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Development of eggs and yolk sac larvae of halibut (*Hippoglossus hippoglossus* L.)

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Summary

Timing of development in halibut eggs and larvae from one female was monitored at 3°, 6°, and 9 °C. Total and viable count of free-living bacteria in the incubators was monitored from hatching until termination of the experiment and water quality was recorded. Differences in development rates were apparent from the first cell divisions. At 9 °C there were significantly more KUPPFER's vesicles in embryos and RNA content was lowest. At 3° embryos had the highest RNA content but often showed incomplete development of the caudal fin. At 6 °C egg mortality was lowest, and larval growth was intermediate. DNA was significantly different between all temperature groups. At 9 °C, larvae grew faster, but developed abnormalities associated with sublethal stressors. A rise in larval mortalities occurred at the same stage of development at 6° and 9 °C. Significantly more jaw deformities occurred at 9 °C than at 6 °C. There was no significant difference in bacterial numbers between groups. An increase in larval mortalities leads to an increased amount of bacteria which preceded an increase in ammonia levels. Optimal temperatures for egg and larval development probably lie between 3° and 6 °C, although there may be different optima for different stages. The experiment was terminated due to uncontrolled temperature fluctuations.

Zusammenfassung

Entwicklung von Eiern und Dottersack-Larven des Heilbutts (Hippoglossus hippoglossus L.)

Die Entwicklungsgeschwindigkeit von Heilbutteiern und -larven eines Weibchens wurde bei 3 °C, 6 °C und 9 °C verfolgt. Die Gesamtkeimzahl und die Zahl freilebender Bakterien im Inkubator wurde vom Schlupfzeitpunkt an bis zum Versuchsende erfaßt und die Wasserqualität bestimmt. Unterschiede in den Entwicklungsraten waren von der ersten Zellteilung an offensichtlich. Bei 9 °C konnten wesentlich mehr Kuppfer'sche Blasen festgestellt werden und die Embryonen hatten den geringsten RNA-Gehalt. Bei einer Inkubationstemperatur von 3 °C hatten die Embryonen den höchsten RNA-Gehalt, zeigten jedoch häufig unvollständige Entwicklung der Schwanzflosse. Bei 6 °C war die Sterblichkeit am geringsten und das Wachstum der Larven lag zwischen den Werten der beiden anderen Temperaturen. Der DNA-Gehalt war in allen 3 Gruppen unterschiedlich. Bei 9 °C wuchsen die Larven am schnellsten, zeigten jedoch Anomalien, die auf subletale Stressoren zurückzuführen sind. Eine Zunahme der Larvensterblichkeit wurde bei 6 °C und 9 °C auf den selben Entwicklungstadium festgestellt. Erheblich mehr Kieferdeformation traten bei 9 °C als bei 6 °C auf. Die Keimzahlen waren in allen Versuchsgruppen nicht signifikant verschieden voneinander. Bei steigender Larvenmortalität nahm auch die Bakterienzahl zu. Diese ging der Erhöhung der Ammoniumkonzentration voraus. Die optimale Inkubationstemperatur für die Eier und Larven des Heilbutts liegen wahrscheinlich zwischen 3 °C und 6 °C; es mögen jedoch unterschiedliche Optima für einzelne Entwicklungstadien existieren. Das Experiment wurde aufgrund unkontrollierter Temperaturfluktuationen beendet.

Résumé

Développement des oeufs et des larves à sac vitellin du flétan (Hippoglossus hippoglossus L.)

Le temps de développement des oeufs et des larves d'une seule femelle a été observé à 3°, 6° et 9 °C. De l'éclosion jusqu'à la fin de l'expérience, on a compté le nombre de bactéries, total et viabilité, qui vivaient librement dans les incubateurs et la qualité de l'eau a été mesurée. Des differences dans le taux de développement sont apparues à partir de la première division des cellules. A 9 °C, il y avait significativement plus de vésicules de Kuppfer dans les embryons et le taux en RNA était plus bas. A 3 °C, les

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embryons avaient le taux en RNA le plus élevé, mais très souvent, le développement de la nageoire caudale était incomplet. A 6° C, la mortalité des oeufs était la plus basse et la croissance larvaire était intermédiaire. Le taux en DNA était significativement différente entre les groupes de température. A 9 °C, les larves grandissaient plus vite, mais développaient des anomalies associées à des facteurs de stress sublétaux. Une augmentation ade la mortalité larvaire apparait au même stade de développement à 6° et 9 °C. Il y avait significativement plus de déformations de la machoire à 9 °C par rapport à 6 °C. Il n' avait pas de difference significative dans le nombre de bactéries entre les groupes. L'augmentation de la mortalité larvaire conduisait à un taux de bactéries plus élevé qui précedait l'augmentation de la teneur en ammoniaque. La température optimale pour le développement des ouefs et des larves se trouve probablement entre 3° et 6 °C, même s'il peut exister des optima différents pour les differents stades. L'expérience a été terminée lorsque des fluctuations de température non-controllées sont apparues.

Introduction

The temperature of incubating eggs affects the rate of embryo development and the condition of the emergent larva (ROSENTHAL & ALDERDICE 1976). Subsequently, temperatures experienced by the primitive halibut larva during the yolk sac stage have been shown to affect growth and yolk conversion efficiencies as well as rates of deformity (PITTMAN et al. 1989). However, it is clear that temperature also influences bacterial activity and the impact of abiotic parameters such as oxygen tension and ammonia (WEDERMEYER 1976). These in turn may also affect larval mortality. This experiment was conducted to monitor correlations between egg and larval development of halibut and the physical and microbial environment in the incubators.

Materials and methods

Water treatment

Sand-filtered water from about 55 m depth was piped to the incubators through mixing valves, which blended two "stock" temperatures (3° and 10 °C). The mixing valves were Satchwell Linear Activator, type ALM 1601, and the heat pump aggregator was an EUW 20 F (Daikin). Cascade filters were used to aerate and equilibrate the water at one atmosphere.

Incubators

A total of 24 incubators were used, nine for the egg stage (three at each temperature) and fifteen for the larval stage (five at each temperature). These were arranged on line so that the same stock water was used for each temperature group. The incubators at 3 and 9 °C were wrapped in rockwool insulation to minimize temperature variation. Only the 3° incubators were insulated during the egg stage, while 6 °C was close to the ambient temperature.

The incubator system was modified from JELMERT and RABBEN (1987). Each incubator was a semiconical, 250 L fibreglass unit with a split inlet valve on the bottom for introduction of new water and extraction of sedimented material and larvae, and a sleeve filter for overflowing water at the top. A lid prevented light from reaching the eggs and the developing halibut (Figure 1).

Egg source and incubation

One female and two males from the broodstock of the Austevoll Aquaculture Station were stripped for eggs and sperm. The eggs (1.4 liters, or about 50,000 eggs) and sperm were transported separately from the broodstock tank, where the temperature was 6–7 °C, to the nine egg incubators. Sand-filtered 6 °C seawater was added to the eggs, which were then fertilized. About 160 ml of the fertilized eggs were placed in each of nine 1-L beakers, and one beaker was placed in each of the incubators for half an hour to attain 3°, 6° or 9 °C before gently inverting them into the incubator. This method was chosen to avoid mortality due to

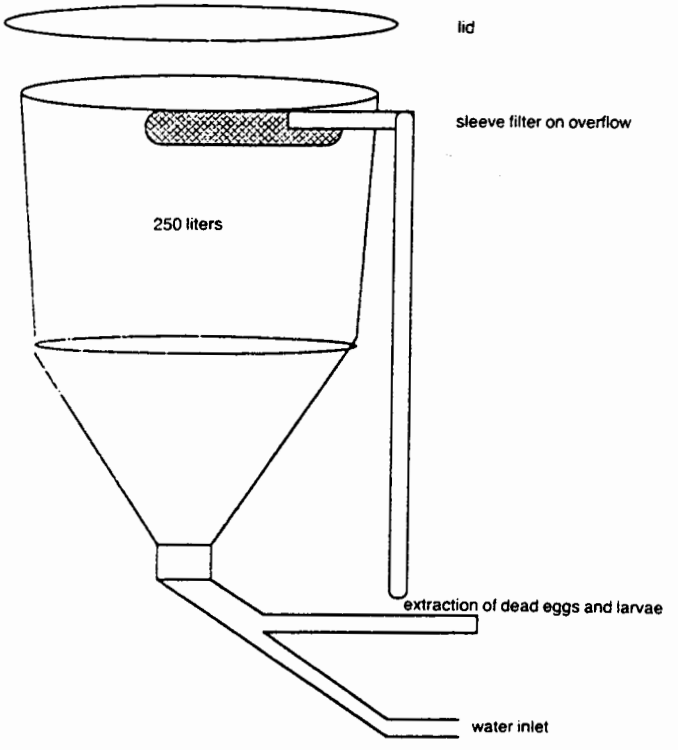

Fig. 1. Schematic diagram of incubator used for halibut eggs (modified from JELMERT & RABBEN 1987)

shock and manipulation according to HOLMEFJORD & BOLLA (1988) and JENSEN & ALDERDICE (1989). A control group had 93 % fertilization of the eggs.

At 50 % hatching, the eggs and larvae were transferred from the three egg incubators at a given temperature and distributed randomly among similar larval incubators at the same temperature. The emergent larvae from one egg incubator at 6 °C and one at 3 °C were not mixed with the others in order to counteract a possible infection.

Egg samples

About twenty eggs were taken every day from each of the incubators to estimate development rate (more often during the first cell divisions), using a red light for illumination of the dark incubators and a sterilized glass pipette for extraction. Each cell division was observed live and eggs were preserved in glutaraldehyde. Thirty eggs from each group were taken every third day, washed in distilled water, measured for diameter and put in a drying oven for 48 hours at 60 °C for dry weight estimation.

Larval samples

Day 0 of larval development was noted when 50 % of the eggs had hatched. Live larvae for sampling were removed every third day from hatching to the end of the experiment. Ten to fifteen larvae were taken from each incubator for morphometric and meristic measurements. Five of these larvae were preserved for scanning electron microscopy and five to ten frozen for dry weight and free amino acid and protein analyses. In addition, six groups of eight larvae were extracted from three incubators in each temperature group once a week and analysed for RNA and DNA, according to procedures in RAAE et al. 1988.

Mortalities

Dead eggs or larvae were removed from the incubators every 2–3 days by stopping the flow and introducing ten liters of salt water (about 45 ppt) for about 10 minutes. Dead eggs and larvae sank and were removed via the bottom valve along with the ten liters of salt water. The material was then filtered and preserved in formalin for mortality counts and counts of jaw deformities. The water flow was restarted and adjusted according to the apparent density of the eggs or larvae.

Scanning electron microscopy

Larvae to be examined by S. E. M. were fixed in 2.5 % glutaraldehyde buffered with cacodylate to pH 7.3, washed three times in cacodylate buffer, postfixed in 2 % cacodylated OsO_4, washed again three times with cacodylate buffer, dehydrated in an ethanol series, dried to the critical point and coated with gold and palladium. A JEOL JSM35 scanning electron microscope was used.

Enumeration of bacteria

For total counts of free-living bacteria, DAPI stain was used (PORTER and FEIG 1980). Sample volumes of 3–5 ml were filtered on 0.2 µm Nucleopore filters that had been prestained with Irgalan Black. Support filters were soaked in the 10 µg/ml stain solution, according to HOFF (1988). Counting was carried out using a Nikon Optiphot epifluorescence microscope at 600x. At least 200 cells were counted in each sample. Total counts were performed twice a week, at the same time as the other samplings.

In addition, viable counts of bacteria (ie. counts of colony-forming units) were conducted once a week on three different media, TSA (Tryptone Soya Broth, Oxoid), TCBS (Tryptone Citrate Bile Salt, Oxoid) and Cytophaga Agar (FRERICHS 1984). All media were made with 70 % seawater. Petri dishes were incubated at 10 °C for at least 10 days.

Water quality

Samples were taken for ammonia and oxygen about twice a week during the larval stage. Water was siphoned from below the surface directly into the sample bottles. Triplicate samples were taken for ammonia and analyzed spectrophotometrically on a Shimadzu UV-160 spectrophometer according to KOROLEFF (1976). Oxygen was measured by Winkler method. Flow rates were maintained between 0.5 and 2-L/min during the experiment and recorded daily. Temperature of the outgoing water from each incubator was recorded daily. Salinity of the inlet water was recorded daily at Austevoll Aquaculture Research Station.

Results

Growth and development of eggs

Development of eggs at 3°, 6° and 9 °C is listed in Table 1. The effect of temperature on development rate was seen from the time of the first cell divisions, when eggs at 3 °C took about 60 % longer to reach the 2-cell stage. By the 8-cell stage the time difference was over 100 % relative to the warmest groups. Incubation time was almost three times longer at 3° than at 9 °C. A linear regression line plotting the incubation time against temperature gave a correlation coefficient of 0.955 and the equation:

$$Y = -65.7928\ X + 772.703945$$

where Y is the time to 50 % hatching and X is the incubation temperature.

Table 1. Egg development (hours) from fertilization to 50 % hatching in halibut eggs incubated at 3, 6 and 9 °C. The replicate incubators at each temperature are A, B and C

Stage	3a	3b	3c	6a	6b	6c	9a	9b	9c
2-cell	8.1	8.8	8.8	6.8	6.8	6.8	4.3	5.5	5.1
4-cell	13.6	13.9	13.9	9.3	10.8	10.8	6.8	7.8	7.5
8-cell	17.9	19.6	19.9	12.6	13.6	13.6	8.4	9.3	9.3
16-cell	24.6	23.5	24.1	15.6	16.8	16.8	10.8	11.8	11.8
32-cell	28.6	28.6	28.6	–	–	–	12.6	13.6	13.6
germring formed	125	118	125	74	74	74	49.5	49.5	49.5
yolk plug closure	330	295	295	144	145	145	103	106	107
heartbeat	536	524	536	297	297	297	191	191	191
hatching	600	600	600	336	336	336	211	211	211

The mean dry weight of the eggs was 1.8 +/- 0.15 mg after fertilization, but 7 days later this decreased in all groups to a mean of 1.64 +/- 0.06 mg. This weight was approximately maintained for the remainder of the egg stage. The mean diameter of the eggs after fertilization was 3.12 +/- 0.1 mm, decreasing to a mean of 3.07 mm with no significant difference between temperature groups.

KUPPFER's vesicles appeared before the yolk plug closure in all groups. There were significantly more KUPPFER's vesicles at 9 °C than at either 6° or 3 °C ($p = 0.0001$, WILCOXON signed rank) but there was no significant difference in the number of vesicles between larvae raised at 3° or 6 °C. The mean number of vesicles at closing of the blastopore was 1.6 +/- 1.42 (3°), 0.60 +/- 0.77 (6°) and 5.44 +/- 3.60 (9°). At the 17-somite stage the mean number of vesicles was 1.66 +/- 1.47 (3°), 1.26 +/- 0.58 (6°) and 5.50 +/- 2.94 (9°). At the initiation of heart activity, the mean number was 0.64 +/- 0.87 (3°) and 2.14 +/- 2.01 (9°).

At the three temperatures studied the embryonic development of the hatching gland took place normally as described by HELVIK 1988. The eggs also appeared to open in the same manner at all temperatures (data not shown) in accordance with the "rim hatching"-model described by HELVIK (1988). However, the rate of post-hatching degeneration of the hatching gland appeared to be temperature-dependent, as illustrated in Fig. 2. Accelerated degeneration was observed at higher temperatures. At 6 °C the hatching gland was visible a week post-hatching.

Egg mortality

The percentage of mortality during the egg and larval stages is displayed in Figure 3, where the three egg incubators and the five larval incubators at each temperature are pooled (for more details see BERGH et al., 1989). The mortality includes the number of halibut sampled each week. Egg mortality was fairly constant throughout embryo development (except in one incubator at 6 °C which was excluded from the figure). Mortality dropped off after day 13 at 3 °C, corresponding with the yolk plug closure.

Growth and development of larvae

Development of larvae in the three temperature groups is shown in Figure 2. All larvae in a temperature group reached the same stage of development at about the same time. In the 9 °C groups, the liver developed quickly into a tight, dark cell mass high on the yolk sac and the intestine generally was curved at the colon, rather than making the normally distinct right angle. As the heart developed, it was apparent that the pericardial cavity was larger and the heart smaller in the 9 °C groups (Fig. 4a). The frequency of these abnormalities decreased with decreasing temperature.

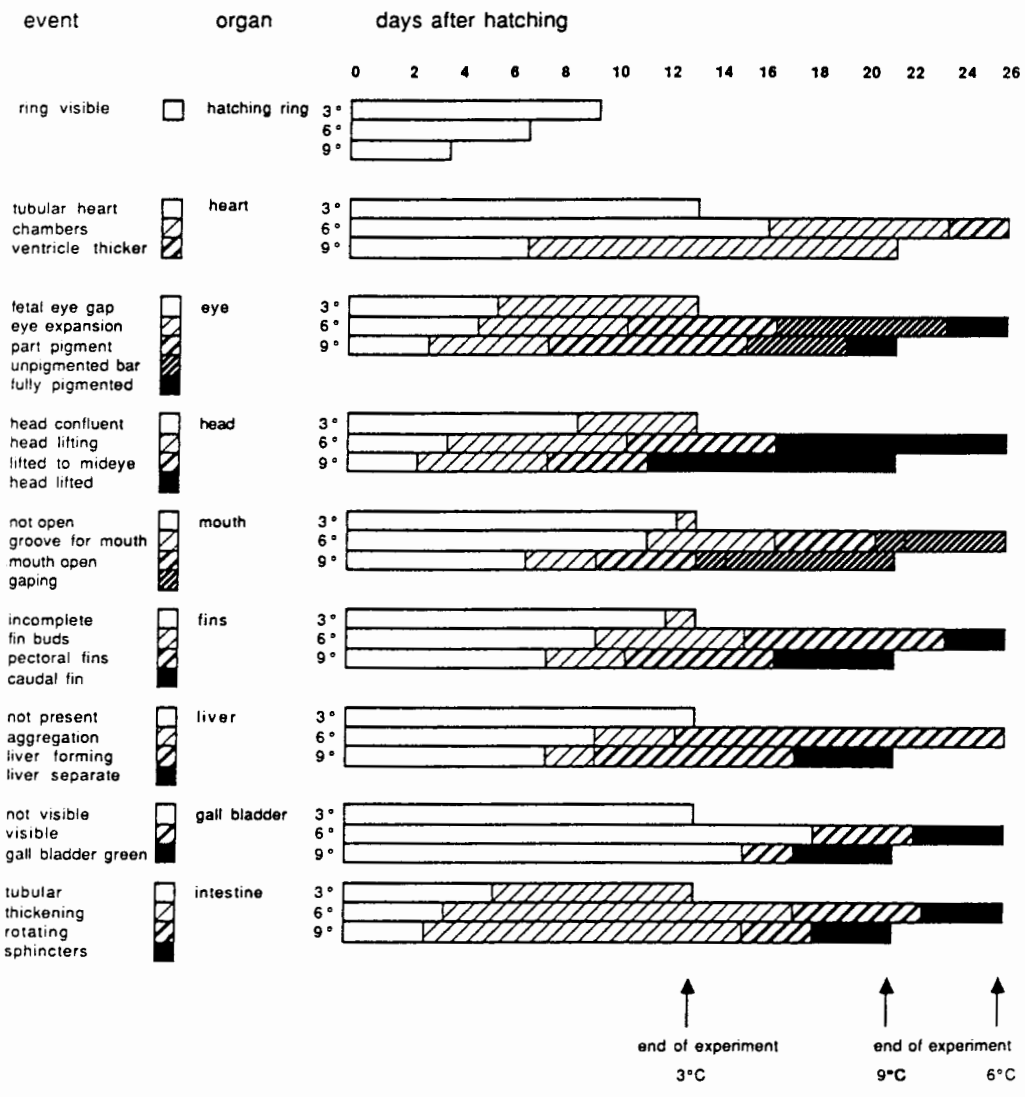

Fig. 2. Morphological development of the halibut larva (in days) at 3°, 6° and 9 °C. The halibut is very primitive at hatching. The eye cup is not completely closed when the larva emerges from the egg (fetal eye gap) but the cup soon expands and forms a circle around the lens. Eye pigmentation is a slow process and there is a period when much of the eye is pigmented with the exception of a distinctly clear bar. There is no opening for the mouth at hatching, although a groove forms between the lifting head and the yolk sac where the mouth will be. The liver is not formed at hatching but a cellular aggregation can be seen on the anterior yolk sac in the period preceding the development and separation of this organ. A more detailed description of the general development of the halibut larvae from hatching to metamorphosis can be found in PITTMAN et al. 1990

Differences between larval development in the temperature groups were seen from hatching onward. Standard length at hatching was significantly different between the temperature groups ($p = 0.0001$, ANOVA), with the larvae at 9 °C being the longest, followed by those from 3° and then those from 6 °C (Fig. 5). The means and standard deviations were 6.12 +/- 0.19 mm (3°), 5.92 +/- 0.21 (6°) and 6.45 +/- 0.21 (9°). The yolk sac length at hatching was significantly less for the 9 °C larvae ($p = 0.0001$, ANOVA) but there was no significant difference between those from 3° and 6 °C. The means and s.d. for yolk sac length were 3.82 +/- 0.24 mm (3°), 3.88 +/- 0.24 (6°) and 3.69 +/- 0.21 (9°). At 3 °C, the yolk sacs

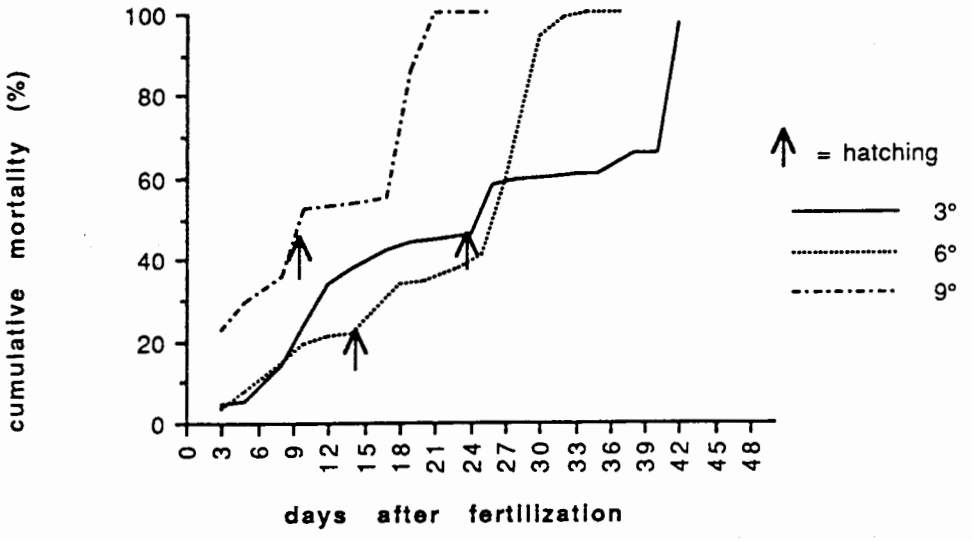

Fig. 3. Cumulative percentage mortality of eggs to hatching and larvae to the end of the experiment at 3°, 6° and 9 °C. Mortality includes the number removed in sampling. The figure excludes one egg incubator at 6 °C where mortality was almost total

filled the peritoneal cavity whereas those at 9 °C often had fairly large spaces between the sac membrane and the outer membrane. At 6 °C, the space was smaller but still visible. In Figure 6, the mean standard length of larvae is plotted against yolk sac length for each of the three temperature groups from hatching to the end of the experiment. Slope was steepest for the 9 °C group, and regression coefficients were 0.92, 0.97 and 0.91 for 9°, 6° and 3 °C, respectively.

The DNA and RNA content of larvae is shown in Fig. 7a and RNA/DNA ratios are plotted in Figure 7b. At hatching, RNA is significantly greater in the 3° than in the 9 °C groups, whereas at 6 °C the RNA is intermediate ($p = 0.0277$, Tukey's H. S. D.). RNA generally increases in all groups from hatching onward. DNA at hatching is significantly different between all groups ($p = 0.000$, Tukey's H. S. D.). DNA increases from hatching only in the 3 °C group, whereas in the 6° and 9 °C groups it remains almost constant during the first 12 days. At 3 °C, 10-day old larvae have significantly more DNA than at 6 °C, ($p = 0.011$, ANOVA) although their stage of development is less advanced. The ratio of RNA/DNA seems to follow a curve, reaching the same levels as at hatching about twenty five days posthatching (6° and 9 °C). Relative protein synthesis (RNA/DNA) for the 9 °C groups is significantly lower than for the others ($p = 0.0086$, Tukey's H. S. D.), while that of the 3 °C groups is significantly higher.

Larval mortality

Larval mortality from the time of 50 % hatch (Figure 3) is given as the pooled percentage for the five larval incubators at each temperature (for more detail see BERGH et al., 1989). The time lag between the increase in larval mortality at 9° and at 6° corresponds to when the head lifts from the yolk sac and there is a groove for the mouth. The increase in mortality at 3 °C corresponds to the time at which the temperature fluctuated nearly 2 °C on a daily basis.

Deformities

An abnormality was often seen in the 3 °C groups during embryo development where the caudal fin fold was not completely expanded and the caudal end of the notochord resembled a small clump (Fig. 4b). The mortality counts also yielded counts of the number of larvae

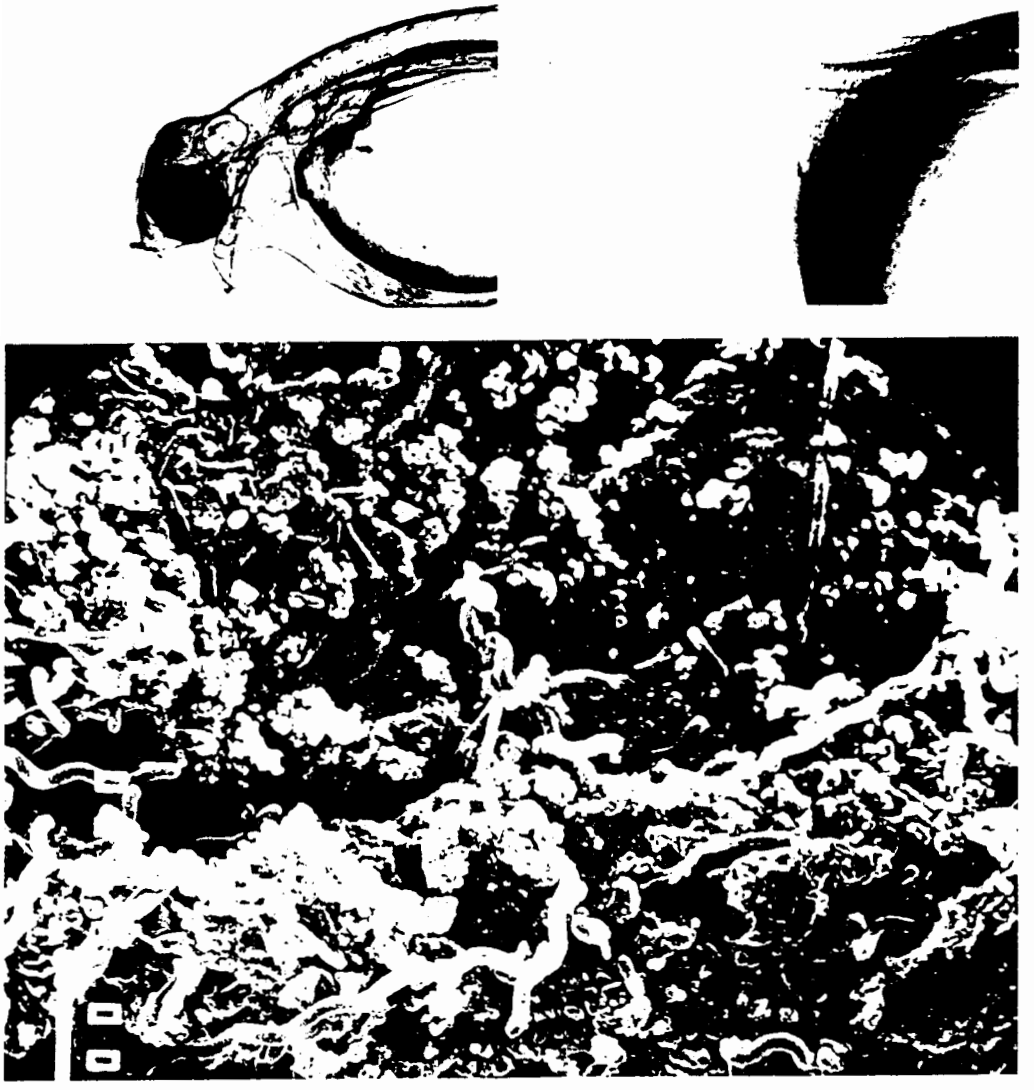

Fig. 4. Problems of development in halibut: a) jaw deformity, large pericardial space, underdeveloped heart and large space anterior to the yolk on a larvae raised at 9 °C; b) incomplete caudal fin development on a larva incubated at 3 °C. This anomaly occurred during embryo development and could affect the notochord, resulting in a stubby foreshortened tail; c) filamentous bacteria infecting a wound on the caudal end of a halibut larva

with jaw deformities per incubator, identifiable from day 12 after hatching at 9 °C and from day 19 after hatching at 6 °C due to differences in rates of mouth development. The ratio of deformities to total mortalities (Table 2) was found for each incubator for the period from which gaping was identifiable. There is a significant difference in the number of jaw deformities at 6° and 9 °C (WILCOXON Signed Ranks Test, $p = 0.059$). The 3 °C group did not develop that far.

Bacteria

Total counts of bacteria are shown in Fig. 8. As a general tendency, total counts increased from about 5×10^5 cells/ml shortly after hatching to $1.5\text{-}2.5\times10^6$ cells/ml when experiments

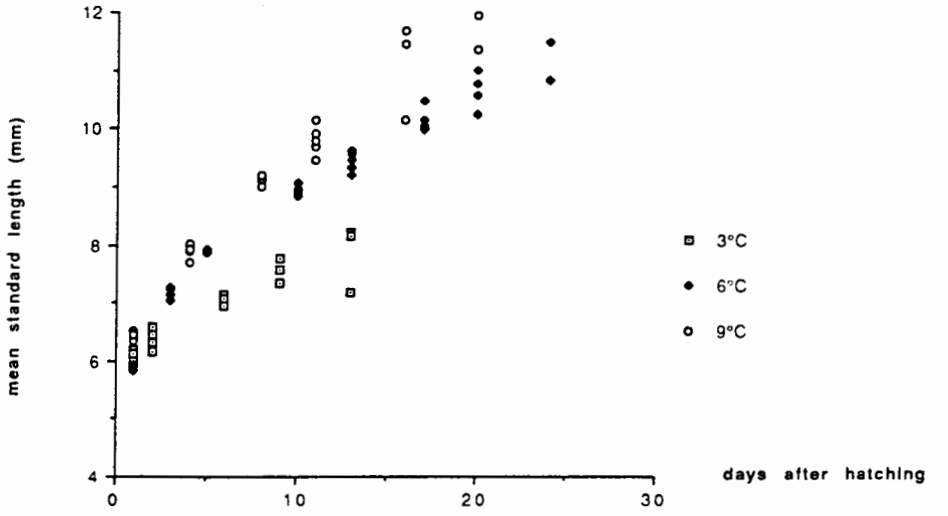

Fig. 5. Mean standard length of halibut larvae grown at 3°, 6° and 9 °C from hatching to the end of the experiment. Each point represents the mean of 10–15 larvae from one incubator

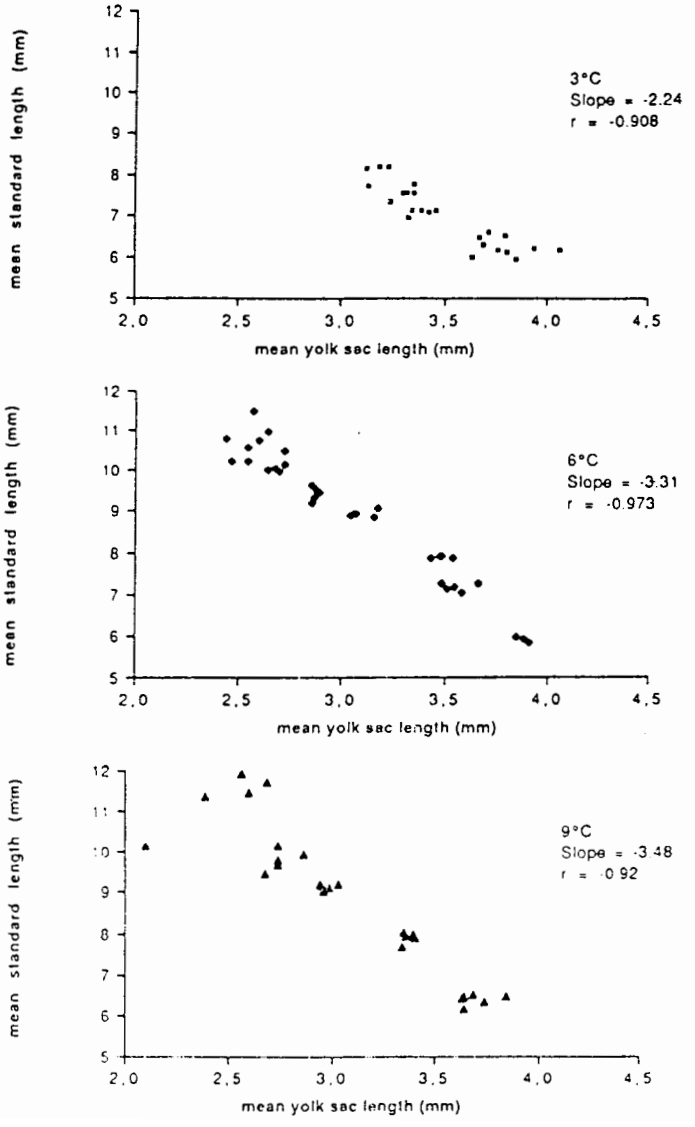

Fig. 6. Mean standard length vs. mean yolk sac length of halibut larvae grown at 3°, 6° and 9 °C from hatching to the end of the experiment. Each point represents the mean of 10–15 larvae in one incubator

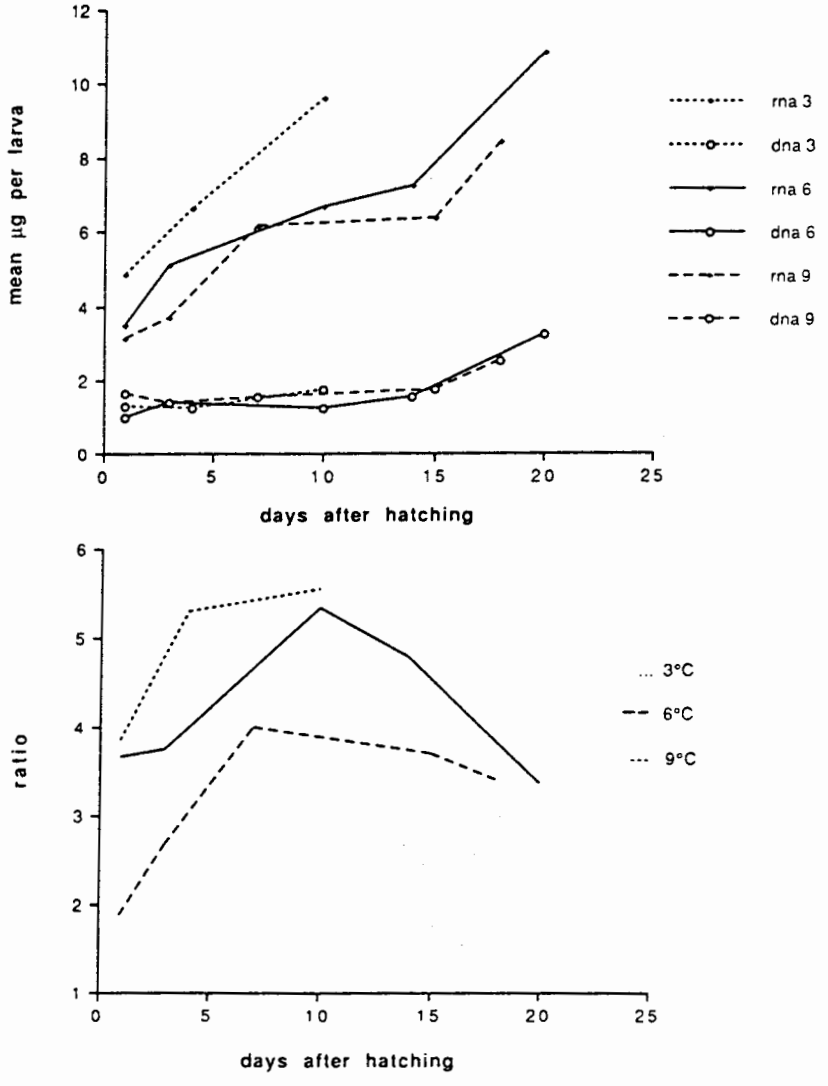

Fig. 7. Rearing halibut yolk sac larvae at 3°, 6° and 9 °C. – a) Mean RNA and mean DNA (in µg per larva) b) RNA/DNA ratios

were terminated. Two of the 9 °C incubators were atypical, with high total counts 10 days after hatching, followed by a decrease. The 3 °C incubators were terminated at an earlier stage in larval development, and total counts did not exceed 1.5×10^6 cells/ml.

Scanning electron microscopy of damaged tail fins revealed wounds colonized by large amounts of surface-growing filamentous bacteria (Fig. 4c). These bacteria were morphologically homogenous, in sharp contrast to the morphologically diverse bacteria colonizing undamaged areas. The bacterial population of the damaged areas was also much denser.

Viable counts of bacteria on the TSA and Cytophaga agar were grossly correlated with total counts, and were in the order of $1\text{-}4 \times 10^4 \times ml^{-1}$. Counts on the TCBS medium were low ($0\text{-}100 \times ml^{-1}$). The characteristic "spreading growth"-type colonies on the Cytophaga agar, typical of many *Cytophaga* sp. and *Flexibacter* sp. strains (Reichenbach 1989), were rare ($0\text{-}100 \times ml^{-1}$) throughout the experiments. Three of the 6 °C incubators showed higher values of this type of colony one day after hatching ($500\text{-}2200 \times ml^{-1}$), but were indistingu-

ishable from the other incubators from day 6 after hatching until termination of the experiment.

Table 2. **Jaw deformities at 6° and 9 °C. The ratio is the total deformity rate for the mortalities from each incubator in a temperature group from the time when jaw deformities were identifiable** (day 12 at 9 °C, day 19 at 6 °C)

Temperature	Ratio deformed/mortalities					mean	s.d.
	(in each of five incubators)						
6 °C	0.32	0.43	0.34	0.30	0.32	0.34	0.06
9 °C	0.72	0.61	0.52	0.35	0.68	0.57	0.15

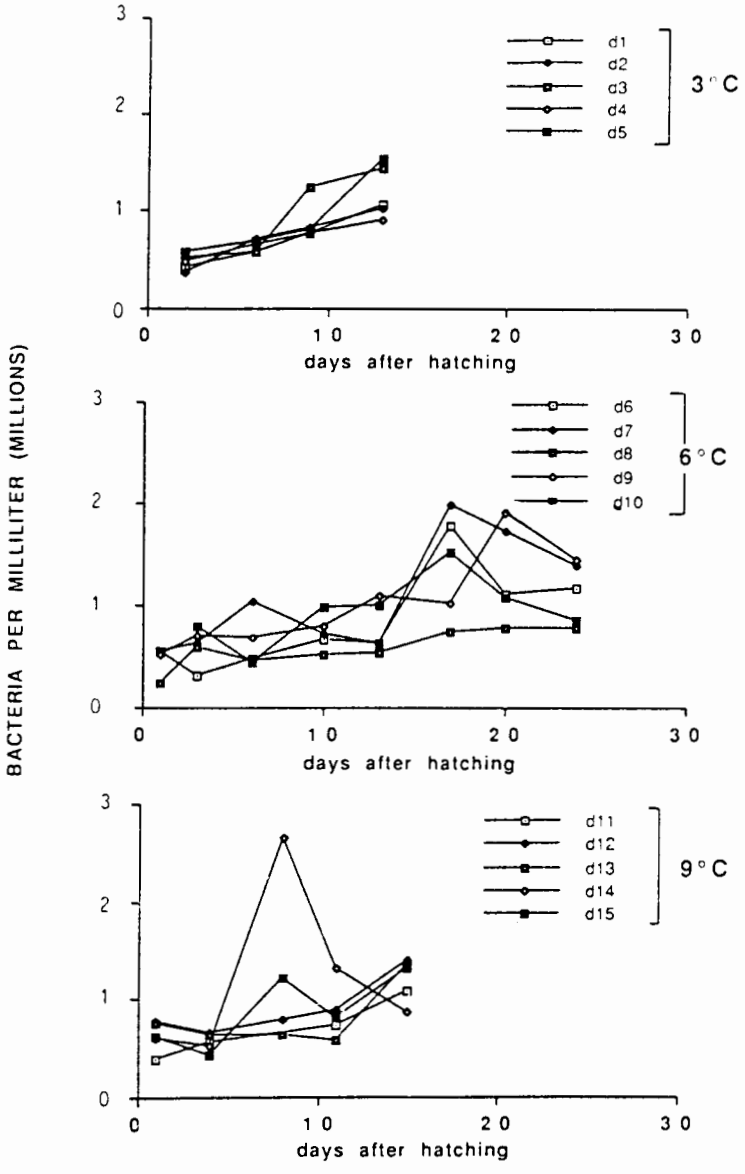

Fig. 8. Total counts of bacteria (in millions) in larval incubators at 3°, 6° and 9 °C (top to bottom). The numbers to the right of the figure are the five larval incubators at each temperature.

Water quality

Mean temperatures in the three groups of egg incubators were 3.0 +/- 0.4 °C, 5.9 +/- 0.2 °C and 8.9 +/- 0.2 °C. Mean temperatures in the three groups of larval incubators were 3.7 +/- 0.5 °C, 6.3 +/- 0.3 °C and 8.9 +/- 0.4 °C. Flow rates in the egg incubators were 1.6 +/- 0.9 L/min (3 °C), 1.8 +/- 0.8 (6 °C) and 1.9 +/- 0.8 (9 °C), whereas in the larval incubators flow rates were 1.0 +/- 0.4 L/min for all temperature groups (for more detail see BERGH et al. 1989). Oxygen was fairly stable at around 10.43 mg/L (3 °C). 9.43 mg/L (6 °C) and 9.14 mg/L (9 °C) as shown in Fig. 9. Total ammonia was generally below 1 µmol/L except after increased mortality when it went over 3.5 µmol/L in 4 incubators (Fig. 10). Salinity was 33.7 ppt +/- 0.06 during the season.

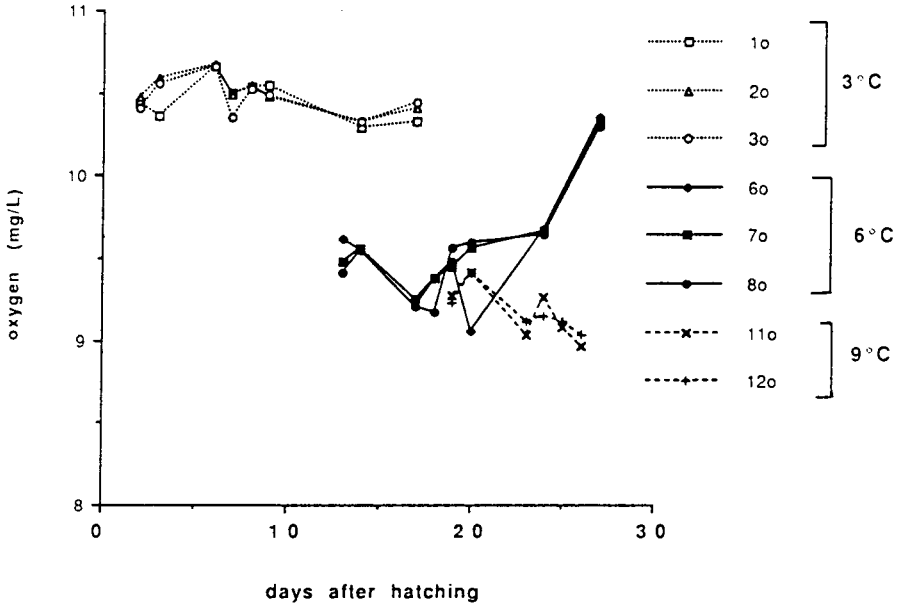

Fig. 9. Oxygen (mg/L) in larval incubators at 3°, 6° and 9 °C

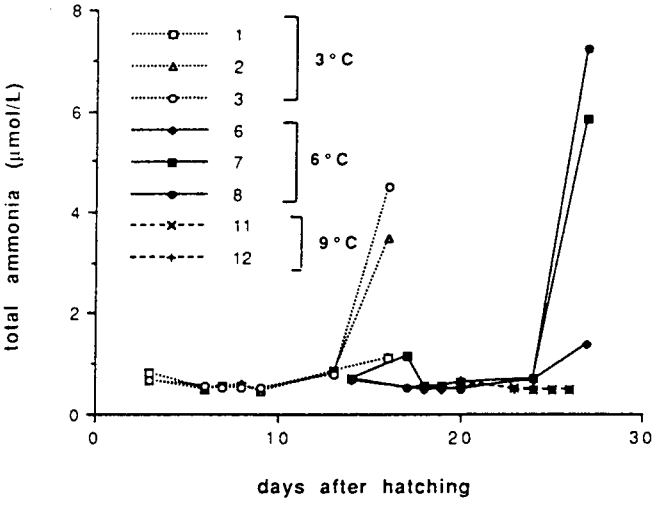

Fig. 10. Total ammonia concentration (µmol/L) in larval incubators

Discussion

Water quality

The incubators acted as true replicates. Total ammonia levels and temperatures were such that no more than 0.4 % could be in the un-ionized toxic form. The levels were below those recommended for chronic exposure (WEDEMEYER et al. 1976) until high mortalities occurred. Halibut eggs are found in nature at salinities close to that used in this experiment (33.8 to 35.0 ppt, HAUG et al. 1986). It is recognized that combinations of salinity and temperature can influence larval morphology and survival, and that optimal salinity is dependent on incubation temperature (VON WESTERNHAGEN 1970). However, our experimental design did not allow investigation of the combined effects of salinity and temperature on halibut eggs and larvae.

Egg development

Development of the eggs showed a difference in rate within the first cell divisions. Hatching took place after 600 hours at 3 °C, 336 hours at 6 °C and 211 hours at 9 °C, corresponding well to the hatching time of 13 days (312 hours) found at about 5.7 °C by FINN (1989). Time to hatching is a linear function of incubation temperature for halibut within the narrow range of this experiment.

Some anomalies were observed during embryo development, such as the appearance of KUPPFER's vesicles (small inclusions of unknown function posterior to the yolk sac which have been observed in developing halibut embryos since ROLLEFSEN, 1934) and, at 3 °C, an incomplete tail development. The mean number of KUPPFER's vesicles was highest in the 9 °C group, whereas many larvae in the 6 °C group had no vesicles. LØNNING et al. 1982 reported one KUPPFER's vesicle in larvae incubated at 5 °C. There was also a decrease in the number of vesicles between the 17-somite stage and the initiation of heart activity in the 9 °C group. However, since there were only three mortality counts during the egg stage at 9 °C, we cannot say whether this was due to selective mortality or resorption. We do however question whether the vesicles are due to (suboptimal) abiotic factors, and whether there is a possible hepatopancreatic function as suggested by ROSENTHAL & FONDS (1973). It also seemed that the nature of the vesicles was different in each of the temperature groups and samples have been taken to examine their histology.

The hatching gland in the halibut has an unusual appearance with individual cells arranged in a belt-like structure ("hatching ring") over the front of the yolk sac. This location results in restricted degradation of the eggshell at hatching so that larvae leave the eggs by opening the top of the egg in a "jack-in-the-box" mechanism (HELVIK 1988). The hatching gland develops from a disc in front of the embryonic head, which is transformed to a ring-structure through cell migration (HELVIK, 1988; HELVIK et al., 1990). Our data show that these mechanisms operate adequately at the three temperatures studied since hatching gland morphology and hatching itself was normal. The belt structure is visible about one week after hatching depending on temperature (Fig. 2), possibly suggesting that there may be cells with another function in addition to the secretion of the hatching enzyme.

Egg mortality

Percent egg mortality was highest at 3 °C and 9 °C, suggesting that these temperatures are near the lower and upper limits for egg development. The exclusion of one egg incubator at 6 °C where mortality was almost complete brings the mortality curve below that of the other temperature groups, although inclusion of the data from this group puts all the curves at about the same level.

Development of our eggs decelerated when temperatures dropped below 3 °C. LØNNING et al. (1982) also found slow development and high mortalities when eggs were incubated at 2.5 °C. Temperatures between 2–3 °C are likely suboptimal for eggs of an ecologically similar species, sablefish (*Anoplopoma fimbria*) (ALDERDICE et al. 1988). Halibut egg mortality in general was greatest before yolk plug closure, agreeing with ROLLEFSEN (1934), FORRESTER & ALDERDICE (1973), BLAXTER et al. (1983) and HOLMEFJORD & BOLLA (1988), and was similar to the egg mortality pattern of the sablefish (ALDERDICE et al. 1988).

Larval growth and development

Development within a temperature group was nearly identical for all larvae, whereas in previous experiments with less temperature control, differences in development stage were noted among larvae from the same incubator (PITTMAN et al. 1987; PITTMAN et al. 1989; PITTMAN et al. 1990).

At hatching, larvae raised at 3° and 6 °C were not significantly different in either yolk sac length, RNA or RNA/DNA ratio, although larvae raised at 3 °C had significantly greater standard length and DNA. This would suggest more efficient use of the yolk nutrients at the lower temperature during egg incubation, or perhaps different temperature optima at different stages of halibut development, as found for salmon by PETERSEN et al. (1977).

There are two ways of growing: either the individual cells become larger or the number of cells increases. The latter is the norm for embryonic development. Both ways will usually lead to an increas in biomasse. However, if one assumes that the amount of DNA is constant for each cell, then only an increase in the number of cells will result in an increase in the amount of DNA. For halibut larvae incubated at 6° and 9 °C, the amount of DNA is relatively constant until about 14 days after hatching and increases thereafter. Since there is an increase in standard length during the first two weeks, then either growth might possibly be attributed to an increase in cell size, or the amount of DNA is not constant per cell. More work is needed.

During the larval stage, the amount of RNA increases from hatching onward, indicating an increase in protein synthesis beyond that of the egg stage. Since this is accompanied by an increase in the RNA/DNA ratio, it would suggest that each cell's capacity for protein synthesis is increasing during the first two weeks after hatching. The RNA/DNA values for halibut larvae are lower than those reported for cod (RAAE et al. 1988), about the same as those reported for winter flounder (BUCKLEY 1981) and slightly higher than those found for salmon (TARANGER 1989). The difference in RNA/DNA of halibut larvae incubated at different temperatures supports the hypothesis of RAAE et al. (op. cit.) that differences in nucleic acid contents reflect environmental differences.

The RNA/DNA ratios decreased to the same level as at hatching about 25 days after larvae emerged from the egg. In BUCKLEY's (1981) study of winter flounder larvae, the lowest ratios were found in starving fish, and were independent of standard length. Cessation of feeding resulted in a decrease in the RNA/DNA ratio of winter flounder and cod within 2 and 4 days respectively, and BUCKLEY proposed that the ratio analysis could be used to detect the early stages of starvation. Although not conclusive, the data presented here for halibut support the idea that exogenous nutrients are required already halfway through the yolk sac stage. This is supported by data on behaviour and development (PITTMAN et al. 1990) and is comparable to the time of first feeding in sablefish (ALDERDICE et al. 1988).

Larval growth showed a rapid increase in standard length in the 9 °C group from the time of hatching, while the 3 °C group showed the slowest increase in standard length. The regression slopes of yolk sac length against standard length show initially a more rapid conversion of yolk to body length at 9 °C. However, it should be noted that within the first ten days after hatching, larvae at 6° and 9° had similar standard lengths but the stages of

development were quite different. Larvae at 9° had more complex hearts, partial pigmentation of the eyes, lifted heads, pectoral fins and developing livers. At a similar stage of development, larvae at 6° were longer. This suggests either more efficient use of the yolk reserves at 3° and 6 °C or greater swimming or metabolic activity at 9 °C. Further analyses of standard lengths and yolk sac lengths, RNA and DNA at particular stages are complicated by the differences in development stages at sampling times.

Larval mortalities

Larval mortality rose rapidly 8 days after hatching at 9 °C and 12 days after hatching at 6 °C, when development had reached approximately the same stage. At this stage, the heart was still tubular, the eyes were partially pigmented, the head was lifted to mideye, there was a cellular aggregation for the liver and a groove at the area of the prospective mouth. This stage in development corresponds to the time at which larvae normally begin to sink passively (PITTMAN et al. 1990), an event which was compensated by increasing the flow rate to lift the larvae. In many systems and experiments (BLAXTER et al. 1983, PITTMAN et al. 1987, NYHAMMER 1990) this period corresponds with the highest mortalities of the entire yolk sac stage.

Our results point to three factors which could possibly contribute to losses of halibut larvae at this stage in aquaculture rearing systems: a) development abnormalities, b) bacteria and c) water flow.

a) The primitive halibut larva begins to form a liver about 10 days after hatching, depending on temperature. Prior to the appearance of the liver, yolk supplies are apparently absorbed directly or via an intermediary layer (KJØRSVIK & REIERSEN 1990), whereas the liver is clearly the mediator of these supplies in the later yolk sac stage (HAUG et al. 1989). It is suggested that if the liver or other digestive organs forming at this time malfunction, the endogenous energy may be misdirected and the result may be increased mortality. This would indicate that larval quality, egg quality and perhaps even broodstock quality play a role in determining the number of halibut larvae suviving beyond two weeks after hatching. Further analysis and histology is needed.

b) Larvae at 8 days (9 °C) and 12 days (6 °C) are capable of drinking (TYTLER & BLAXTER 1988) and have an opening mouth area or stomodeum (PITTMAN et al. 1989). Thus they no longer have a sufficient barrier to prevent ingestion of bacteria, which if pathogenic could contribute to mortality. This would indicate stricter requirements for hygiene in the rearing of halibut larvae and the importance of monitoring the bacterial population and species composition in the incubators. Our samples were not suited to detection of bacteria in the larval intestine, but alternative methods are being examined.

c) There is some evidence that high flow rate could cause increased larval mortality and increased bacterial activity (OPSTAD & BERGH, unpub. data) but the variable flow rates in this experiment make the calculation of bacterial growth rates impossible. Water exchange rates in this experiment exceeded the level at which OPSTAD and BERGH obtained high mortalities (one full exchange per day). Larvae in the present experiment also developed opaque spots, which had previously been shown under scanning electron microscopy to be areas where the tissue was worn down, in some cases to the cartilage (PITTMAN et al. 1989). The incidence of this feature was higher in these incubators than it had been in previous years (PITTMAN & STRAND, unpub. data). If the rate of flow is indeed a contributing factor, this may explain the coincidence of the periods of passive sinking, counteracting flow rates and increases in mortality. It may also have contributed to the general wear on the fins and snout, allowing bacterial colonization of the wounds.

Larval mortality in the abovementioned experiments also includes three other periods of high mortality: one period is associated with hatching, another around 25–35 days after

hatching (around 50 % yolk absorption) and a last period around 45–60 days after hatching (at end of yolk sac). If 50 % yolk absorption does indeed coincide with the transition from endogenous to exogenous food supplies, then it is possible that some of the mortality may be attributed to an energy deficit, as suggested by BUCKLEY (1982) for winter flounder. However, this period also coincides with the time at which light is first introduced to the developing larvae and this may be an additional stress factor. Again, if first feeding may take place half way through the yolk sac stage then mortality at the end of the yolk sac stage may be due to starvation.

Deformities

Two deformities were observed, incomplete caudal development (at 3 °C) and gaping (at 9° and 6 °C). Caudal development was affected during embryo development and many have been due to the incidence of very cold temperatures (less than 3 °C) during the egg stage.

Gaping was visible from day 12 after hatching (9°) and from day 19 (6°). The significant difference in the frequency of jaw deformation at 6° and 9 °C emphasizes the importance of temperature control in the early larval stages. The one incubator at 9 °C with the lowest rate of jaw deformity in that group (34.9 %) was noted as having the lowest temperature from days 7 to 11 after hatching, and the raw data notes that these larvae were slightly less developed than the others. This supports findings which associate gaping in halibut larvae with warm temperatures (BOLLA & HOLMEFJORD 1988 and PITTMAN et al. 1989). For a further discussion of normal and abnormal development in halibut larvae, see PITTMAN et al. (1990). Many of the dead larvae in all groups had narrow tail fins which may be the result of flow rates damaging the fragile fins and a subsequent colonization by bacteria. There is also a possibility that a combination of several sublethal stressors is involved. Many of the abnormalities reported here, such as yolk, organ and mouth defects, have been described in other fish larvae as responses to sublethal environmental stressors (ROSENTHAL & ALDERDICE 1976).

Bacteria

We were not able to find statistically significant differences among the bacterial populations of different temperature groups. Both total count and viable count data indicate that factors such as flow rate, larval number and larval mortality, are more important in determining bacterial activity and numbers in the incubators. The lower total counts in the 3 °C incubators were probably caused by the termination of these experiments at an earlier stage in larval development. There is also evidence (HOLMEFJORD, pers. comm.) that larval density greatly influences bacterial counts but the latter is not correlated with larval mortality. OPSTAD and BERGH (unpubl. data) have shown differences in bacterial population composition and bacterial activity as a response to differences in incubator flow rate, and, probably, larval mortality.

BOLINCHES & EGIDIUS (1987) reported that the viable count of bacteria in TCBS agar was an underestimation compared to the number of vibrios assessed by isolation on a less selective medium (Marine agar), but TCBS was still deemed the best selective medium available for vibrio counts. The TCBS counts found by us indicated low numbers of *Vibrio* sp. in the incubators. This is in agreement with BOLINCHES & EGIDIUS (op. cit.) who found low vibrio counts before the first feeding of larvae.

The low number of colonies typical of *Cytophaga* sp. and *Flexibacter* sp. indicates that these were relatively rare among the free-living bacteria in the incubators. However, as only free-living bacteria were measured, the larvae may still have been infected by these bacteria. It has been shown earlier that these genera are present among bacteria colonizing halibut eggs (HANSEN & OLAFSEN 1989). HANSEN & BERGH (unpub.) have found that a pathogenic Flexibacter strain is dominant among the surface bound bacteria of some egg groups.

Conclusions

For halibut, temperature during egg incubation affected rates of development, growth, and mortality as well as rates of protein synthesis and cell division at hatching. However, the developing embryo at 3 °C could be afflicted by incomplete caudal development and suffered higher mortality than at 6 °C, suggesting that 3 °C is near the lower limit for our halibut eggs. Development was slowest, but mean RNA and relative protein synthesis (RNA/DNA) and yolk size at hatching were highest in larvae raised at 3 °C. The halibut raised at 9 °C had high egg mortality but appeared to have developed furthest at hatching although the time required to reach this stage was shortest. These larvae quickly developed abnormalities associated with sublethal stressors, such as small hearts and livers, and large peritoneal and pericardial spaces.

Relative protein synthesis (RNA/DNA) was highest at 3 °C and lowest at 9 °C for larvae throughout the experiment. Three to four weeks after hatching, RNA/DNA levels showed a tendency to return to amounts found in the emergent larva. The data suggest that the period between 25 – 30 days after hatching (50% yolk absorption) is the time at which uptake of exogenous nutrients is required to continue a level of protein synthesis above that found at emergence. More study is required to determine whether delays in initial food presentation result in reduced fish size and survival, as found by HEMING et al. (1982).

Patterns of larval mortality have been similar over a number of years and experiments, though unfortunately much data is as yet unpublished. Based on this and other studies, the following factors are proposed as contributing to the four periods of high larval mortality found in aquaculture systems at temperatures between 3° and 9 °C:

a) mortality at hatching may be due to poor embryo quality, problems with the hatching process, bacteria and/or oxygen levels;

b) mortality about 10–14 days after hatching may be due to malfunction of the developing organs, ingestion of pathogenic bacteria and/or flow rates;

c) mortality about 25–35 days after hatching may be due to an increasing requirement for exogenous nutrients, stress in conjuction with the introduction of live prey, the transition from darkness to light, system constraints which make the larvae crowd together in one place and/or deterioration in water quality;

d) mortality 45–60 days after hatching may be due to starvation.

No primary cause of death of the larvae can be identified. As most mortality took place at the same stage of development at 6° and 9 °C, before the increase in amount of free-living bacteria, these were not the cause. There is some evidence that surface-bound bacterial infections were involved, as the filamentous bacteria found in fin deformities could be of importance. An increase in total bacteria was in turn followed by a large increase in ammonium concentration, probably caused by bacteria metabolizing compounds from dead larvae, which are rich in proteins and amino acids, the surplus nitrogen being excreted. Thus a cycle is started, where larval death leads to increased bacteria numbers and increased ammonia levels which then weaken the remaining larvae, allowing further mortality and bacterial growth.

Although an optimal temperature for the early life stages of halibut has not been identified, the data indicate that 9 °C is supraoptimal at the egg and larval stages, that 3 °C may be near the lower limit for embryo development but seems to have some advantages for growth potential, and that 6 °C was often not significantly different from 3 °C. It is possible that there are different temperature optima for different stages of halibut development.

Further investigation is continuing to add dry weight, specific growth rates, amino acids, protein content, histology, pathology, yolk conversion factors and characterization of bacterial isolates to the data already treated here. The identification of critical stages where environmental stability is important for favourable development of halibut larvae seems to be equally important for the development of commercial halibut farming.

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