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Early ontogeny of the Atlantic halibut *Hippoglossus hippoglossus* head

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An ontogenetic sequence of Atlantic halibut *Hippoglossus hippoglossus* larvae, reared in intensive culture conditions, was cleared and stained and histologically processed to determine normal cranial chondrification for specimens ranging from 0 to 41 days post-hatch (dph). Twenty-six cranial cartilaginous structures were described, at daily intervals post-hatch. The ontogenetic trajectory, composed of alternating steps and thresholds, was interpreted as saltatory. In comparison with other flatfishes, *H. hippoglossus* exhibits delayed onset of chondrification. From 9 dph onwards, the ontogenetic trajectory resembles more than that of the turbot *Psetta maxima* than that of the common sole *Solea solea* or the summer flounder *Paralichthys dentatus* and winter flounder *Pseudopleuronectes americanus*. *Hippoglossus hippoglossus* with the gaping-jaw malformation, common in intensively cultured individuals of this species, were examined histologically. The reason larvae cannot close their mouth, as their yolk-sac resorbs, seems to be related to the fusion of the interhyal to the hyosymplectic and ceratohyal with which it is normally articulated.

Key words: chondrification; gaping jaw; Pleuronectiformes; saltatory ontogeny.

INTRODUCTION

Pleuronectiforms, such as the Atlantic halibut *Hippoglossus hippoglossus* (L.), are characterized by a complex remodelling of the skull that is associated with a habitat shift from a pelagic to a demersal life style (Schreiber, 2006). The asymmetrical shift of the skull during early ontogeny implies important modifications of jaw structure and development (Morrison & MacDonald, 1995; Hunt von Herbing, 2001; Francis & Turingan, 2008), eye migration (Keefe & Able, 1993; Kvenseth *et al*., 1996; Saele *et al*., 2006; Schreiber, 2006), neurocranium remodelling as well as changes in the epicranial portion of the dorsal fin (Wagemans *et al*., 2002; Saele *et al*., 2006).

Detailed studies on the early development of the pleuronectiform head skeleton have been conducted on turbot *Psetta maxima* (L.) (Wagemans *et al.,* 1998), common sole *Solea solea* (L.) (Wagemans & Vandewalle, 2001) and winter flounder *Pseudopleuronectes americanus* (Walbaum) (Hunt von Herbing, 2001). Only Morrison &

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MacDonald (1995) have compiled a cranial chondrification sequence (albeit incomplete) for *H. hippoglossus* in an attempt to describe the jaw development in yolk-sac larvae. Other studies provide only partial information on the appearance of cartilaginous structures during cranial ontogeny of *H. hippoglossus* (Blaxter *et al*., 1983; Pittman *et al*., 1990; Kjørsvik & Reiersen, 1992; K. Pittman, L. Berg & K. Naas, unpubl. data). Saele *et al*. (2004) described cranial osteological development of *H. hippoglossus* larvae but only from first feeding through metamorphosis. Therefore, a detailed description of the complete cranial chondrification sequence of *H. hippoglossus* is lacking.

Skeletal deformities of the jaw, cranium, vertebral column and caudal fin are a major problem in intensive aquaculture of *H. hippoglossus* (Pittman *et al*., 1989, 1990; Ottesen & Bolla, 1998; Olsen *et al*., 1999; Lewis *et al*., 2004; Lewis & Lall, 2006; A. Jelmert & K. Naas, unpubl. data). Malformations occurring during the development of feeding structures have been identified as one of the major sources of larval mortality in intensively cultured *H. hippoglossus* (Morrison & MacDonald, 1995; Saele *et al*., 2004). The underlying causes of these developmental malformations are uncertain, although salinity, temperature, larval density and bacterial and viral infections have been proposed. It has been suggested that mouth-gaping (the primary malformation), around 45 days post-hatch (dph), resulted from either bacterial infection in the snout region (Morrison & MacDonald, 1995), premature breakdown of the oral septum (Pittman *et al*., 1990), or maternal hereditary malformation (Saele, 2002). Minor changes in the sequence of chondrification and ossification, however, could also result in malformations. A complete description of the normal developmental sequence is necessary as a baseline against which to compare the abnormalities that typically arise during intensive culture of marine fishes, including *H. hippoglossus*.

The present study describes the normal early development of the chondrocranium (neurocranium and splanchnocranium) in intensively cultured *H. hippoglossus* before 45 dph. Special attention was placed on the formation and development of the feeding structures during the 9–23 dph window, a period of major anatomical changes. The sequence of chondrification of head structures was used to identify critical developmental stages and compare their temporal expression relative to that of other pleuronectiforms.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS AND SPECIMEN PREPARATION

Larval *H. hippoglossus* were obtained from the Institute of Marine Research, Austevoll Research Station, Norway. Specimens were cultured at Austevoll following standard practices (Mangor-Jensen *et al*., 1998) and were sampled as described below. Handling of animals complied with the Principles of Animal Care, publication no. 86–23, revised in 1985, of The National Institutes of Health and with the institutional animal care guidelines of the Institute of Marine Research, Norway.

Developmental timing (age) is given in dph and day-degrees post-fertilization (d°). Two rearing series were processed (S1 and S2): S1 was explorative and consisted of larvae reared in 2003, ranging from 0 to 41 dph [79·5–373·8 d° post-fertilization and mean standard length (L_S) ranging from 4·9 to 13·8 mm], sampled 11 times during this period. From this series, a coarse developmental window was identified during which larvae undergo major chondrogenesis, between 12 and 19 dph. Therefore, a more selective sampling schedule was developed

within this time window and a second series, S2, was processed. S2 comprised fish that had been reared in 2004, sampled daily from 9 to 23 dph (143·2–234·5 d° post-fertilization), with the exception of 22 dph. Mean L_S ranged from 8·5 mm at 9 dph to 11 mm at 23 dph. Specimens were fixed in neutral buffered formalin, renewed after 24 h and transferred subsequently to 70% ethanol (Presnell & Schreibman, 1997).

SKELETAL PREPARATION

Specimens ranging from 0 dph to 41 dph were cleared and double stained (C&S) using alcian blue for cartilage and alizarin red-S for bone. Larger specimens were stained according to the protocol of Dingerkus & Uhler (1977), modified by Potthoff (1984). Smaller specimens (0–23 dph), too delicate for this procedure, were cleared and stained only for cartilage following Liu & Chan (2002). Because ossification starts after 41 dph, the alizarin red-S stain for ossified bone was unnecessary. C&S specimens were examined under a MZ16A Leica stereomicroscope (www.leica-microsystems.com) and all cranial structures were identified in specimens from each sampling day. Terminology of the cartilaginous cranial elements follows that of Wagemans *et al*. (1998); branchial nomenclature is taken from Nelson (1969).

HISTOLOGICAL PREPARATION

Serial sections of the head were prepared for five specimens of the S1 series (5, 12, 19, 26 and 41 dph) and 14 specimens of the S2 series (9–21 and 23 dph). Specimens were processed in a Shandon Citadel 2000 automated tissue processor (www.thermoscientific.com). They were dehydrated in graded ethanol solutions (20–100%), transferred to xylene/ethanol and finally pure xylene. Specimens were then transferred to a 50/50 xylene/paraffin solution and impregnated with melted paraffin under a vacuum. Larvae were embedded in Paraplast Plus (www.mccormickscientific.com) and sectioned at 7 μm intervals. Sections of S1 specimens were stained using standard haematoxylin and eosin. Cartilaginous and bony structures were revealed in S2 using the Hall's and Brunt's Quadruple (HBQ) stain (Hall, 1986). Stained sections were photographed with a CCD camera mounted on a Leica DMLB microscope and connected to Northern Eclipse software (www.empix.com). Images of each specimen were stacked and aligned using the Reconstruct v. 1.0.7.3 software (Fiala, 2005). The differential colouration obtained using HBQ staining enabled the use of semi-automatic structure recognition (regional wildfire boundary recognition) in Reconstruct for 3D reconstructions of chondrocrania and splanchnocrania from 9 to 23 dph.

ANALYSES

Descriptive statistics [Pearson coefficient of correlation (r) and Spearman rank coefficient of correlation (r_s)] were performed using SAS 9.1.3 (www.sas.com).

The sequence of chondrification was converted to a curve of cumulative number of newly formed chondrocranial elements. These cumulative counts were plotted against the age of specimens (dph); this curve is referred to as an ontogenetic trajectory. Paired structures were counted as one element. This skeletal ontogenetic trajectory was then compared to four other pleuronectiforms: *S. solea* (Wagemans & Vandewalle, 1999), *P. maxima* (Wagemans *et al*., 1998), summer flounder *Paralichthys dentatus* (L.) (Martinez & Bolker, 2003) and *P. americanus* (Hunt von Herbing, 2001). Sequences of chondrification were reconstructed based on the published descriptions and the interpretation of the illustrations presented by each study's authors.

RESULTS

From 0 to 9 dph (143 d°; 8·7 mm mean L_S), the head of the larva remained tightly attached to the yolk sac and only started aligning with the body axis from 10 dph

onwards. At 0 and 2 dph, the head of the larva does not present any visible external structure, but is distinguishable from the rest of the body by its oval shape. Only the transparent crystalline lens of the eye is visible at 2 dph in a few specimens, the complete transparent eye being identifiable at 5 dph (7·0 mm mean L_S). Cartilaginous structures first appear in C&S specimens at 9 dph (7·8 mm mean L_S). In histological sections, the first cartilaginous clusters are seen at 10 dph (149·8 d°; 8·9 mm mean L_S). There was a high degree of consistency between C&S and histology (Fig. 1), with a strongly significant correlation between the number of structures observed using both techniques ($r = 0{\cdot}9584$; $n = 14$ specimens; $P < 0{\cdot}001$). The slight discrepancy between the two techniques may be due to fluctuation in dye content and minor interindividual variation among specimens. The anatomical description (from 0 to 41 dph) that follows is based primarily on C&S specimens and complemented with data from histological sections.

DAY 0 (79·5 d°) TO DAY 7 (132·2 d°)

The larvae present no evidence of cephalic chondrification in either C&S specimens or histological sections.

DAY 9 (143·2 d°)

Cartilage associated with the neurocranium is visible at this stage in C&S specimens. The otic capsules are visible due to clusters of chondrocytes around the semi-circular canals situated posteriorly to the eye and forming a U-shaped structure, the concavity of which is directed dorsomedially. At this stage, this paired structure is far less dense than at later stages.

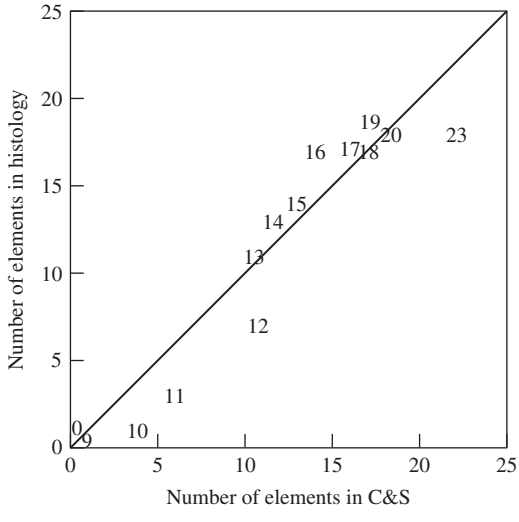

FIG. 1. Congruence between the number of chondrocranial elements of *Hippoglossus hippoglossus* identified from both histological and cleared and stained (C&S) techniques at equivalent days post-hatch (dph). Numbers correspond to the age of the specimen (dph) for which numbers of elements are compared. The line represents a theoretical equal number of elements identified by both techniques.

DAY 10 (149·8 d°)

The trabecula communis and the trabecular bars are clearly visible on histological sections and in C&S specimens. The trabecular bars are visible between the eyes as two thin bars connecting anteromedially at the tip of the notochord and extending posterolaterally. The trabecular bars are slightly dorsal to the anterior part of the notochord. The trabecular bars extend anteromedially to fuse between the eyes and form the trabecula communis. Together, the trabecula communis and the trabecular bars form a Y-shaped structure, the foot of the Y extending anteriorly and the arms of the Y extending posterodorsally. The cartilage density of the trabecula communis decreases anteriorly towards the eyes. The chondrocyte masses of the otic capsules are denser than in earlier stages and their shape is better defined. They are composed of three elements: a U-shaped tube (formed around a semi-circular canal), of which the concavity is directed medially with two 'pill-shaped' clusters of chondrocytes near the extremities of the U-shaped tube [Fig. 2(a)].

At 10 dph, the Meckel's cartilage is the first structure of the splanchnocranium to appear. Meckel's cartilages are small transverse bars on either side of the head. These structures form in the membrane still attaching the head to the yolk sac.

DAY 11 (156·2 d°)

The trabecula communis now extends anteriorly to the level of the anterior margin of the eyes. The paired Meckel's cartilages are dark blue, suggesting the presence of relatively dense cartilage. They extend posteriorly to the middle of the eye and are rounded anteriorly following the curvature of the head. The palatoquadrate, the second component of the suspensorium to appear at this stage, is not as dense as the Meckel's cartilage. The small rod-like palatoquadrate is positioned posteriorly to the Meckel's cartilage and appears as a cluster of chondrocytes positioned ventrally to the posterior margin of the eye. The palatoquadrate forms an angle of *c*. 120° with the Meckel's cartilage. A small ceratohyal cartilage (= 'hyoid bar' of Wagemans *et al*., 1998) is present posterior to the palatoquadrate [Fig. 3(a)].

DAY 12 (162·9 d°)

The trabecula communis continues to develop anteriorly where it slightly expands laterally and curves dorsally. Posteriorly, the trabecula has curved so that the posterior trabecular bars are now in the vertical axis, extending posteriorly on each side of the notochord. The elements inside the otic capsules are denser and are supported by the thin parachordal plates forming the floor of the otic capsule. The Meckel's cartilages have elongated anteriorly towards the midline. The palatoquadrate has extended posteriorly, tilted mediodorsally, following the posteroventral margin of the eye. The palatoquadrate overhangs the anterior part of the newly formed hyosymplectic. The hyosymplectic, presenting the same angle as the palatoquadrate, is approximately triangular in shape, having a narrow anterior portion and a fanning posterior part. Ventrally to the hyosymplectic, the ceratohyal cartilage develops anteromedially towards its homologous counterpart. The first and second ceratobranchials form on each side of the newly formed first basibranchial [Figs 2(b) and 3(b)].

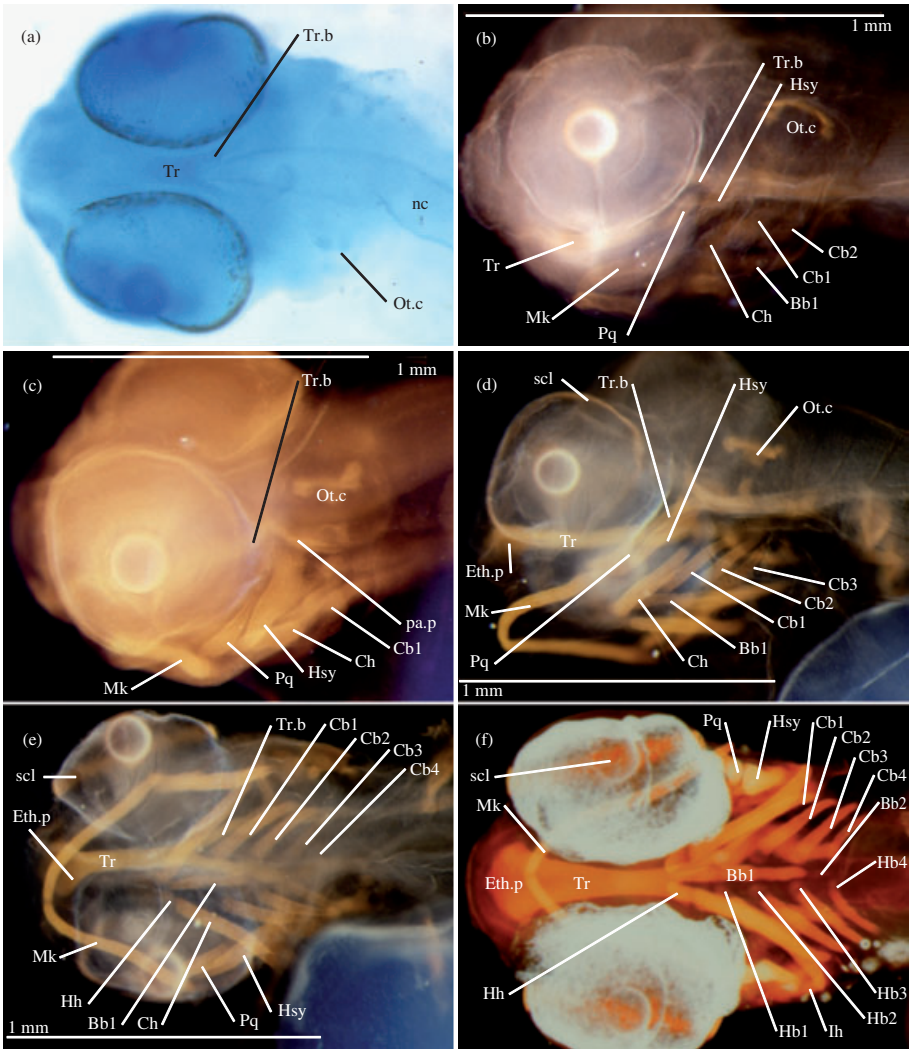

FIG. 2. Cranial morphology of *Hippoglossus hippoglossus* larvae. (a) Dorsal view of a 10 days post-hatch (dph) cleared and stained larva. (b) Lateral view of a 12 dph cleared and stained larva. (c) Lateral view of a 16 dph cleared and stained larva. (d) Lateral view of an 18 dph cleared and stained larva. (e) Ventral view of an 18 dph cleared and stained larva. (f) Ventral view of a 23 dph cleared and stained larva. (a)–(f) Cartilages stained with alcian blue; contrast and brightness have been enhanced and colours inverted to increase the distinction of cartilages [orange in (b)–(f)]. Bb1-2, basibranchial 1-2; Cb1-4, ceratobranchial 1-4; Ch, ceratohyal cartilage; Eth.p, ethmoid plate; Hb1-4, hypobranchial 1-4; Hh, hypohyal; Hsy, hyosymplectic; Ih, interhyal; Mk, Meckel's cartilage; nc, notochord; Ot.c, otic capsule; Pa.p, parachordal plate; Pq, palatoquadrate; scl, sclerotic plates; Tr, trabecula communis; Tr.b, trabecular bar.

DAYS 13–14 (169·5–175·9 d°)

As the head of the larva detaches from the yolk sac and uncurls, it leaves more space for the hyoid and branchial arches to develop. Therefore, the most characteristic feature at this stage consists of the rapid development of the branchial arches.

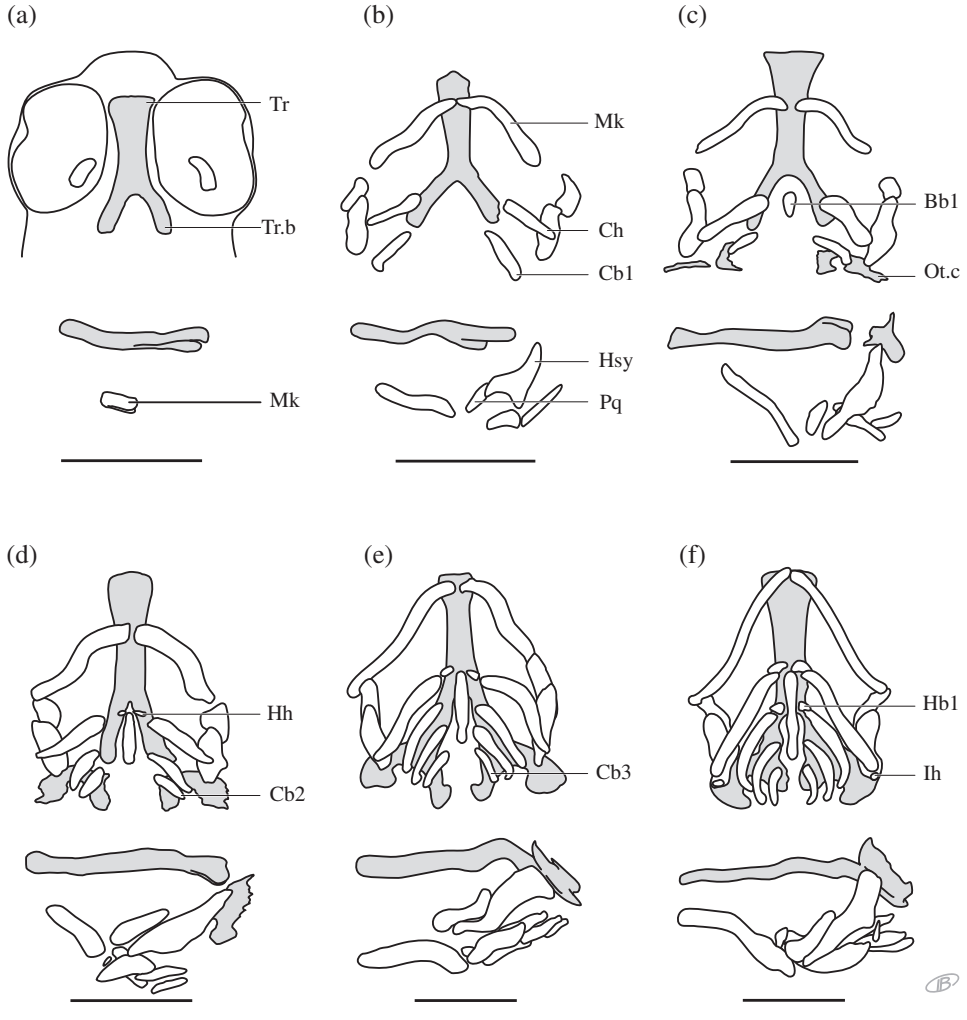

FIG. 3. Cranial developmental series of *Hippoglossus hippoglossus* from (a) 11, (b) 12, (c) 13, (d) 14, (e) 16 and (f) 19 days post-hatch (dph). Ventral (top) and lateral (bottom) views are based on 3D reconstructions from histological sections [discrepancies from cleared and stained specimens are in the earlier stages (11 and 12 dph, see Fig. 1)]. Only median and left bilateral structures have been represented on lateral views. Scale bars indicate 0·5 mm. Neurocranium is in grey. Bb1, basibranchial 1; Cb1-3, ceratobranchial 1-3; Ch, ceratohyal cartilage; Hb1, hypobranchial 1; Hh, hypohyal; Hsy, hyosymplectic; Ih, interhyal; Mk, Meckel's cartilage; Ot.c, otic capsule; Pa.p, parachordal plate; Pq, palatoquadrate; Tr, trabecula communis; Tr.b, trabecular bar.

Elements composing the arches are much longer and denser than previous stages. The angle between the Meckel's cartilage and the palatoquadrate is reduced to *c*. 90°. The size and shape of the palatoquadrate have not changed significantly. The posterior fan-shaped portion of the hyosymplectic has expanded. At 14 dph, the ethmoid plate has appeared at the anterior extremity of the head and consists of an anterior distal enlargement of the trabecula communis. The parachordal plates thicken and enlarge [Fig. 3(c), (d)].

DAY 15 (182·3 d°)

Until 14 dph, all elements were clearly isolated from one another, but at 15 dph the suspensorium and the hyoid arch have expanded enough to articulate with one another. The Meckel's cartilage has elongated ventroposteriorly up to the posterior rim of the eye. As a result, it meets the palatoquadrate at a 90° angle. The hyosymplectic attains its definitive shape with a posterodorsal fan-shaped process, the *processus opercularis*, and an anteroventrally extending part, the *pars symplecticus*, positioned ventrally along the palatoquadrate. The *processus opercularis* extends dorsally to meet the parachordal plate whereas the *pars symplecticus* extends almost up to the Meckel's cartilage, slightly curving ventrally. The parachordal plates have enlarged, clearly presenting a *fenestra basicapsularis*, and fuse anteriorly with the trabecular bars at the *commissura basicapsularis anterior*, forming, with the ethmoid plate, a single long cartilaginous element from the rostrum to the otic capsule. The third ceratobranchials appear.

DAYS 16–17 (188·8–195·3 d°)

All previously described structures are denser at 16 dph. Sclerotics appear around the eyes as a succession of square cartilaginous plates. The interhyal cartilage is the last element of the hyoid arch to appear at 17 dph. At this stage, the interhyal is a faint cluster of chondrocytes on the distal extremity of the ceratohyal cartilage, serving as an articulation between the ceratohyal cartilage and the hyosymplectic. The hypohyals also appear at this stage in C&S specimens, although histological reconstructions show traces of hypohyals as early as 14 dph [Fig. 3(d)]. Hypohyals are present as chondrocyte condensations at the proximal end of each ceratohyal cartilage, anterior to the first basibranchial [Figs 2(c) and 3(e)].

DAYS 18–19 (201·8–208·2 d°)

The trabecula communis is broad. The ethmoid plate, located at the anterior extremity of the trabecula, has widened into a definite club-shape. The *pila occipitalis* starts to be visible. It forms the ventroposterior margin of the otic capsule. The fourth ceratobranchials are visible at 18 dph and the arch clearly develops at 19 dph. As the interhyal becomes denser, the dorsal part of the hyosymplectic changes shape slightly. The *pars opercularis* thickens and develops a posterior extension to articulate on the interhyal [Figs 2(d)–(e) and 3(f)].

DAYS 23 (221·3 d°) AND 26 (241·1 d°)

At 23 dph, a cartilaginous plate, the tectum posterius, now covers the top of the otic capsule. The Meckel's cartilage is rounded dorsally so that the anterior portion is more dorsal than the posterior extremity. The posterior extremity of the Meckel's cartilage is approximately hook-shaped, forming the retroarticular and coronoid processes between which the palatoquadrate articulates. By 26 dph, the retroarticular process has curved around the palatoquadrate. The distal end of the ceratohyal cartilage thickens into a ball-shaped structure at the level of the elongated interhyal. The second basibranchial is now visible between the third and fourth branchial arches. The first to third hypobranchials appear at 23 dph [Figs 2(f) and 4(a)].

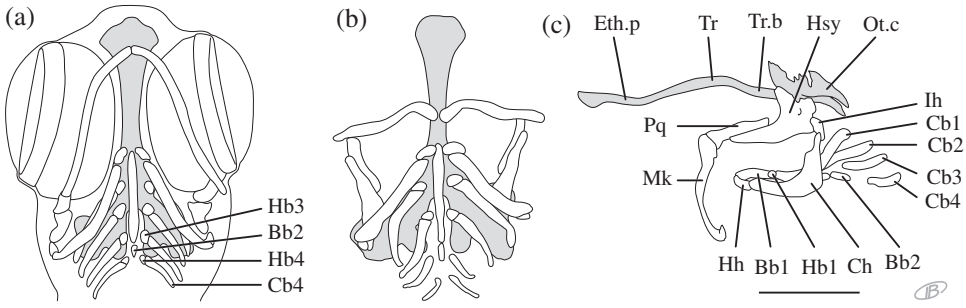

FIG. 4. Cranial morphology of 23 days post-hatch (dph) *Hippoglossus hippoglossus* larvae. (a) Ventral view of a normally developed larva. (b), (c) Ventral and lateral views of a larva with a gaping-jaw malformation. Scale bars indicate 0·5 mm. Bb1-2, basibranchial 1-2; Cb1-4, ceratobranchial 1-4; Ch, ceratohyal cartilage; Eth.p, ethmoid plate; Hb1, 3-4, hypobranchial 1, 3-4; Hh, hypohyal; Hsy, hyosymplectic; Ih, interhyal; Mk, Meckel's cartilage; Ot.c, otic capsule; Pq, palatoquadrate; Tr, trabecula communis; Tr.b, trabecular bar.

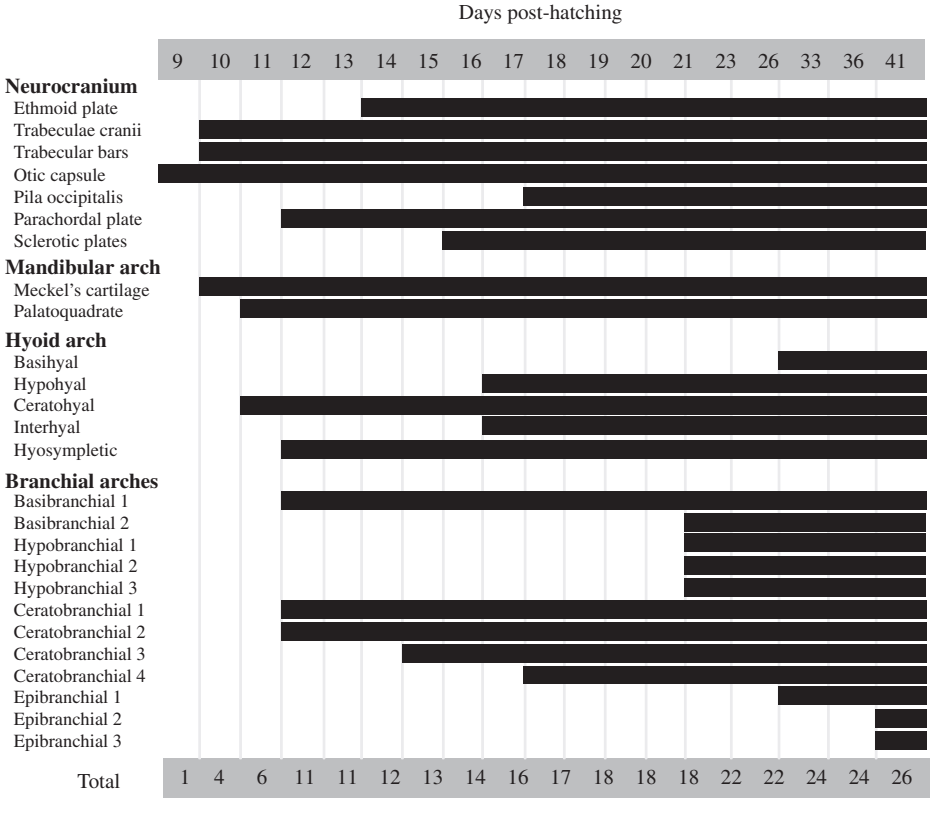

FIG. 5. Chondrification sequence of neurocranium and splanchnocranium of *Hippoglossus hippoglossus* larvae based on cleared and stained specimens from 9 to 41 days post hatch (dph), including the total number elements (counts) per dph.

DAYS 33–41 (312·3–373·8 d°)

The basihyal is visible as a mass of chondrocytes with little matrix located anteriorly to basibranchial 1. The otic capsule is almost closed. Epibranchial 1 can be seen at 33 dph, at the dorsal end of the first branchial arch. The coronoid process of the Meckel's cartilage elongates anteriorly. Epibranchials 2 and 3 are visible by 41 dph.

SEQUENCE OF CHONDRIFICATION

The sequence of chondrification for the 26 elements (four unpaired and 22 paired) of the neurocranium and splanchnocranium of *H. hippoglossus* larvae was reconstructed based on C&S specimens (Fig. 5). The sequence was also reconstructed using observations from the histological technique applied to specimens 9 to 23 dph as shown in Figs 3(a)–(f) and 4(a). It is to be noted, however, that the sequence reconstructed based on the C&S specimens and the one based on histological sections are not perfectly congruent ($r_s = 0{\cdot}9900$; $n = 19$ elements; $P < 0{\cdot}001$). The otic capsule displays the greatest shift in the sequence in terms of rank; the otic capsule ranks first with the C&S specimens being visible at 9 dph, whereas it ranks 9·5 based on histological sections being identifiable at 13 dph.

In Fig. 6, the ontogenetic trajectory of *H. hippoglossus* is constructed based on its sequence of chondrification (Fig. 5) and is compared to four other pleuronectiforms. Ontogenetic trajectories are based on the cumulative number of newly formed cartilaginous cranial structures from 0 to 41 dph. The ontogenetic trajectory of *H. hippoglossus* is divided into four phases (A to D of Fig. 6): (A) an initial step (period of slow ontogenetic development; Balon, 1981), (B) a threshold (period

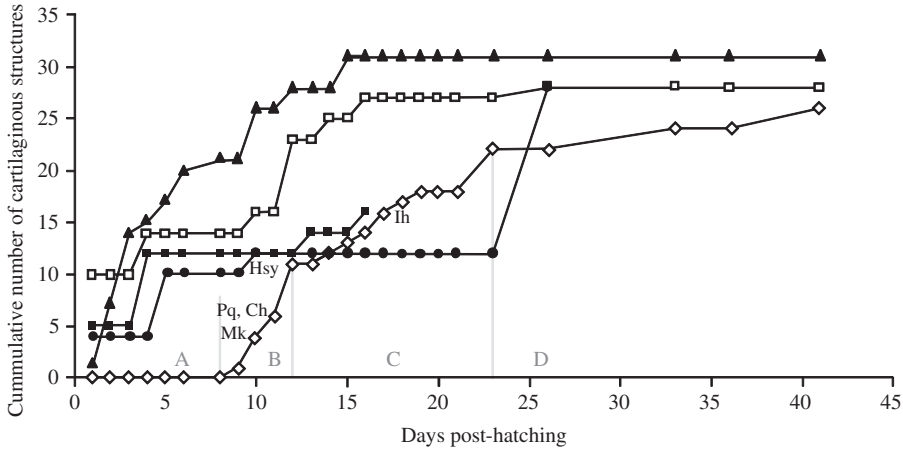

FIG. 6. Ontogenetic comparison of cumulative number of cartilaginous elements for five pleuronectiform species. *Hippoglossus hippoglossus* (◇; this study), *Solea solea* (□; Wagemans & Vandewalle, 1999), *Psetta maxima* (▲; Wagemans *et al.,* 1998), *Paralichthys dentatus* (■; Martinez & Bolker, 2003) and *Pseudopleuronectes americanus* (●; Hunt von Herbing, 2001). Data points represent samples for which the number of cartilaginous elements is available. The ontogenetic trajectory of *H. hippoglossus* is divided into four phases: two steps (A and D), one threshold (B) and one gradual phase (C). The onset of chondrification for the Meckel's cartilage (Mk), palatoquadrate (Pq), ceratohyal cartilage (Ch), hyosymplectic (Hsy) and interhyal (Ih) is reported on the ontogenetic trajectory of *H. hippoglossus*.

of abrupt changes; Balon, 1981), (C) a gradual phase and (D) a terminal step. The limits of these phases were identified by notable changes in the slope of the trajectory. The first phase is a post-hatching step that lasts 8 days (phase A; Fig. 6): no cranial elements are forming. The second phase (phase B; Fig. 6) is initiated with the onset of chondrification (9 dph) and corresponds to a threshold that lasts 4 days. During this phase, the number of structures increases rapidly until major structural elements are present (12 dph), including two components of the suspensorium (palatoquadrate and hyosymplectic) and the Meckel's cartilage. A gradual, non-saltatory ontogenetic phase (phase C; Fig. 6) occurs between 12 and 23 dph as the finer details of cranial chondrification take place and all structures start articulating with each other. The components of the suspensorium increase in size. The structural integrity of the hyoid arch is assured by the paired ceratohyals and hyosymplectics. The completion of the branchial apparatus from 21 dph onwards occupies the ending part of phase C. The last phase of the trajectory (phase D; Fig. 6) is a step lasting from 23 to 41 dph.

GAPING JAW

'Gaping jaw' malformation (an abnormal jaw development often seen in cultured species of *H. hippoglossus*) was observed histologically and in whole C&S specimens in order to identify the source of the malformation. Gaping jaw was due to an articulation anomaly either on a single side or on both sides of the head. In contrast to most vertebral malformation which could be observed at different degrees (weakly to strongly deformed), gaping jaw is either present or absent. In gapers, the neurocranium and branchial arches present a normal pattern [Fig. 4(a), (b)]. As is clearly visible in a 23 dph specimen [Figs 4(c) and 7(b)], however, the Meckel's cartilage is oriented vertically and the palatoquadrate is only slightly angled anteroventrally. With the exception of its orientation, the Meckel's cartilage does not differ visibly in its general morphology. Concerning the hyoid elements, however, it appears that gaping jaw specimens have excess chondrocytes on the ventral and dorsal extremities of their interhyal, yielding a continuum in consecutive histological sections from

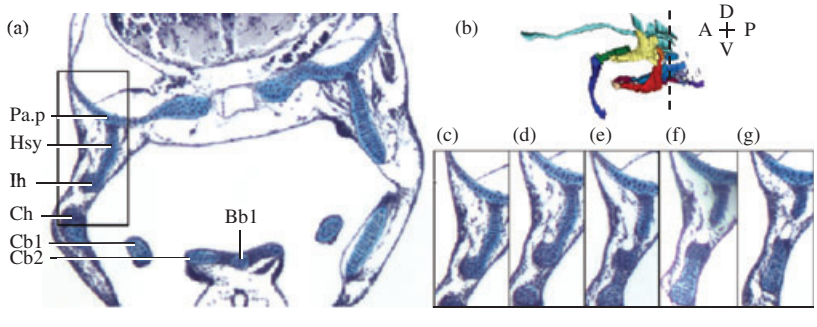

FIG. 7. Cranial anatomy of a 23 days post-hatch *Hippoglossus hippoglossus* larva with a gaping-jaw malformation. (a) Histological section through the posterior part of the head showing parts of the hyoid and branchial arches. (b) Positioning of the sections on a 3D reconstruction [same specimen as in Fig. 4(c)]. (c)–(g) Hyosymplectic, close-up of left side, from anterior [(c), same as in (a)] to posterior (g). Sections are stained with the Hall's and Brunt's Quadruple stain. Bb1, basibranchial 1; Cb1-2, ceratobranchial 1-2; Ch, ceratohyal cartilage; Hsy, hyosymplectic; Ih, interhyal; Pa.p, parachordal plate.

the dorsal end of the hyosymplectic to the distal end of the ceratohyal cartilage [Fig. 7(a), (c)–(g)]. The same observations could not be made with absolute certainty on C&S specimens, probably because of the very low chondrocyte density at these extremities. There are, therefore, histological indications that among gapers, the interhyal is fused to the hyosymplectic and to the ceratohyal cartilage or that the interhyal does not differentiate properly from the original chondrocytes.

DISCUSSION

Chondrocranial developmental sequences in teleosts vary not only among orders but also among phylogenetically close relatives (Cubbage & Mabee, 1996; Adriaens & Verraes, 1997; Koumoundouros *et al*., 2000; Mabee *et al*., 2000; Faustino & Power, 2001; Vandewalle *et al*., 2005; Ristovska *et al*., 2006), rendering it difficult to establish a general bauplan for teleosts. Moreover, although skull development generally presents a significant priority to feeding structures and then to respiratory structures (Koumoundouros *et al*., 2000), developmental patterns depend greatly upon the acquisition of structures filling functional requirements imposed by environmental and behavioural constraints (Mabee *et al*., 2000). Despite these confounding factors, it is still possible to establish patterns among species having similar life-history strategies and comparable cranial architecture. Therefore, *H. hippoglossus* cranial chondrification is compared here with that of other pleuronectiforms.

A general view of the neurocranium and splanchnocranium of *H. hippoglossus* brings to light the relative simplicity of their chondrocranial anatomy. *Hippoglossus hippoglossus* is unusual in the delayed appearance of the first cartilaginous element (9 dph). By comparison, other species that present no cartilaginous cephalic structure at hatching all developed at least one structure by 1 dph and by 9 dph they had at least 10 cephalic structures (Wagemans *et al*., 1998; Wagemans & Vandewalle, 1999; Hunt von Herbing, 2001; Martinez & Bolker, 2003). When comparing ontogenetic rates (Fig. 6), however, *H. hippoglossus* develops at approximately the same rate as other pleuronectiforms, perhaps even faster than flounders (*P. dentatus* and *P. americanus*). Therefore, contrary to some earlier reports (Lønning *et al*., 1982), *H. hippoglossus* larvae do not have a slow development but rather a delayed development due to their very premature state at hatching. This is consistent with previous research on *H. hippoglossus* larvae, which indicates that this species hatches prematurely in comparison to other pleuronectiforms (Lønning *et al*., 1982; Kjørsvik & Reiersen, 1992). Although *H. hippoglossus* development is delayed compared to other pleuronectiforms, it is important to note that the present study detected earlier appearances of cranial structures than any previous work on this species. The oldest larvae studied here (41 dph) present a total of 26 cartilaginous elements, and ossification was not observed during the period studied. According to Saele *et al*. (2004), the first ossified structures are observed 5 days after first feeding (*i.e.* 48 dph).

THE NEUROCRANIUM

The order of appearance of cranial structures follows that of most teleosts in the way that the neurocranium and the splanchnocranium appear simultaneously

(Ristovska *et al*., 2006). The first element of the neurocranium to appear in *H. hippoglossus* is the otic capsule at 9 dph (143 d°, 62 day-degrees post-hatch, ddph). This is earlier than that reported by Kjørsvik & Reiersen (1992) and Morrison & MacDonald (1995) who found, respectively, the first cartilaginous structures at 12 dph (72 ddph) and 13 dph (65 ddph). The basal support of the neurocranium is evident at 10 dph with the appearance of the trabecular bars, fused rostrally to form the trabecula communis. This is considered to be a tropibasic conformation of the skull, characterizing species with a laterally compressed head, higher than wide, generally having large ocular globes close together (Wagemans & Vandewalle, 1999). Considering the adult morphology of *H. hippoglossus* and its benthic ecology, it might have been expected that these fish would have a platybasic skull conformation, *i.e.* a dorso-ventrally compressed skull characteristic of some benthic teleosts. Although Vandewalle *et al*. (2005) stated that phylogenetically close species may present both skull conformations all pleuronectiforms compared in this study have the tropibasic skull type. Therefore, the pelagic larvae of *H. hippoglossus*, as in other pleuronectiforms (Wagemans & Vandewalle, 1999), require a skull construction belonging to the tropibasic type, thus emphasizing the major transformations they undergo throughout their development. Since Hunt von Herbing (2001) and Martinez & Bolker (2003) only described the feeding structures of *P. americanus* and *P. dentatus*, the neurocranial structures were inferred by the analysis of their illustrations but could not be used when comparing date of appearance. In *S. solea* and *P. maxima*, however, the cranial support appears respectively at 1 and 2 dph. In *H. hippoglossus*, the trabecula communis–trabecular bar complex curves ventrally during early growth and develops into the ethmoid plate at 14 dph. In *S. solea* and *P. maxima*, the ethmoid plate appears much earlier, at 5 and 3 dph. The parachordal plates are well developed by 12 dph, forming the 'floor' of the otic capsule, but do not present a *fenestra basicapsularis* and fuse to the trabecular bars until 15 dph. Finally, the *pila occipitalis* develops at 18 dph. In *S. solea*, the parachordal plates, present from 1 dph, widen at 4 dph and fuse to the trabecular bars at 6 dph. They also exhibit the *fenestra basicapsularis* at this moment. Contrary to most pleuronectiforms, but similar to *P. maxima*, however, parachordal plates do not fuse medially to form the basal plate.

THE SPLANCHNOCRANIUM

Hippoglossus hippoglossus does not exhibit the classical teleost splanchnocranium order of appearance as reported by Vandewalle *et al*. (2005); *i.e.* the Meckel's cartilage generally appearing first, with or without hyoid elements, followed by the ceratohyal cartilage and hyosymplectic and ending with the palatoquadrate and the branchial basket. Although the Meckel's cartilage is the first splanchnocranial element to appear at 10 dph, it is followed the next day by the palatoquadrate and the ceratohyal cartilage. Therefore, the cartilaginous suspensorium in *H. hippoglossus* is completed before the appearance of the hyosymplectic. In the other pleuronectiforms to which *H. hippoglossus* was compared, *P. dentatus* shows the classical viscerocranial ontogeny. The remaining three species either have a palatoquadrate appearing before the hyosymplectic (*P. maxima*) as in *H. hippoglossus* or present all suspensorium structures simultaneously as several hyoid structures, including the hyosymplectic (*S. solea* and *P. americanus*). The Meckel's cartilage in *H. hippoglossus* grows from 10 dph as a pair of small transverse bars on each side of the rostral

end of the head and develops anteriorly and posteriorly to fuse at the Meckel's symphysis and articulates with the palatoquadrate. The posterior part of the Meckel's cartilage will differentiate at 23 dph as the *processus retroarticularis* and the *processus coronoideus* to accommodate articulation with the palatoquadrate; this occurs by 8 dph in *P. maxima*. The palatoquadrate, appearing at 11 dph, develops into a longitudinal rod-like structure, ventrally curved so that its posterior end rises dorsally along the dorsal surface of the hyosymplectic.

The paired ceratohyal cartilages are the first elements of the hyoid arch to appear at 11 dph. At 12 dph, the hyosymplectic appears. It then takes 5 days until all the other elements of the hyoid arch appear together (17 dph). In the meantime, the hyosymplectic develops its functional aspect with its posterior fan-shaped *processus opercularis* and an anteroventral extending portion, the *pars symplecticus*. This enables the onset of articulation when the hyoid arch is completed with an interhyal at 17 dph. The interhyal acts as a structural linkage between the hyosymplectic and the ceratohyal cartilage, enabling articulation for the lateral and downward opening of the buccal cavity (Véran, 1988).

The branchial system appears at 12 dph and is composed of basibranchial 1 and ceratobranchials 1 and 2, with hypobranchials 1 and 2 appearing at 23 dph. Kjørsvik & Reiersen (1992) observed the first branchial structures at 16 dph. Thus, the skeletal elements involved with feeding develop around the time of first exogenous feeding. Similarly, the development of the branchial apparatus that is required to switch from cutaneous respiration to branchial respiration develops before the resorption of the yolk sac (*i.e.* 26 dph). This is consistent with the findings of K. Pittman, L. Berg & K. Naas (unpubl. data), who reported branchial activity at 180 d° (15 dph in the present study). The branchial arches continue to develop to include four ceratobranchials, three hypobranchials and three epibranchials by 41 dph. No fifth branchial arch and no pharyngobranchials were seen before the end of the ontogenetic series that was sampled here: the branchial arches were not completely developed at 41 dph.

ONTOGENETIC TRAJECTORY

The chondrocranial ontogeny of *H. hippoglossus* appears to be characterized by different tempos of development: slow steps, a gradual phase and a fast threshold. This presence of steps and threshold are characteristic of saltatory ontogeny (Balon, 1981). In the present study, steps represent periods of relative stagnation in the cumulative number of cartilaginous structures, whereas thresholds represent sudden increases in the cumulative number of cartilaginous structures. When longer periods of time are analysed, stepwise trajectories (alternation of steps and thresholds) are recovered (Balon, 1981; Kováč, 2002); however, owing to the focus of this study on a single morphological system during a relatively short period of time, a single threshold and two steps were identified. Furthermore, in contrast to the theory of saltatory ontogeny which stipulates that a trajectory is composed solely of steps and thresholds, a phase of gradual changes has been recognized for *H. hippoglossus*. The four phases identified in the ontogenetic trajectory of *H. hippoglossus* have been recognized in the remaining four species of pleuronectiforms analysed but with various onsets and rates (Fig. 6). In comparison to *H. hippoglossus*, phase A (Balon, 1981) is shorter in *P. americanus*, *P. dentatus* and *S. solea*, whereas it is absent in *P. maxima*. In *P. americanus*, *P. dentatus* and more obviously in *S. solea*, however, numerous

elements are already present at hatching. Phase B is present in all species with the exception that it occurs earlier in ontogeny around 3–4 dph for the other pleuronectiforms. The gradual phase C is also occurring in *P. maxima*, *S. solea* and *P. dentatus*, but it occurs earlier in ontogeny. The trajectories available for *P. dentatus* and *P. americanus* do not cover the extent of the trajectories available for the remaining three species. In *P. americanus*, the trajectory suggests an important threshold between 23 and 25 dph, which might either correspond to the gradual phase C or simply reflects the coarser resolution of the original data (Hunt von Herbing, 2001). The terminal step of phase D is present in all species, with the exception of *P. dentatus* for which data are not available past 16 dph. In *P. maxima* and *S. solea*, phase D starts earlier than in *H. hippoglossus*. The overall trajectory of *H. hippoglossus* appears to begin later and end later than for other pleuronectiforms. *Solea solea*, *P. americanus* and *P. dentatus* display similar early ontogenetic trajectories. *Psetta maxima* has a unique trajectory having a single structure at 1 dph but completing chondrification by 13 dph. The trajectory of *H. hippoglossus* is closest to that of *P. maxima* with the exception of an important delay in time and a slower terminal rate.

The recognition of the four phases suggests a developmental delay for the onset of chondrification in *H. hippoglossus* but a relatively faster rate of development to complete the chondrocranium. The threshold phase could also be identified as a critical period during the development of *H. hippoglossus* because numerous (12) cartilaginous elements are formed over a short period of time (4 days). Threshold phases have been recognized as critical periods for the functional integrity of larval fishes and specifically for the feeding system (Liem, 1991; Kováč, 2002). Already at 12 dph, the integrity of the cranial structures of *H. hippoglossus* is ensured by the presence of robust elements (trabecula communis, trabecular bars, otic capsules and parachordal cartilages) protecting the neural and sensory organs in development, as well as by an almost complete framework for hyomandibular function (Meckel's cartilage, palatoquadrates, hyosymplectics and ceratohyals) and respiration (first basibranchial and first two branchial arches). The final step probably enables the mouth structures to become functional as first feeding approaches.

GAPING JAW

Intensive culture of *H. hippoglossus* is still constrained by low cumulative survival over the yolk-sac incubation and first feeding period and by an often high (and unpredictable) level of skeletal malformation (Olsen *et al*., 1999). A large proportion of yolk-sac larvae exhibit abnormal jaw development ('gaping jaw') and abnormal eye migration at metamorphosis. It has been suggested that some of these malformations might be caused by temperature and salinity changes, by water flow or by bacterial infection (Pittman *et al*., 1990; Morrison & MacDonald, 1995; Saele, 2002).

Morrison & MacDonald (1995) applied cartilage staining and electron microscopy to describe jaw development and to determine the cause of 'gaping jaw'. They found that the anterior parts of the ethmoid and Meckel's cartilage were bent apart. These larvae cannot survive past first feeding since they have used up their yolk-sac reserves and cannot switch to exogenous feeding. It was suggested that this condition was associated with an abrasion of the head followed by invasion of pathogens. The study of Morrison & MacDonald (1995), however, was conducted in small volume multi-well dishes, an environment which can tell little about the root causes of such

malformations in large volume silos, which are the standard in intensive culture of *H. hippoglossus*. Morrison & MacDonald (1995) also noticed that the protractor hyoideus muscle was clearly contracted in a 29 dph gaping *H. hippoglossus*; thus they linked the bending of the Meckel's cartilage to a weakening of the membrane surrounding the Meckel's symphysis, the function of which, according to these authors, was to prevent the premature opening of the mouth before cartilaginous structures were properly formed. The present study, however, clearly shows that all structures are in position for mouth articulation by 17 dph. Some specimens in the study had the gaping jaw malformation. Histological investigation of these specimens indicates that the malformation is due to a defect in the development of the interhyal. Consecutive histological sections revealed a probable fusion of the interhyal at its two extremities, *i.e.* the dorsal end with the hyosymplectic's *processus opercularis* and the ventral end with the distal portion of the ceratohyal cartilage (Fig. 7). If this is true, then the interhyal cannot fulfil its major function in the articulation of the mouth, normally allowing the slight lateral expansion of the buccal cavity as well as the lowering of the Meckel's cartilage (Véran, 1988; Liem, 1991; Adriaens & Verraes, 1994; Wilga *et al*., 2000; Hernandez *et al*., 2002). At this stage, the mouth has no ossified element and is solely composed of the Meckel's cartilage. To open the mouth, the protractor hyoideus muscle contracts and pulls on the lower jaw, but because the fused hyosymplectic–interhyal–ceratohyal complex is not able to articulate, the Meckel's cartilage will be forced to bend down. A Meckel's cartilage bent downwards results in the gaping of the jaw. This happens when larvae open their mouth to wear away the oral membrane, as observed by K. Pittman, L. Berg & K. Naas (unpubl. data), as soon as they are able to articulate their jaw. This is consistent with observations of 19 dph gapers by Pittman *et al*. (1990). Furthermore, Francis & Turingan (2008) have demonstrated that lower jaw depression and elevation changed from a hyoid-based to an opercular-based mechanism prior to the onset of metamorphosis in southern flounder *Paralichthys lethostigma* Jordan & Gilbert. Nonetheless, this fusion must be studied in greater detail to determine whether it is truly responsible for the gaping jaw syndrome and, if so, what causes it. A potential avenue of investigation would be to test whether suboptimal conditions induce subtle rank shifts or timing changes in the chondrification sequence of the hyoid elements during the threshold phase which might be responsible for this skeletal malformation. Lewis & Lall (2006) have already suggested that rapid development over a short period of time (which to some extent corresponds to a threshold) during the prometamorphic stages of *H. hippoglossus* might be associated with axial skeleton malformations in later development.

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