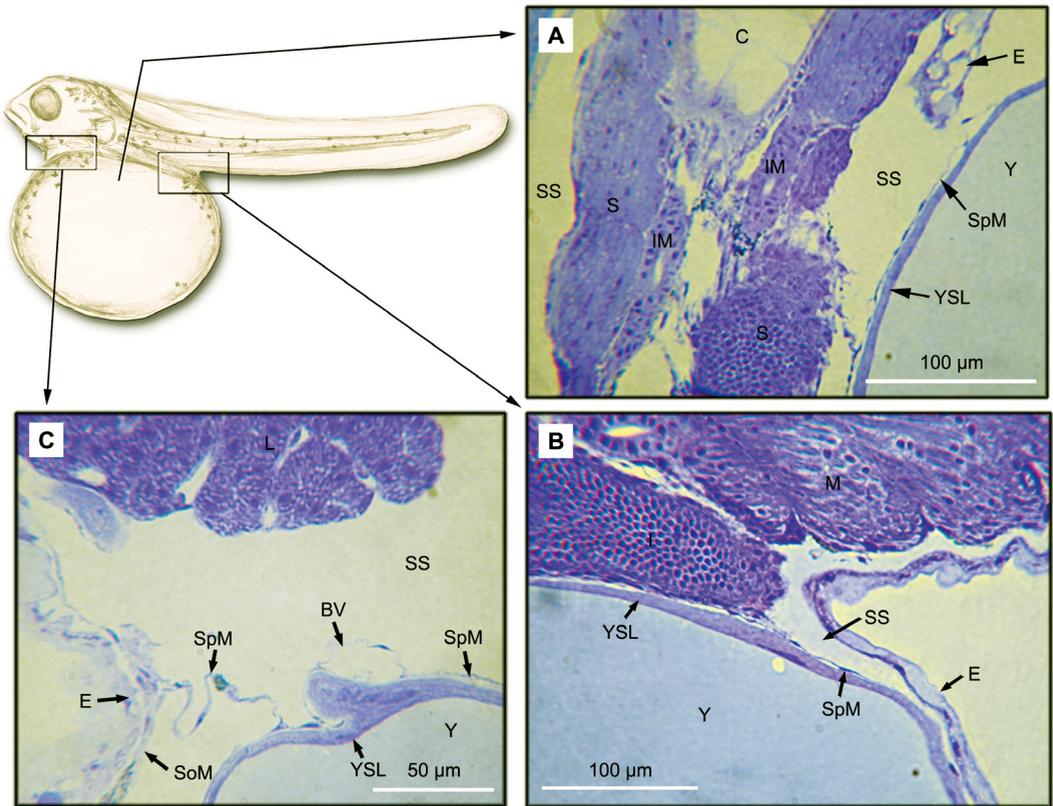


Figure 7 from Skjærven et al. (page 202). Histological sections stained with toluidine blue showing the fate of the splanchnic mesoderm and yolk syncytial layer of yolk-sac larvae of plaice (*Pleuronectes platessa*, series A). A: Transverse section of an embryo (15 DPF). The splanchnic mesoderm covers the dorsal part of the yolk syncytial layer and the yolk. Note that the splanchnic mesoderm ended lateral to the embryonic tissue, and does not continue around the entire yolk-sac. B: Saggital section of a larva (15 DPF, days post fertilization). The splanchnic mesoderm covers the dorsal part of the yolk syncytial layer and the yolk, but ended caudal as a loose cell layer close to the anus. C: Saggital section of anterior region of a larva (20 DPF). Splanchnic mesoderm attached to the somatic mesoderm which underlies the epidermis in the cranial area of the yolk-sac. Note the blood vessel evident in the splanchnic mesoderm. BV: blood vessels, C: Chorda, E: epidermis, IM: intermediate mesoderm, I: intestine, L: liver, M: muscles, SoM: somatic mesoderm, S: somite, SpM: splanchnic mesoderm, SS: subdermal space, Y: yolk, YSL: yolk syncytial layer.

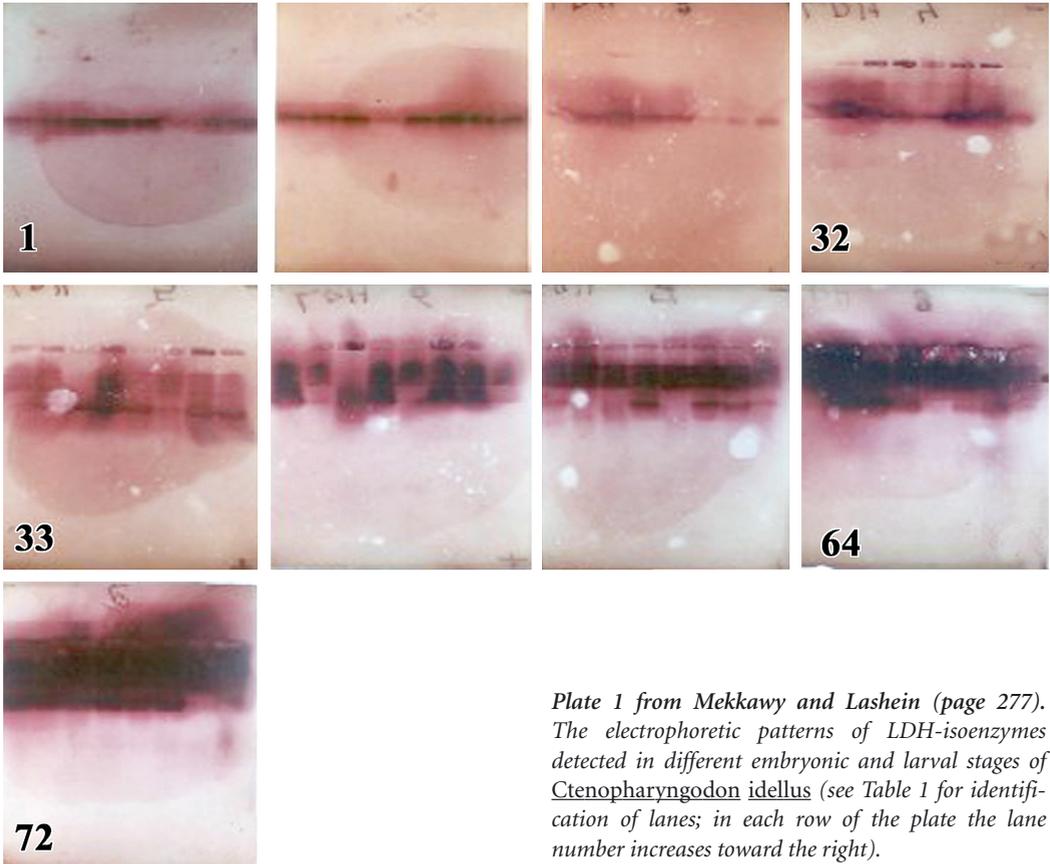


Plate 1 from Mekkawy and Lashein (page 277).
The electrophoretic patterns of LDH-isoenzymes
detected in different embryonic and larval stages of
Ctenopharyngodon idellus (see Table 1 for identifica-
tion of lanes; in each row of the plate the lane
number increases toward the right).

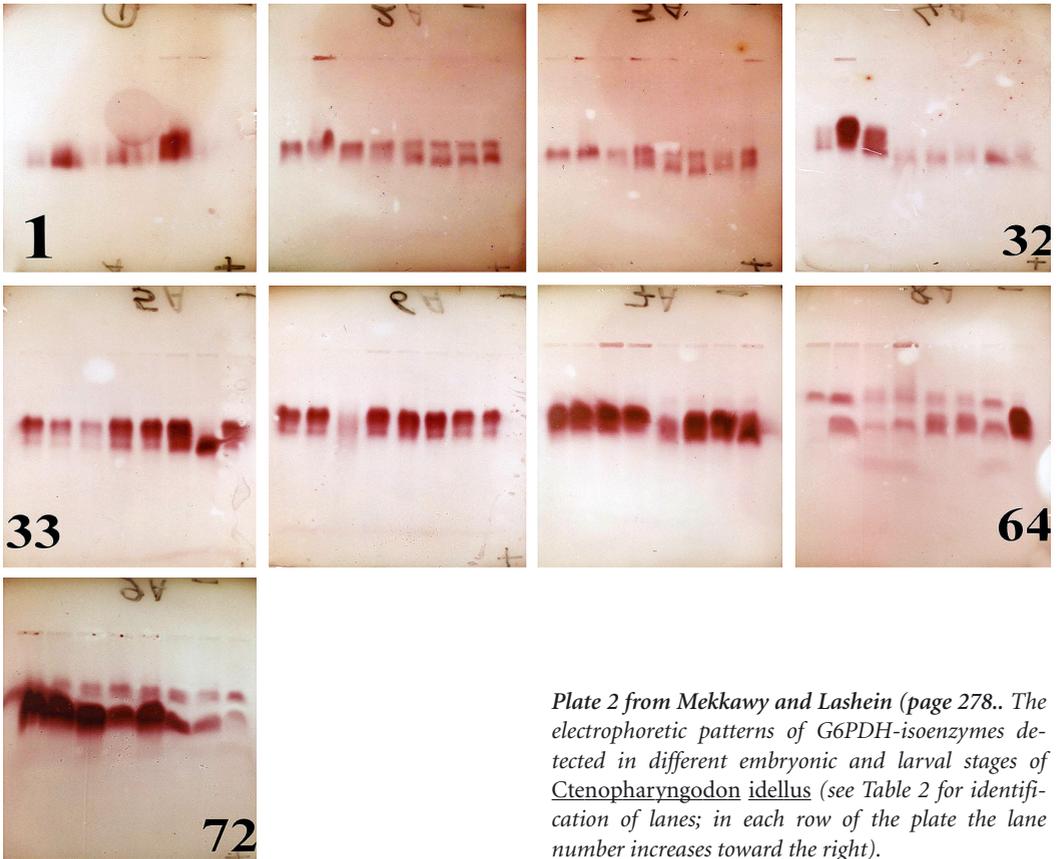




Plate 3 from Mekkawy and Lashein (page 282). 16-hour, 20-hour and 3-day post-hatching stages of *Ctenopharyngodon idellus* showing somite and organ differentiation, eye pigmentation and yolk reduction.

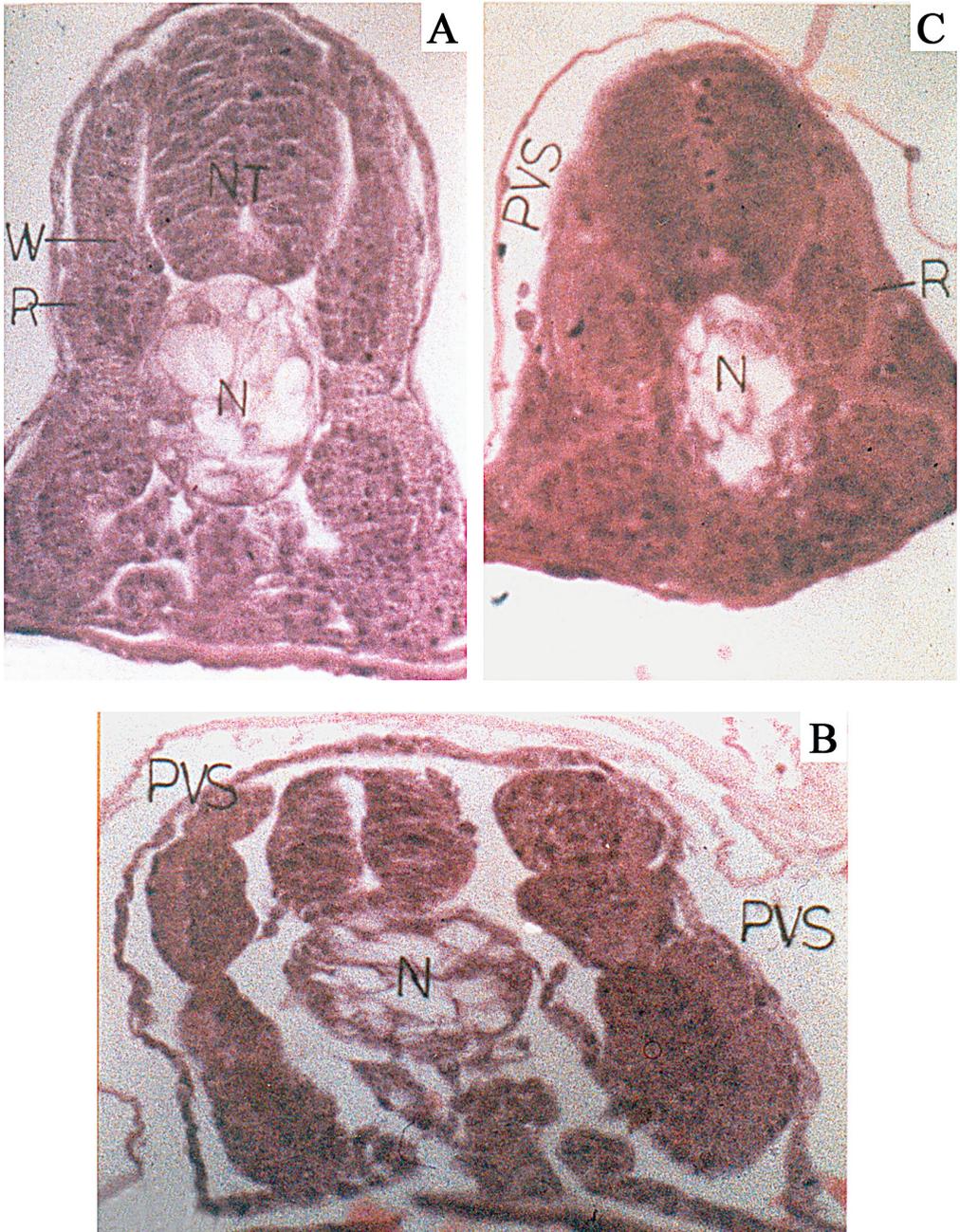
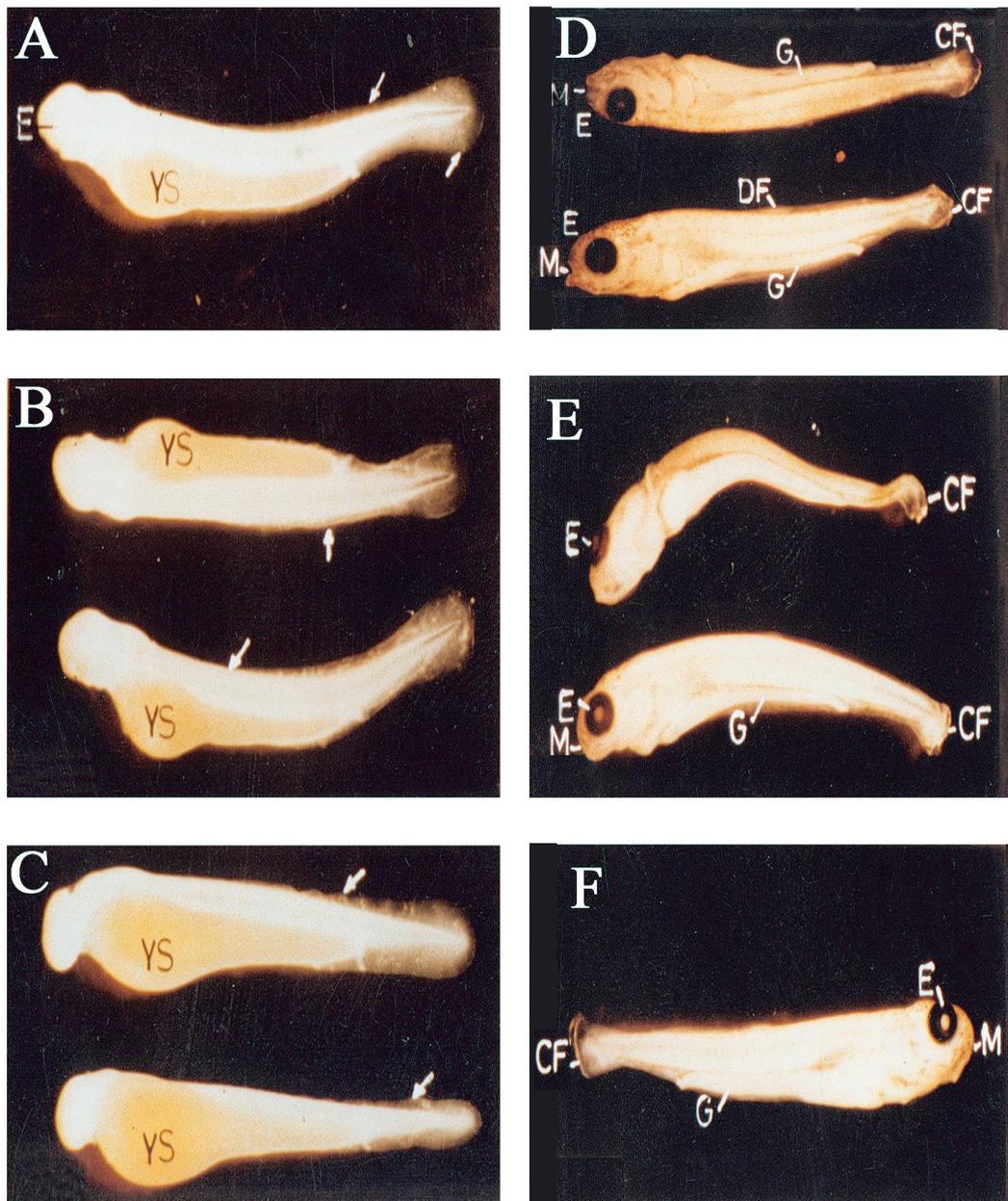


Plate 4 from Mekkawy and Lashein (page 283). Transverse sections at the trunk level of 18-hour embryo of *Ctenopharyngodon idellus* treated with cadmium (B) and lead (C) in comparison with the control (A). See the reduced perivitelline space, PVS; disorganized peripheral red myogenic cells, R and distorted notochord, N (Pb less toxic than Cd). W: white muscles; NT: neural tube.



1DPH-stage

5DPH-stage

Plate 5 from Mekkawy and Lashein (page 285). Control (A, D), Cd-100 ppm (B, E) and Pb-100 ppm (C, F) treated 1-day and 5-day post-hatching larvae of *Ctenopharyngodon idellus* showing spinal-vertebral curvature, paleness of body and necrosis of caudal fin (CF). E: eye, M: mouth, G: gut, YS: yolk sac, DF: dorsal fin.

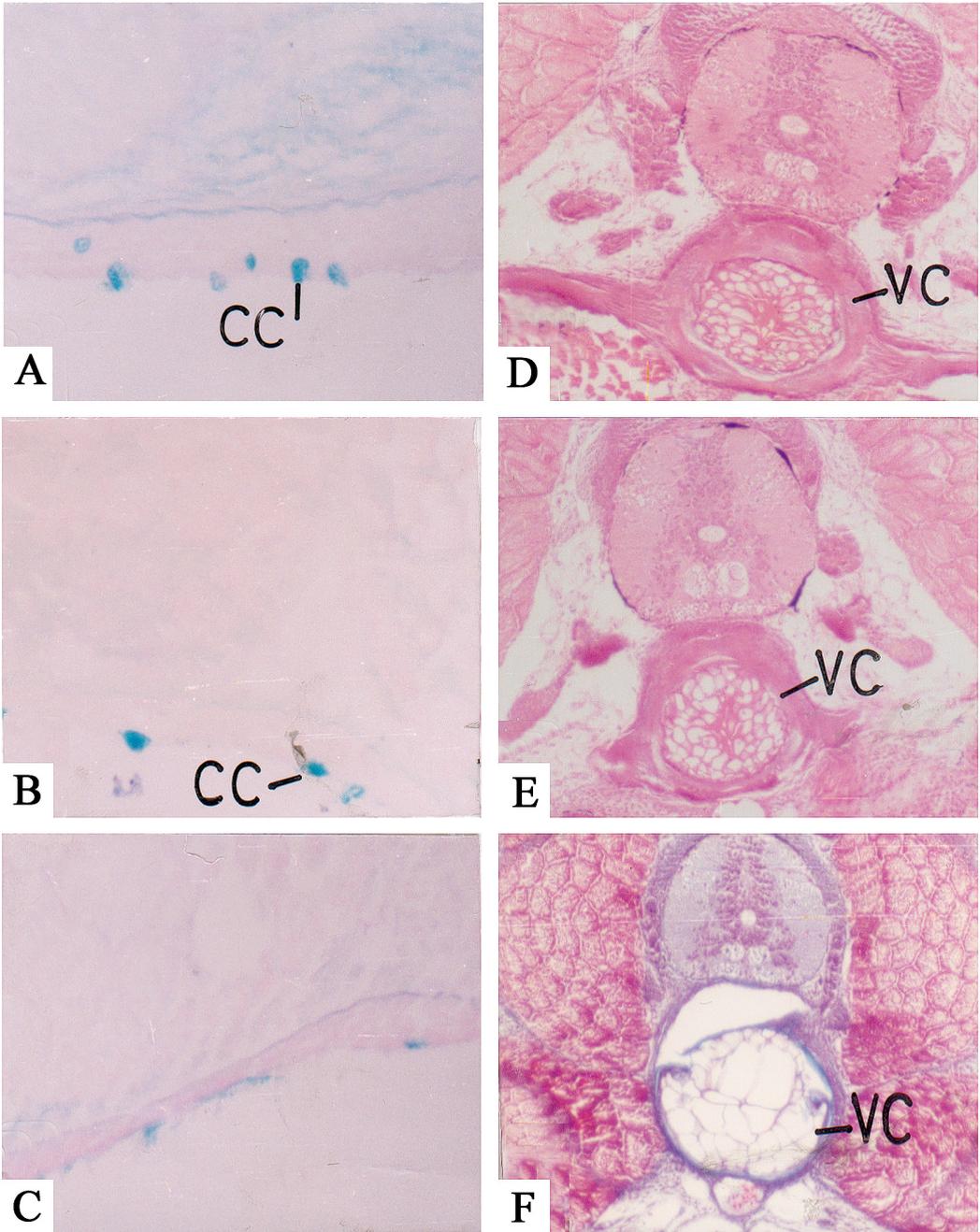


Plate 6 from Mekkawy and Lashein (page 288). Transverse sections of the control (A), Cd-treated (B) and Pb-treated (C) 14-day post-hatching larvae of *Ctenopharyngodon idellus* showing chloride cells (CC) in the skin and the corresponding vertebral centrum (VC) deposition (D, E, F respectively).