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Larval Development of the Oriental Lancelet, *Branchiostoma belcheri*, in Laboratory Mass Culture

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We are successfully maintaining a laboratory colony of the lancelet *Branchiostoma belcheri* bred in the laboratory. Based on living individuals in this mass culture, morphological characteristics from the seven-day larval to benthic juvenile stages have been studied. Most striking was that later larval development of *B. belcheri* showed great individual variation even in a rather stable culture environment. Metamorphosis first occurred on 60 days post fertilization (dpf) and was continuously observed throughout the present study up to 100 dpf. Morphological traits such as the number of primary gill slits and body size at the start of metamorphosis are apparently affected by culture condition. Body size measured in the largest individuals showed nearly linear growth at 0.087 mm/day. The variability found in larval development calls for caution when developmental stages and chronological ages are compared between populations. However, the developmental flexibility of this animal also raises the possibility that growth and sexual maturation could be controlled artificially in captivity.

Key words: chordate, amphioxus, larval development, laboratory culture, metamorphosis

INTRODUCTION

Since the hallmark study of Kowalevsky (1867), the lancelet (amphioxus) has been studied intensively, especially its development and morphology, producing a large literature (reviewed by Gans, 1996). However, lancelet studies seem not to have kept pace with recent advances in studies on ascidians and model vertebrates. This is apparently caused by difficulties in getting research material. Relying on seasonal laboratories set up once a year, embryos for developmental study can be obtained in Florida, USA (Holland and Holland, 1993), Qingdao, China (Wu *et al.*, 1994), and the French Mediterranean (Fuentes *et al.*, 2004), but larvae cultured without special care generally die within a few weeks after fertilization.

Studies on later larval stages, including the metamorphic period, were so difficult that more than a century passed from the first description of *B. lanceolatum* by Willey (1891) until Stokes and Holland (1995) described development from hatchlings to early juveniles in *Branchiostoma floridae*. The former study was accomplished by daily collection in the wild (Lankester and Willey, 1890; Willey, 1891). The study on *B. floridae* (Stokes and Holland, 1995) was accomplished by adopting the laboratory culture method for later larvae attempted by Gilmour (in Holland and Holland, 1993), in which animals were maintained in Petri dishes with daily changes of food and water.

Holland and Yu (2004) offered useful instructions for obtaining experimental specimens of *B. floridae* based on previous studies. However, to make lancelets a laboratory animal, a more convenient method for long-term culture at large scale is still required to establish laboratory colonies. Along these lines, some groups, including us, are improving culture methods (Wu *et al.*, 2000; Fuentes *et al.*, 2004; Wang *et al*., 2006, 2007; Yasui *et al.*, 2007), and Wang *et al.* (2006) and Zhang *et al*. (2007) successfully obtained a second laboratory generation.

Our culture system also resulted in keeping alive a first mass laboratory generation and provided opportunities to study the long-term development of *B. belcheri* in the laboratory. Based on living individuals, we describe development from the 7-day-old larval stage to the early juvenile stage in detail. The present study compliments previous studies on normal development from the fertilized egg to the early larval stage by Hirakow and Kajita (1990, 1991, 1994) in the same species but from Qingdao, China (but see Zhang *et al.*, 2006) and completes the description of the early life of *B. belcheri*.

MATERIALS AND METHODS

Adult *Branchiostoma belcheri* with mature gonads were collected by dredging in the Ariake Sea, Kumamoto, Japan, in June

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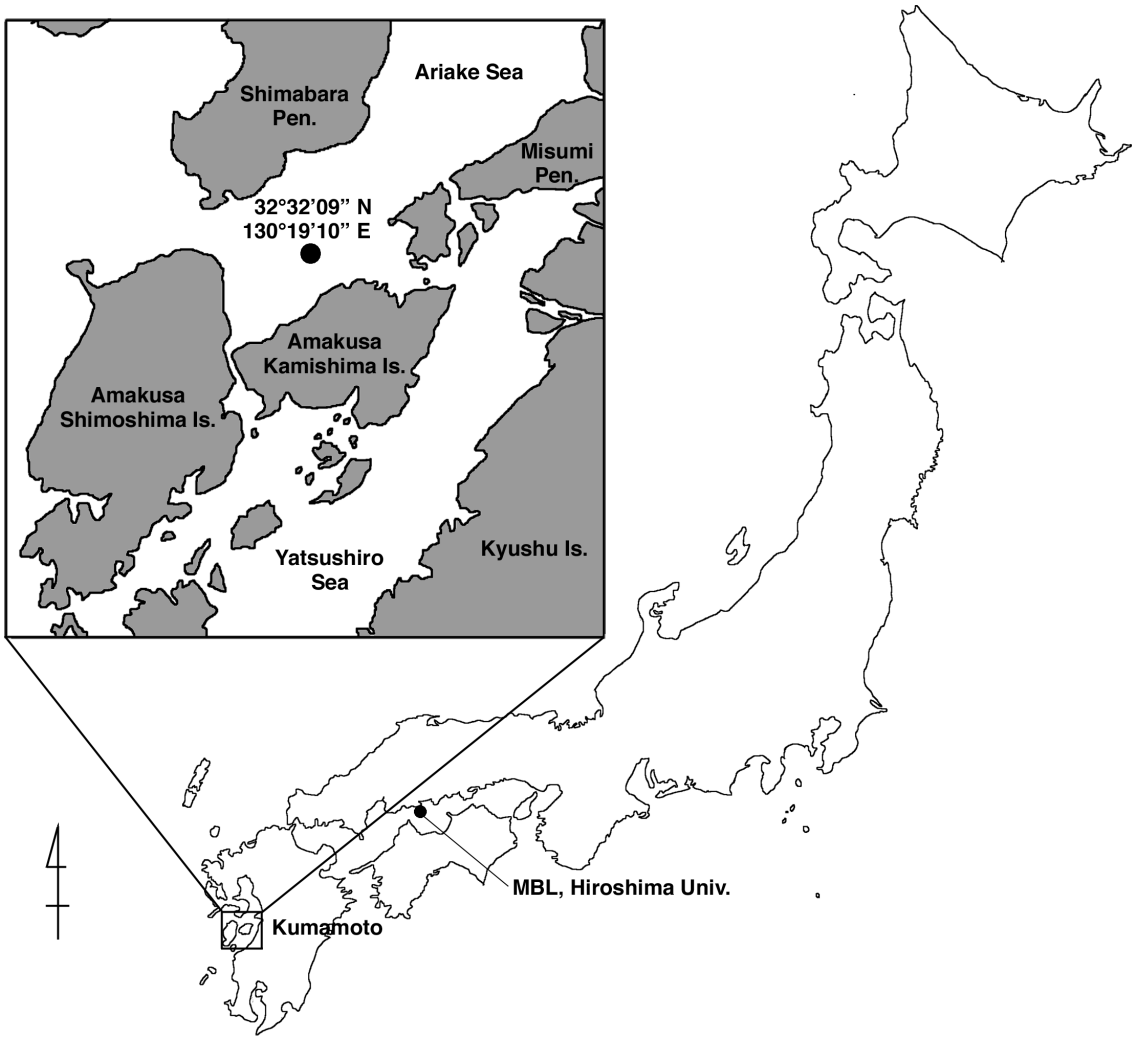

Fig. 1. Outline map of Japan showing the location of the collecting site (enlargement) in Amakusa, Kumamoto. At the mouth of the Ariake Sea about 20–30 m in depth, 100–200 individuals were collected by one trial of dredging.

2005 (Fig. 1). Some of the animals released gametes in the laboratory from July to August (Yasui *et al.*, 2007). Using hatched embryos found in the morning on 23 August 2006 and probably spawned by two females, we started a mass culture.

The mass culture is being carried out in a 33-L fish glass tank (W, 29 cm; L, 45 cm; H, 31 cm) filled with about 30 L of filtered seawater (FSW) that is changed weekly, under natural light and dark regime. The water is aerated by a 14-cm-long airstone on the bottom. The water was at room temperature (28–33°C in August, 27–32.5°C in September, and 19.5–29.5°C in October) until mid October and was then controlled at 23–25°C with an air conditioner. When embryos grew to larvae just opening the mouth, they were fed with a mixture of unicellular algae, *Chaetoceros gracilis* NRIA (roughly 30,000 cells/mL) and *Dunaliella* sp. (roughly 5,000 cells/mL). The algae were purchased from the National Research Institute of Aquaculture, Fisheries Research Agency of Japan, and are being cultured in our laboratory. To characterize and measure developing animals, we randomly selected five to ten individuals during early development and selected five largest individuals during later development. Sampling intervals were about every 5 days, every day, and every 10 days for larval development, metamorphosis, and juvenile development, respectively. Selected individuals were photographed and measured alive under the microscope. Total body length was represented by the largest animals among those selected, because of the great individual variation in the colony.

We also performed a supplementary tube culture using 50-mL centrifuge tubes starting at the neurula stage. In this culture, tubes were filled with FSW containing the same algal mix as that for the mass culture, but 12 times as dense. Each tube initially contained 10 individuals and was rotated vertically at 3 rpm at room temperature. At the daily water change, animals were observed and transferred into new medium under a dissection microscope. Other conditions were the same as those of the mass culture. The culture was terminated 82 days after fertilization because most individuals had died.

RESULTS AND DISCUSSION

Growth pattern

In our mass culture, rapidly growing individuals showed nearly a linear growth curve (Fig. 2). The growth rate calculated from the curve (Fig. 2) was 0.087 mm/day, higher than the 0.05 mm/day estimated for planktonic larvae of the same species in the waters around Ehime, Japan (Ueda and Kamakura, 2005). However, if we had obtained the growth rate by random sampling, it might have come close to the estimate for the Ehime population. Although it is known that

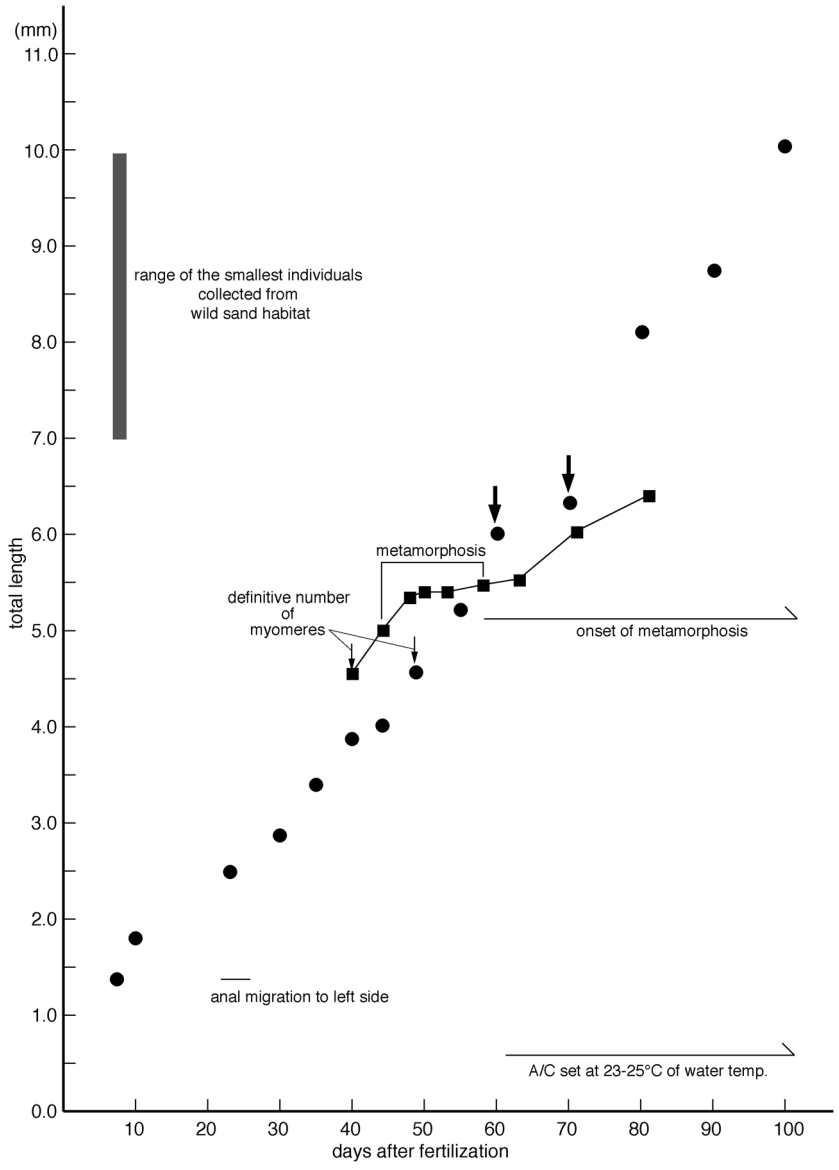

Fig. 2. Growth pattern of cultured larvae and juveniles. Solid circles are for the largest animals selected from the mass culture and solid squares for an individual cultured in a 50-mL tube. Both exhibit possible growth retardation during metamorphosis (large arrows). The number of myomeres reaches the adult state before metamorphosis.

the embryonic and early larval development of lancelets is synchronized within a spawn, which was the case in the present culture, later larval development in our culture varied markedly among individuals. Metamorphosis was first observed at 60 dpf in larvae 5.6–6.1 mm long with 16–18 gill slits, and thereafter we continuously observed metamorphosing larvae.

In the size plot, a weak plateau was found during metamorphosis (large arrows in Fig. 2). To confirm this, the development of an individual in tube culture was individually traced, and we detected developmental retardation during metamorphosis, although this should be confirmed by tracing additional individuals. In a field collection on 26 October 2005 in the Ariake Sea, Japan, we obtained by dredging two juveniles 9.5 mm long. This size corresponds to that of the majority of the smallest benthic animals collected so far from the Ariake habitat (Henmi and Yamaguchi, 2003), and therefore we stopped the present study on 100 dpf, when the largest individuals exceeded 10.0 mm in length.

External appearance of developing larvae and metamorphosed juveniles

Staging for developing larvae and juveniles with easily identifiable characters is supplied as on-line Supplementary Table 1.

Larvae prior to metamorphosis

Larvae at 7 dpf are slender in appearance with three primary gill slits on the right side (Fig. 3A). The anteriormost gill is opposite the posterior half of the mouth, which opens on the left side (Fig. 4A). The anterior end of the nerve cord is dilated, forming the cerebral vesicle, and in the nerve cord

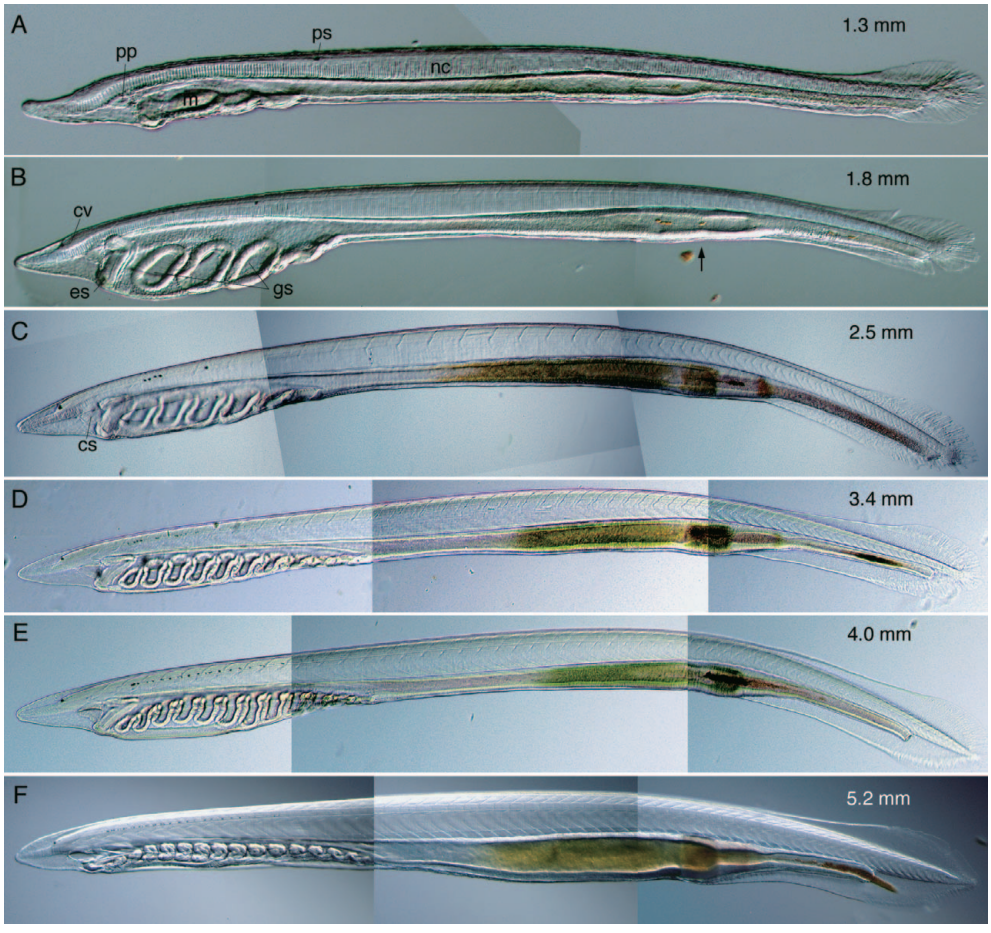

Fig. 3. Left lateral view of premetamorphic larvae. All the larvae are shown as the same size. **(A)** Seven days post fertilization (dpf), **(B)** 12 dpf, **(C)** 23 dpf, **(D)** 35 dpf, **(E)** 44 dpf, and **(F)** 55 dpf. Arrow in B indicates constriction between midgut and hindgut. During this period, larvae grow to four times their initial size. Cv, cerebral vesicle; cs, ciliated surface; es, endostyle; gs, primary gill slits; m, mouth; nc, notochord; pp, preoral pit; ps, pigment spot.

a pigment spot is visible at the level slightly posterior to the third gill slit. The notochord under the cerebral vesicle curves ventrally, and at the posterior end of the curve is located the opening of the preoral pit (Fig. 4A). Opposite the anterior margin of the mouth, the primordium of the endostyle has developed vertically (Fig. 4A); at this level, the body wall protrudes laterally on both sides, showing a triangular appearance in ventral view. At the ventral margin of the protrusion on the left side, the mouth papilla (Andersson and Olsson, 1989), evident when larvae lie upon the left side of the body (arrow in Fig. 4A), appears at 3 dpf and is observed until 30 dpf. Rostral papillae (Lacalli *et al.*, 1999) are also discernible at the dorsal and ventral margins, from the level of the anterior end of the nerve cord to ventral to the mouth (arrows in Fig. 4B). In the alimentary canal, the future midgut region (in which ciliary movement makes the food cord twist counterclockwise, viewed from the tail) is dilated, and the anus opens on the right side. The caudal fin, which has formed with an elongate array of single cells, is well developed and shows an elliptical outline.

In 12-day-old larvae, the anterior three primary gill slits are well developed on the right, and the forth and fifth are forming ventrally (Fig. 3B). The body becomes thickened dorsoventrally, especially in the pharyngeal region. The middle third of the notochord is also thickened. In the nerve cord, in addition to the first pigment spot, the anteriormost spot (part of the frontal eye of Lacalli *et al.*, 1994) and a spot in between have appeared. Owing to the growth of the nerve cord, especially in the middle third, the diameter of the cerebral vesicle becomes almost the same as in the middle region. In the pharyngeal region, the first primary gill slit is located opposite to the mouth, and the second at the level of the posterior margin of the mouth. Oral spines, which appeared by 2 dpf, are well developed and project about three-fourths of the dorsoventral diameter of the mouth (Fig. 4C). The dorsal and ventral spines project alternately. Among the dorsally projecting rostral papillae, one located at the level of the anteriormost pigment spot becomes prominent. The endostyle becomes chevron-shaped, and the preoral pit is expanding ventrally, forming a ciliated surface. In the alimentary canal, the boundary between midgut and hindgut is constricted (arrow in Fig. 3B). The region anterior to the constriction will become the iliocolonic ring.

In 23-day-old larvae, there are six primary gill slits, of which the anterior three are located opposite the mouth (Fig. 3C). There are four pigment spots in the nerve cord between the first and anteriormost ones. The anus migrates during this period from the original right side to the left side (Stokes

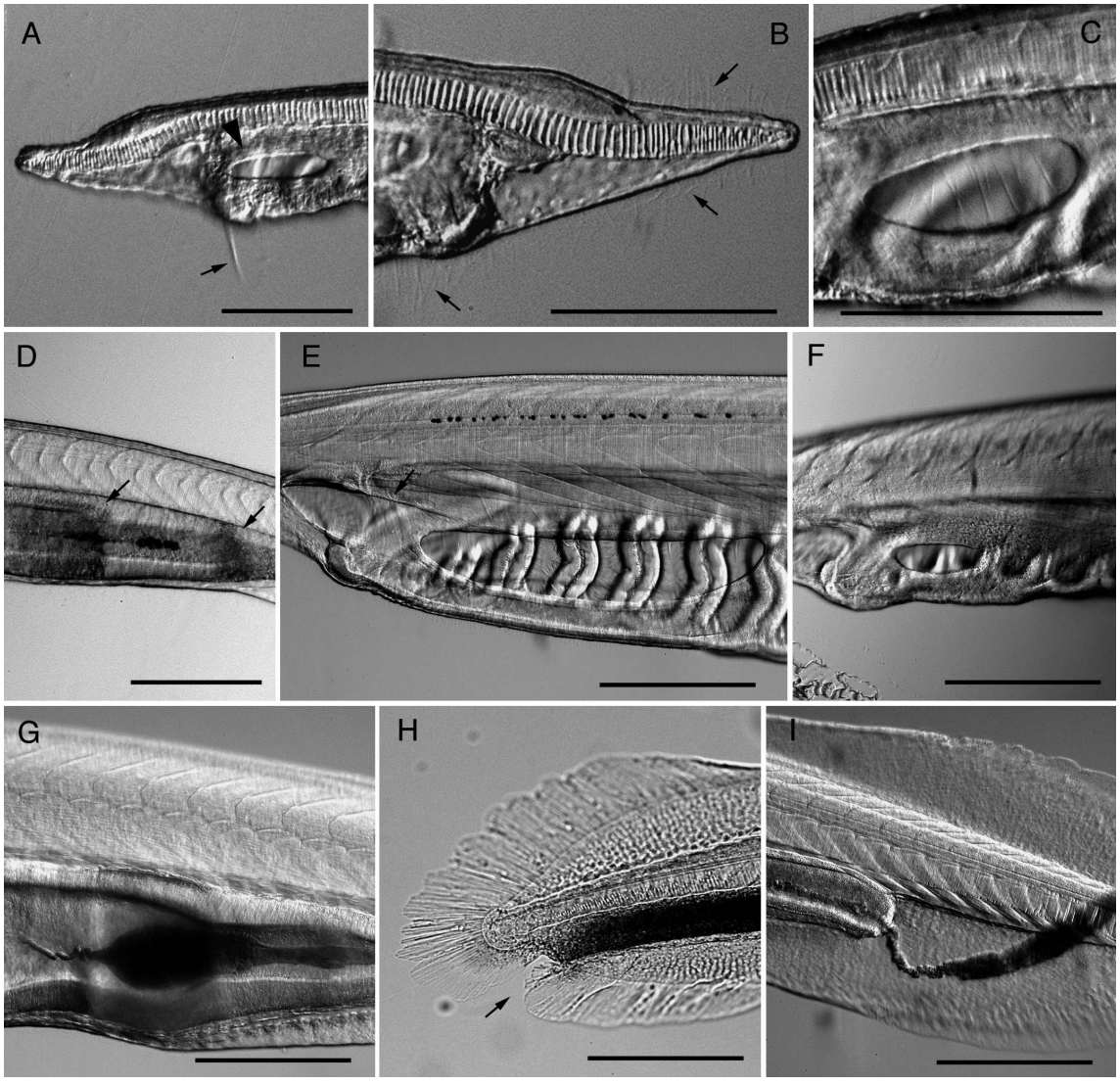

Fig. 4. Larval characters prior to metamorphosis. **(A)** Mouth papilla projects from anterior left angle of pharyngeal region (arrow), and vertically located endostyle is found on right wall of pharynx (arrowhead) in a 7-day larva. **(B)** Rostral papillae project from the dorsal and ventral surface, from ventral to the mouth to the anteriormost pigment spot (arrows) in a 10-day larva. Note the prominent papilla at the level of the pigment spot. **(C)** Long oral spines across the mouth opening in a 15-day larva. **(D)** Pigmented bands at site of the future iliocolonic ring and anterior ampulla of hindgut (arrows) in a 23-day larva. **(E)** Integumental fold, developing anterior to the mouth, that delineates dorsal margin of preoral cavity (arrow). Wide mouth projects oral spines from its margin in a 55-day larva. **(F)** Small mouth of a premetamorphic larva cultured in a 50-mL tube. **(G)** Twist of food cord in iliocolonic ring and anterior ampulla of hindgut in a 55-day larva. **(H)** Migrating anus located in the midplane making an incision in caudal fin in a 23-day larva. **(I)** Anal opening located at the destination in a 55-day larva. Scale bars, 0.1 mm.

and Holland, 1995). When the anus reaches the median plane during its migration, the caudal fin is incised at the anal opening (Fig. 4H). Soon after completion of the anal migration, however, the incision of the caudal fin disappears (Fig. 4I).

From 30 to 55 dpf, larvae grow 1.9 times in length (from 2.8 to 5.2 mm), but their overall appearance is similar, except for the following (Fig. 3D–F). Primary gill slits increase from nine to 14 in number. The timing of new slit formation seems not to be constant, but occurs periodically. The number of myomeres reaches the adult state, 64–66 myomeres (n=5) (see Henmi and Yamaguchi, 2003) prior to the onset of atrial formation by 55 dpf. The anteriormost gill slit becomes reduced its size in the dorsal half in 35-day-old larvae (Fig. 3D). Pigment spots in the nerve cord appear posterior to the first one, which is usually distinguishable by its larger size. The cells constituting the wall of the prospective iliocolonic ring and part of the anterior ampulla of the hindgut become brownish in color (arrows in Fig. 4D). Food particles (mostly unicellular algae) move fast caudally in the esophageal region and are accumulated anterior to the prospective iliocolonic ring, where they are trapped into a food cord that is twisted by ciliary movement (Fig. 4G). By 55 dpf, five or six gill slits are opposite the mouth, and anteriorly an integumental fold (arrow in Fig. 4E) appears dorsally to the transforming preoral pit, which ventrally forms a triangular depression called the preoral cavity (Stokes and Holland, 1995). The diameter of the cerebral vesicle is

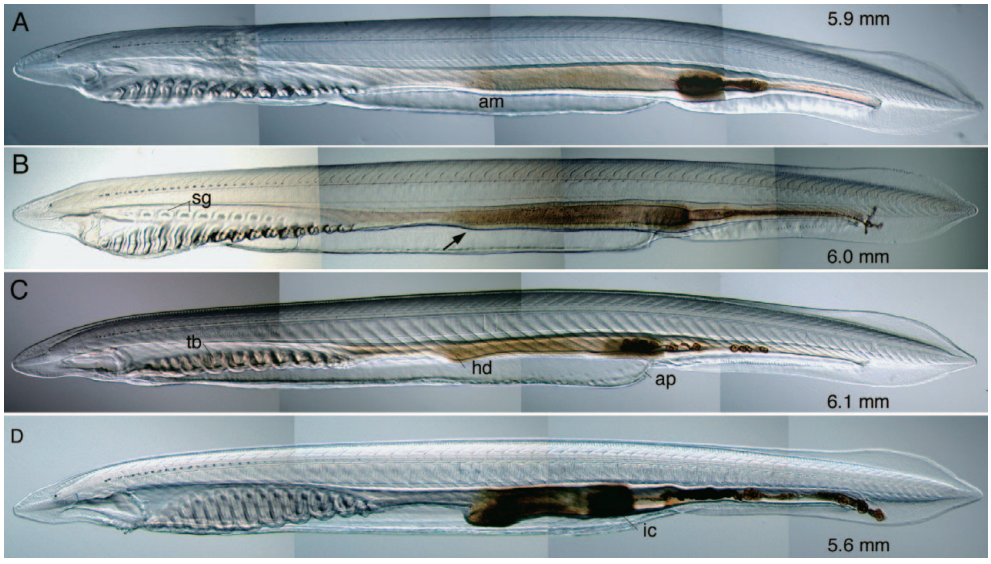

Fig. 5. Left lateral view (right mirror image in A) of animals during metamorphosis in the mass culture. All larvae are shown as the same size. **(A)** First appearance of secondary gill row on right side; 60 dpf. **(B)** Atrial chamber is expanding anteriorly; budding of hepatic diverticulum is noticeable (arrow); 63 dpf. **(C)** Migration of the primary gill row is almost complete, and pharynx is covered completely with atrium; 71 dpf. **(D)** Tongue bar has fused with ventral margin of gill slits on both sides, and future right preoral cirri are protruding; 67 dpf. Am, atrium; ap, atriopore; hd, hepatic diverticulum; ic, iliocolonic ring; sg, secondary gill slits; tb, tongue bar.

relatively reduced and smaller than that of the other parts of the nerve cord. The outline of the caudal fin becomes spear-like. During this period, larvae in the tube culture showed a small mouth occupying the width of two gill slits when they reached the adult number (64) of myomeres (Fig. 4F).

Larvae developing the secondary gill row (onset of metamorphosis)

The secondary gill row appears in animals with 16–18 primary gill slits, 5.6–6.1 mm long, from 60 dpf onwards (Fig. 5A). Just before the epithelial thickening of the secondary gill slits, a canal-like atrial chamber has formed from the level of the second or third posterior gill slit to the anterior midgut region. The posterior end of the canal opens as the atriopore. Dorsal fin-ray chambers are also forming (Fig. 6A). Soon after the appearance of the secondary gill row, forward extension of the forming atrium by fusing subatrial ridges (Lankester and Willey, 1890) proceeds from the former anterior limit of the canal, but remains the mouth region (Fig. 5B). At the former anterior limit, a temporary septum is formed in the chamber (Fig. 6B). The atriopore is located at the anterior margin of the future iliocolonic ring. When the secondary gill row appears, a row of preanal fin-ray chambers become visible in the fin as a line of small vesicles (Fig. 6C) that have a quite different appearance from the initial stage of the dorsal fin-ray chambers (Fig. 6A).

The dorsal margin of the mouth continues anteriorly as an intigumental fold that overhangs the preoral cavity. The anterior margin of the mouth slightly shifts medially, and a knob, which will become a velar tentacle on the right side (arrow in Fig. 6D), is visible at the midpoint. At the posterodorsal corner of the preoral cavity, Hatschek's pit is differentiated and is surrounded by the ciliated surface of the future wheel organ that forms the first finger. In the posterior primary gill slits that lie on the floor, a small pit opens on the lateral wall of each slit (arrows in Fig. 6E). These pits may be the outlets of excretory tubules, but this needs further investigation.

Animals with a fully formed atrium

About four days after the first appearance of the secondary gill row, fusion of the subatrial ridges is complete, forming the atrium. In the atrial chamber, the anterior primary gill slits are still located on the right side. Bisection of the secondary slits by tongue bars takes place earlier than in the primary slits, and that in primary slits starts during the migration onto the left side. Some of the anterior and posterior primary slits do not form tongue bars and will disappear (Fig. 5C). Accordingly, the first paired gill slits, which have been bisected by a tongue bar, become 9 to11 pairs. As soon as the atrial chamber is formed, five preoral cirri are easily visible at the ventral oral margin and will become those of the right preoral hood (Fig. 6F). The preoral hood on the left side soon forms five to six counterparts. The larval mouth faces forward and transforms into the velum, which projects tentacles. By translocation of the mouth and development of the preoral hood on either side, the vestibule (=buccal cavity, preoral cavity) is fully established (Fig. 5D). On the internal wall of the vestibule, fingers of the ciliated wheel organ are added ventrally (Fig. 6H).

In the nerve cord, pigment spots become numerous in the pharyngeal region, showing a metameric pattern of two accumulations in each myomere, and a few spots have also scattered in the caudal region (Figs. 5, 6G). At the caudal end of the nerve cord, the caudal ampulla is visible (Fig. 6I). Besides the mouth, the most striking change during this period is in the alimentary canal. The budding of the hepatic diverticulum occurs at the same time as the completion of the atrium (Fig. 5C). The prospective iliocolonic ring was

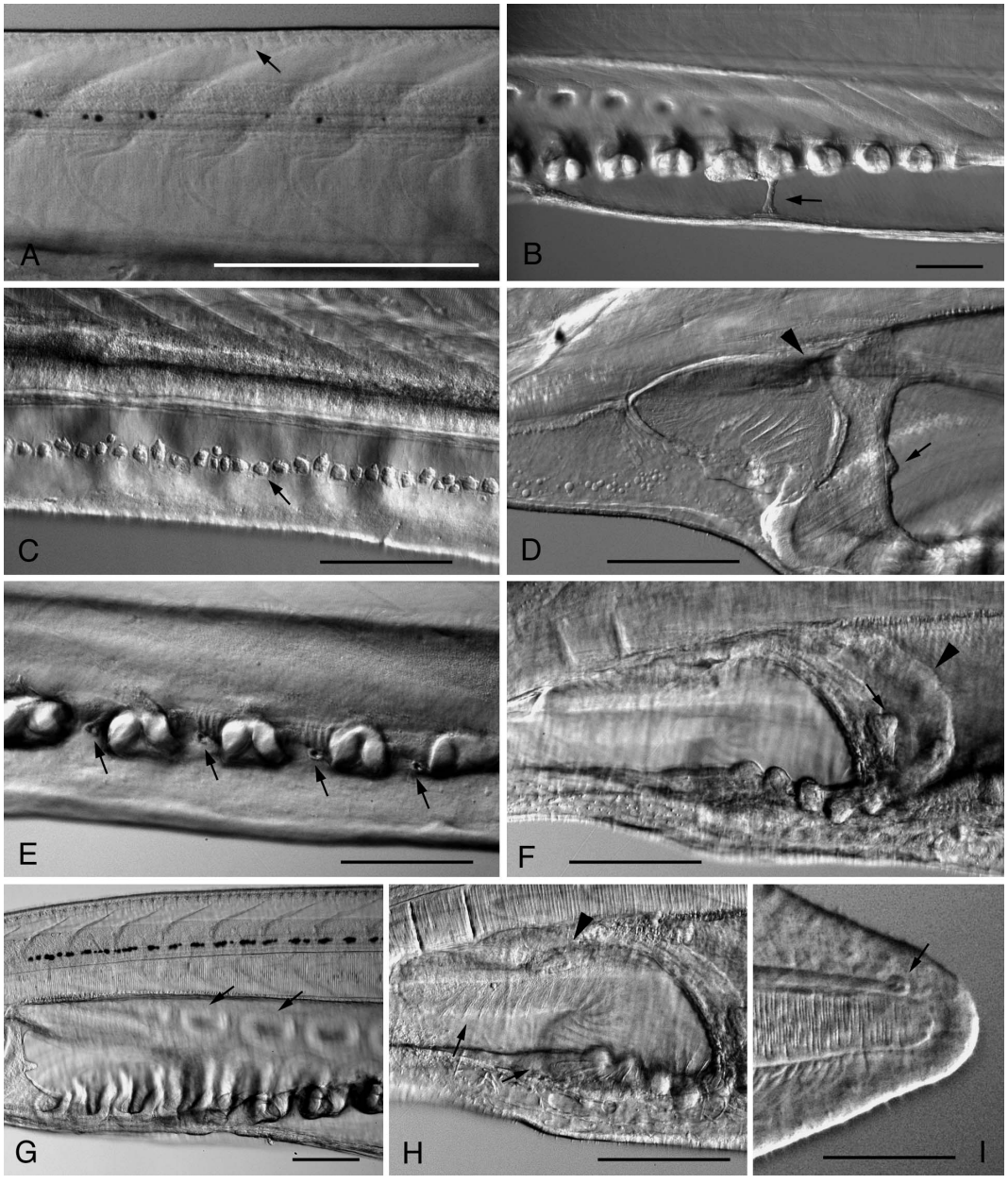

Fig. 6. Characters of animals during and post-metamorphosis. **(A)** Appearance of dorsal fin-ray chambers (arrow) in a 60-day larva. **(B)** Atrial septum appears transiently during metamorphosis (arrow). **(C)** Appearance of preanal fin-ray chambers (arrow), which show a shape distinguishable from that of dorsal ones, in a 63-day individual. **(D)** Preoral cavity in a 63-day individual. Guitar-pick-shaped prospective wheel organ, Hatschek' pit (arrowhead), and first right velar tentacle protruding from anterior margin of mouth (arrow) are seen. **(E)** Small pits on lateral wall of posterior gill slits (arrows) in a 60-day individual. **(F)** Photomontage of mouth showing basal skeleton extending from skeleton of preoral cirri (arrowhead) and right velar tentacle (arrow) in a 71-day individual. **(G)** Tongue bar formation in secondary gill slits (arrows) in an individual during metamorphosis. **(H)** Preoral cavity with wheel organ extending two fingers (arrows) and Hatschek's pit (arrowhead) in a 71-day individual. **(I)** Caudal ampulla of nerve cord (arrow) in a 71-day individual. Scale bars, 0.05 mm in I, 0.1 mm in the others.

located posterior to the atriopore previously, however, it is located anteriorly at this period. In juveniles, the atriopore is located at the level of the anterior part of the intestine (hindgut). Since the position of the myomere at the level of the atriopore is not changed, this relative movement of the atriopore is due to modification of the alimentary canal. The budding hepatic diverticulum is pigmented, as is the iliocolonic ring (Fig. 5D).

Development of juveniles

After formation of the atrium, the most conspicuous change is dorsoventral thickening of the body (Fig. 7). The ratio of dorsoventral thickness/total length increases about 8% after metamorphosis. Gill slits bisected by a tongue bar have elongated dorsoventrally at a posterior oblique angle (Fig. 7A). First synapticles that connect a tongue bar to both adjacent gill bars develop in the ventral region of the gill slits and move upward, and then second synapticles appear

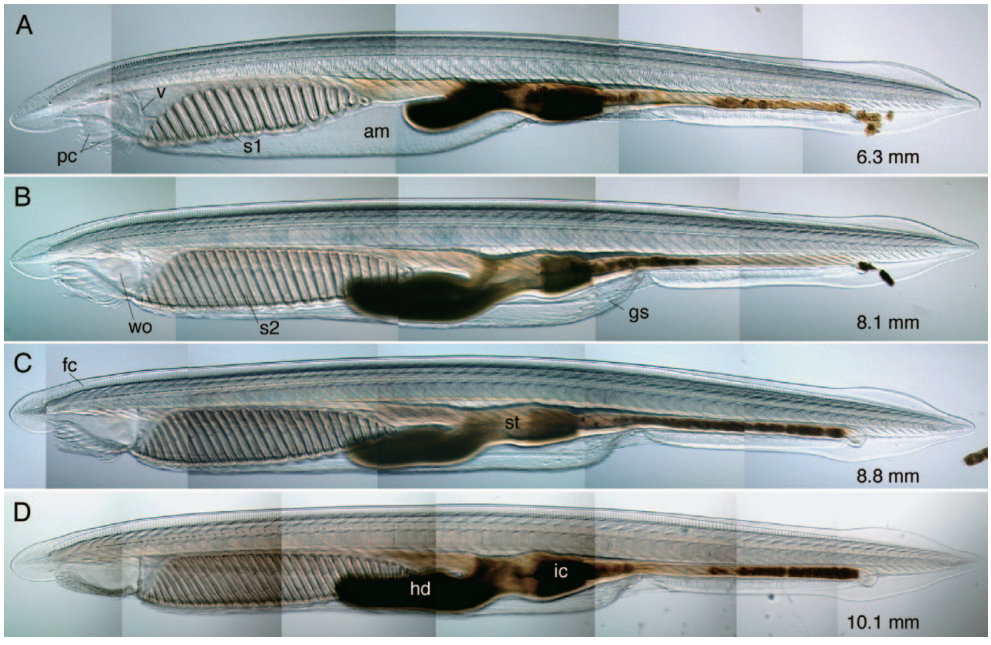

Fig. 7. Left lateral view of juveniles after metamorphosis. All juveniles are shown as the same size. **(A)** An individual just starting the formation of tertiary gill slits and first synapticles (s1) between gill and tongue bars; 70 dpf. **(B)** Second synapticles (s2) have appeared in gills, and glandular stripes (gs) in posterior wall of atrium; 80 dpf. **(C)** 90 dpf. **(D)** Overall appearance becomes similar to that of adult; 100 dpf. Am, atrium; fc, fin-ray chamber; hd, hepatic diverticulum; ic, iliocolonic ring; pc, preoral cirri; st, stomach; wo, wheel organ; v, velum.

ventrally (compare Fig. 7A with 7B). The number of gill slits, now bisected by a tongue bar, increases rapidly during the first 10 days after metamorphosis and reaches 16. Thereafter, a new gill slit adds at the posterior angle of the pharynx about every 10 days.

In accordance with the migration of the primary gill slits onto the left side, the endostyle, which was formerly located vertically on the right wall of the pharynx, has come to lie on the floor and is not visible in lateral view.

The stomach becomes conspicuous as a constriction between the branching of the hepatic diverticulum and iliocolonic ring (Fig. 7C). Pigmentation in the hepatic diverticulum and iliocolonic ring is affected by food ingestion and becomes pale when food is insufficient. The hepatic diverticulum is well developed by 100 dpf, reaching the posterior third of the pharynx, and shows various outlines among individual animals (Fig. 7D). On the internal wall of the atrium near the atriopore, glandular strips (Ruppert, 1997) are developing (Fig. 7B). In the vestibule, the tiny fourth finger of the wheel organ is formed ventrally on the left side in the largest individuals. Kölliker's pit, observed just above the anteriormost pigment spot in the cerebral vesicle on the left side, opens at the level of the first fin-ray chamber. On the wall of the pit, numerous cilia are actively moving.

Modification of the oral region

During short metamorphic period, the lancelet mouth, which first opens on the left lateral body wall, shifts its position almost perpendicular to the body axis, transforming into the velum,. From the first appearance occupying the level of the first myomere, the mouth expands posteriorly and extends from the third to eighth myomere. Posterior expansion of the mouth reaches its maximum from 55 to 60 dpf. An integumental fold limiting the preoral cavity dorsally extends posteriorly and merges with the dorsal margin of the mouth. Around 7 dpf, a line parallel to the ventrolateral margin of the mouth becomes visible; this line is a ciliated band that will become the peripharyngeal band and epipharyngeal groove in the adult anatomy.

When the row of secondary gill slits appears, the anteroposterior diameter of the mouth becomes reduced within a few days. During this transformation, a future right velar tentacle appears on the anterior margin (Fig. 8A). Oral spines are still well developed, projecting from the original margin of the mouth except the anterior vertical margin (Fig. 8A). When secondary gill slits are bisected by a tongue bar, the anteroposterior diameter of the mouth reduces significantly, showing a pointed posterior angle at which the left and right preoral hoods will meet caudally (Fig. 8B, C).

Skeletal rods of the future right preoral cirri are discernible along the ventral margin of the oral region (Fig. 8A, B), and in some individuals they start to project as cirri during the mouth reduction. Curiously, the basal skeleton of the preoral cirri extends posterodorsally, bearing knobs along the posterior margin of the mouth that will become velar tentacles on the left side (Fig. 8D). When the left preoral hood fully covers the vestibular region, it catches up with the right counterpart in development and immediately forms five to six preoral cirri corresponding to those on the right side (Fig. 8E). New preoral cirri are added anteriorly, reaching 10 in number on both sides by 70 dpf, and papillae and intercirral membranes become visible by 80 dpf (Fig. 8E). The number of oral cirri did not change in our observations once 10 cirri had developed.

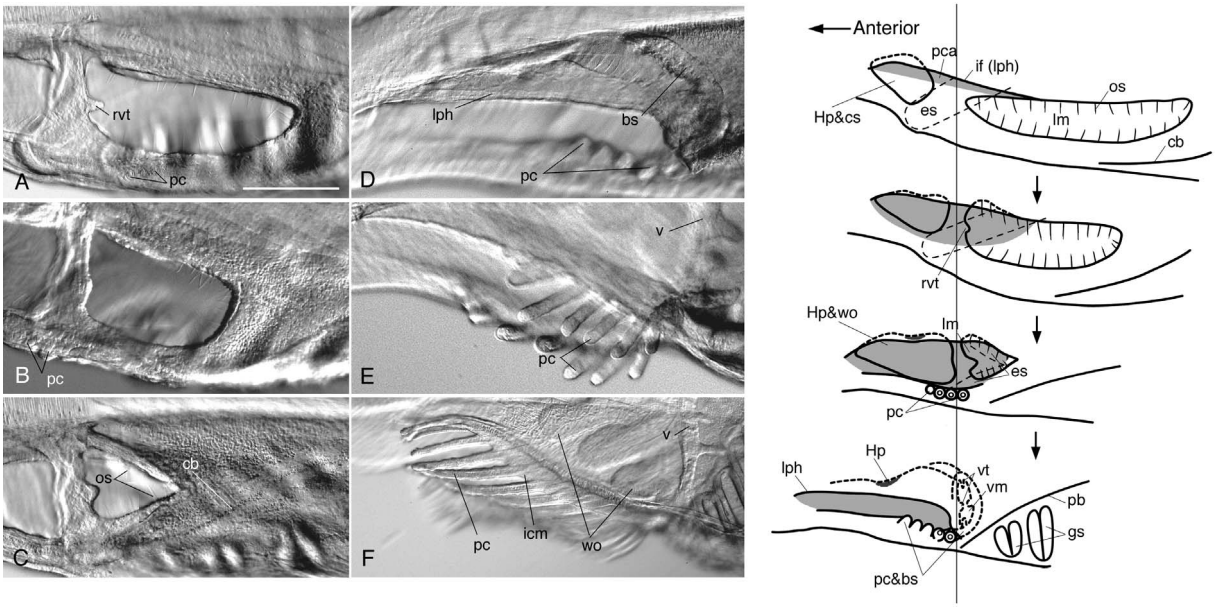

Fig. 8. Transformation of oral region during metamorphosis. Shading in the scheme denotes preoral cavity (pca). A–C, photomontage of oral region, in which integumental fold (future left preoral hood) is out of focus. **(A)** Decrease in the anteroposterior diameter of the mouth by a medial shift of anterior margin soon after formation of secondary gill row. Oral spines, future right velar tentacle (rvt), and initial development of future right preoral cirri (pc) are visible. **(B)** Further reduced mouth. Note anterior shift of future right preoral cirri (rpc). **(C)** Final stage of laterally located mouth. Oral rim still projects many oral spines (os). **(D)** Mouth transforming into velum, which is covered by left preoral hood (lph). Preoral cirri are protruding and posteriorly their basal skeleton (bs) extends along the proximal margin of velum. **(E)** Catch-up growth of left oral hood with preoral cirri in metamorphosed individual. **(F)** Oral region in 80-day larva showing preoral cirri with papillae and intercirral membrane (icm), wheel organ (wo), and velum (v) with thin tentacles. Cb, ciliated band; es, endostyle; gs, gill slit; Hp, Hatschek's pit; if, integumental fold; lm, larval mouth; pb, peripharyngeal band; vm, velar mouth; vt, velar tentacle. Scale bar, 0.1 mm.

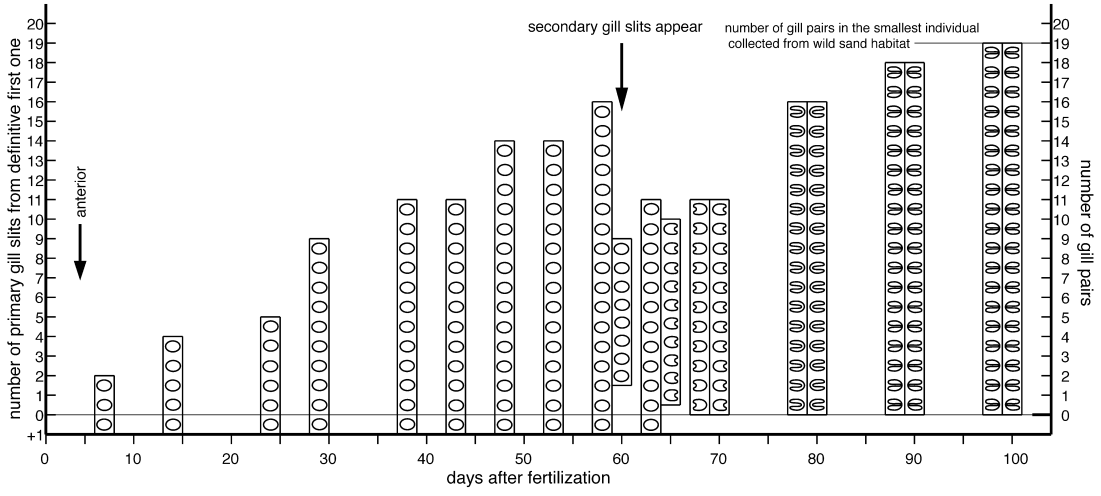

Fig. 9. Typical changes in number of primary and secondary gill slits, and of paired bisected gill slits, observed in the mass culture. Original first gill slit (numbered as +1) in primary gill row disappears, and thus numbering starts from the second. Patterns in columns show schematic shapes representing gill slits.

Gill formation

Gill formation in lancelets is complicated (Willey, 1891). The first gill slits that appear on the right side are called primary gill slits. These will move onto the left side, and the actual right ones, called secondary gill slits, appear dorsally to the primary ones on the right side at the initial stage of metamorphosis. Gill slits added posteriorly after the complete migration of the primary gill row are called tertiary gill slits.

The first primary gill slit becomes open about 40 hours after fertilization, the second one by 2 dpf (Hirakow and Kajita, 1994), and the third by 3 dpf (not shown). Thereafter, formation of the fourth primary slit waits at least until 8 dpf (Fig. 9). Although the precise pattern of how primary slits appear after the forth is unknown, each slit until the tenth seems to form constantly, after which two slits seem to be added periodically. Each newly formed primary slit is first

located in the floor of the pharynx, then gradually faces the right side with dorsal expansion.

The second gill slits, around eight in number, first appear simultaneously when the number of primary gill slits reaches 16 to 18 (Fig. 9). The row of secondary slits lies dorsally to the primary gill row, obliquely relative to the dorsal margin of the primary slits. The initial, anteriormost secondary slit is located at the level between the third and forth primary slits, and soon a new secondary slit is added anteriorly between the second and third primary slits. This sequence is the same as in *B. lanceolatum* (Willey, 1891). Posterior addition of secondary slits also occurs, and one or two new slits appear by the completion of bisection by tongue bars.

Downgrowth of a tongue bar starts earlier in the secondary slits when a new anterior slit is added than in the primary gill slits when these are moving onto the left side. Although it is unknown precisely when tongue bars start to form in primary slits, they do not fuse with the ventral margin of the slit when the primary gill row arrives at its destination. The anteriormost and posteriormost silts of the primary row do not develop tongue bars. When the migration is complete, three to six primary slits disappear depending on the individual; individuals with a large number of primary slits lose more slits. Judging from the secondary slits, those in the primary gill row having no counterparts in the secondary row may disappear. After the primary and secondary gill slits have paired and are bisected completely by tongue bars, tertiary gill slits start to form simultaneously on both sides without pause, unlike Willey's description of *B. lanceolatum* (1891). By 100 dpf, the number of total gill slits on each side is 19, which is the same number as in wild juveniles of equivalent body size.

Atrial chamber

Downgrowth of the metapleur on either side is first visible in the posterior region of the pharynx about 5 to 10 days before the appearance of the secondary gill row and extends posteriorly toward the anterior limit of the iliocolonic ring. Although we did not detect where fusion of the subatrial ridges (Willey and Lankester, 1890) started, prior to the appearance of the secondary gill row, a narrow canal covering a few posterior primary gill slits is formed from the posterior pharyngeal region to the level of the anterior limit of the iliocolonic ring. The anterior end of the canal is usually closed and the posterior end opens as the atriopore. Fusion of the subatrial ridge proceeds anteriorly from the primary anterior limit of the canal and becomes complete when the mouth opening faces anteriorly as the velar mouth. The primary anterior limit of the canal remains for a while as a septum during atrium formation, but in metamorphosed animals the atrium becomes a single chamber, losing the septum. Since all observed individuals formed the septum around the posterior third to forth gill slits, this division of the forming atrium seems to be the normal condition.

The location of the atriopore shifts caudally relative to the alimentary canal from the anterior limit of the iliocolonic ring to the posterior end of the anterior ampulla of the intestine. There occurred several abnormal fusion types of the subatrial ridges, raising multiple chambers, and rarely herniation of the hepatic diverticulum, which caused animal death. Once the atrial chamber has formed, it enlarges rapidly to allow enough room for the developing pharynx and midgut-hepatic diverticulum complex.

Individual variation in larval development

The mass culture in the present study revealed great individual variation in development after the synchronous development during the embryonic and early larval stages. Even at 100 dpf, slowly growing individuals were at the premetamorphic stage, 3 to 4 mm long, while rapidly growing ones reached larger than 10 mm and were adult-like in appearance. Slowly growing individuals frequently died, but many of them were able to undergo metamorphosis with a remarkable delay. Developmental variation was also found between the mass culture and the supplementary tube culture. While metamorphosis started from 60 dpf onwards in the former, when larvae were about 6 mm long with 16–18 primary gill slits, it began at 45 to 50 dpf in the latter, where larvae were 4.9 to 5.4 mm long with 12–13 primary gill slits. Only food density was designed to change between the two culture methods. Larvae in tube culture showed a small mouth (Fig. 4F), which may affect the timing of metamorphosis. We suggest that developmental variation in the glass tank is caused by differences in individual ability to ingest food, or in other words, that animals competed for food. Despite synchronous spawning from mid-June to early July, benthic individuals smaller than 10 mm were found from October to the following May in the Ariake Sea (Henmi and Yamaguchi, 2003). This implies that larvae in the wild also show great individual variation in growth, with the time that each starts benthic life depending on its growth rate. This developmental flexibility during later larval life calls for caution in comparing larval status even between populations of the same species.

Larval development in *B. belcheri* showed flexibility that probably depends on the surrounding environment. This may be the case for other *Branchiostoma* species, because the wild populations of *B. lanceolatum* studied by Willey (1891) and of *B. nigeriense* studied by Webb (1957) also showed individual variation in primary and secondary gill slits at the onset of metamorphosis. *Branchiostoma nigeriense*, especially, changes its growth pattern dramatically depending on its surrounding environment, *e.g*., between brackish Lagos Lagoon and the open sea (Webb, 1957). *Branchiostoma floridae* living in warmer water grows more rapidly than *B. belcheri* in Japan, and thus its pelagic life lasts only 3–4 weeks (Stokes, 1996). Holland and Yu (2004) pointed out that the higher the temperature, the more frequently lancelets spawn. The present study suggests that it is possible not only to control the timing of sexual maturation, but also to obtain any desired developmental stages of lancelet species throughout the year, by controlling artificial conditions. The supplementary data for this article can be found online at "http://dx.doi.org/10.2108/zsj.24.787.s1".

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