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The Development of the Enteropneust Hemichordate *Balanoglossus misakiensis* Kuwano

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ABSTRACT—We describe development from fertilization to metamorphosis of the enteropneust hemichordate *Balanoglossus misakiensis*. This is the first report to describe the complete development of an indirect-developing hemichordate under laboratory conditions. Mature adults were induced to spawn by shifting the temperature of seawater from 23 to 28°C. Eggs (200 µm diameter) were enclosed within a non-mucilaginous membrane, and dispersed readily in seawater. After artificial insemination, a fertilization envelope was elevated from the egg surface beneath the egg membrane; this was followed by the formation of the first and second polar bodies within the envelope. Zygotes cleaved at 20-min intervals to form blastulae, and gastrulation started 9 h after fertilization. Embryos hatched 1 day after fertilization to become typical feeding tornaria larvae. The larvae metamorphosed 7–10 days after fertilization without undergoing the first (Müller) or forth (Krohn) stage of indirect-developing hemichordate development. Larvae that were not fed failed to metamorphose. Juveniles completed adult body formation within a week of settling in sand at the bottom of the culture tube. We discuss heterochronical modifications of *B. misakiensis* development, and make the case for this species as a potential model organism for the investigation of indirect-developing hemichordates.

Key words: hemichordate, acorn worm, indirect development, tornaria, metamorphosis

INTRODUCTION

Recent progress in molecular biology has expanded our knowledge of bilaterian phylogeny, and revealed the genetic programs that regulate the development of their phyletic body plans. Peterson *et al*. (2000) proposed that the latest common ancestor of deuterostomes and protostomes was an indirect developer that had larval stages and developed an adult body via post-larval developmental processes, which included stepwise regulatory mechanisms that produced *Hox* gene-mediated regional specifications. In addition, Peterson *et al*. (2000) suggested that modifications of such post-larval processes resulted in the present diversity of bilaterian adult body plans.

Hemichordates are one of three deuterostome phyla. Recent phylogenetic studies have led to the suggestion that echinoderms and hemichordates are sister groups; such studies have included molecular analyses of 18S ribosomal RNA (Turbeville *et al*., 1994; Wada and Satoh, 1994; Halanych, 1995) and analyses of a unique codon reassignment in mitochondrial translation that is found in only these two groups (Castresana *et al*., 1998). Although typical members of the two phyla exhibit indirect development with dipleurula larva, the development and morphology of the adult organisms are distinct. Metamorphosis of hemichordates is less drastic than that of echinoderms: echinoderms transform from a bilateral larva into a pentamerous juvenile, the structure of which is derived mainly from the left side of the larva; by contrast, hemichordate juveniles inherit the fundamental body axis, as well as most of the body structure, directly from the larva. These observations suggest that hemichordates may retain ancestral features of deuterostomes, not only in their adult morphology, but also in their development.

The development of hemichordates has been less investigated than that of other deuterostomes, particularly the later larval stages of indirect developers; this is largely because it is difficult to collect adults, obtain mature gametes, and culture larvae. Six sequential larval stages have been described for indirect-developing enteropneusts, namely, the Müller, Heider, Metschnikoff, Krohn, Spengel, and Agassiz stages (Hadfield, 1975; from van der Horst

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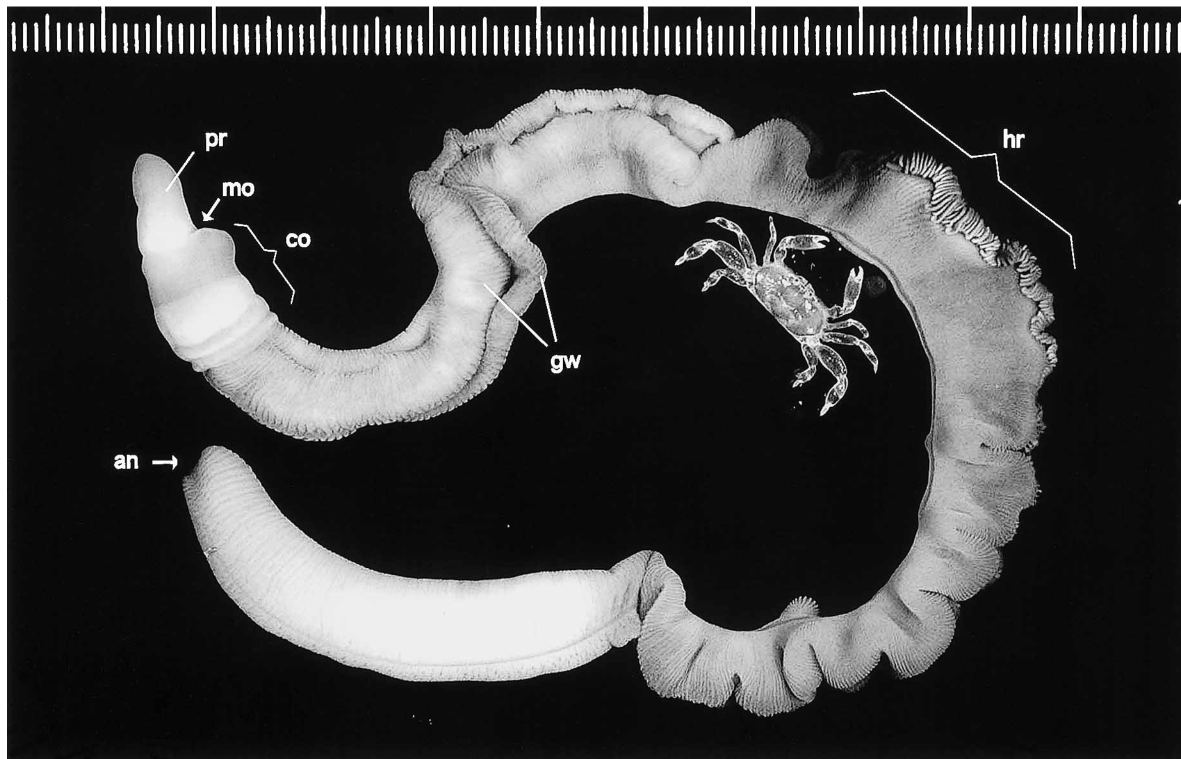

Fig. 1. A living *Balanoglossus misakiensis* adult and the symbiotic crab *Pinnixa balanoglossana*. The scale is marked in millimeter. an, anus; co, collar; gw, genital wing; hr, hepatic region; mo, mouth; pr, proboscis.

1932–1939). Descriptions of later tornaria stages and subsequent metamorphosis have been based on observations of planktonic larvae that have been collected from the ocean. *Balanoglossus clavigerus* is exceptional among indirect-developing hemichordates in that its development has been described from the fertilization to metamorphosis, by Stiasny (1914a, b). Stiasny's study relied on natural spawning, and there has been little progress since in following the complete development of indirect-developing hemichordates.

B. misakiensis (Fig. 1) was presented first by Kuwano in 1902, after which van der Horst (1930) described its adult morphology in detail. Miyashita (1925) described the size and color of the eggs of this species, but its development is unknown. The specimens of *B. misakiensis* were collected near the Misaki Marine Biological Station on the Pacific coast, but these animals have not been collected from the habitat in recent two decades. In 1997, a new population of *B. misakiensis* was discovered along the coast of the Japan Sea (Sakai *et al*., 2001). We used this population to investigate the development of this species. We successfully carried out artificial insemination in the laboratory, and cultured the resultant larvae to the juvenile stage in 2002, exactly one hundred years after Kuwano's presentation. In this paper, we describe the development of *B. misakiensis*. This is the first report of the development of an indirect-developing hemichordate under laboratory conditions, starting from artificial induction of spawning to adult body formation.

MATERIALS AND METHODS

Adult *B. misakiensis* were collected at a depth of 3 m at Masuho-ga-ura beach, Togi, Ishikawa, Japan, in July and August between 1999 and 2002. Specimens were transported in a cooling box to a laboratory at Kanazawa University to prevent their spontaneous spawning. They were held in an aquarium within which seawater was circulated at ~23°C. Mature individuals were induced to spawn by shifting the temperature from 23 to 28°C, and fertilization was carried out artificially.

Embryos were cultured in artificial seawater (Jamarin U, Jamarin Laboratory) at 23–25°C in Petri dishes without agitation. Hatched larvae were transferred to 50 ml polypropylene tubes at a concentration of ~0.5 larvae/ml and were cultured with gentle stirring. After the larvae had established a functional digestive system, they were fed a mixture of single-celled algae, *Chaetocerous gracilis*, *Dunaliella sp.*, *Isochrysis galbana*, and *Pavlova luteri*, all of which were provided by the National Research Institute of Aquaculture, Fisheries Research Agency of Japan. Agassiz-stage larvae were cultured with grains of sand to induce metamorphosis. Routine washing was carried out and the seawater was changed every few days.

RESULTS

Collection and culture

As there was little information about the population of *B. misakiensis* in the area of Masuho-ga-ura beach, we started to collect specimens at various sites along the beach between June and September to estimate both the breeding season and the distribution of the adult organisms. Although specimens were found in sandy bottoms over a relatively

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wide area at a depth of 2–10 m, mature individuals were restricted to a narrow band along the beach at a depth of 3 m in the breeding season (July–August). The tidal range of the Japan Sea is so narrow that mud flats are not formed in the area, and the aforementioned habitat lies in a subtidal zone.

Because *B. misakiensis* had not previously been cultured in a laboratory, we used a simple aquarium with closed circulation. Most specimens lived for more than a year in these conditions, and mature individuals retained their reproductive ability for about 2 months.

Induction of spawning

The genital wing of both male and female *B. misakiensis* developed fully during the breeding season. Therefore, it was possible to determine the sex of individuals based on their appearance: female genital wings were filled with grayish-pink ovaries, whereas those of males contained numerous small whitish-yellow pouches of sperm.

Two reports have described the artificial induction of spawning in hemichordates, both of which described the use of a change in the temperature of seawater. In one report, a temperature change down to 22°C after a 7–8 h preincubation period at 27°C induced *Saccoglossus kowalevskii* to spawn (Colwin and Colwin, 1962); in the other, a temperature change up to ~26°C after at least 6 h of preincubation at 22°C induced *Ptychodera flava* to spawn (Tagawa *et al*., 1998). Based on these observations, we found that changing the water temperature from 23 to 28°C could induce *B. misakiensis* to spawn. All mature *B. misakiensis* spawned within 4 h of the temperature change, and males and females spawned independently.

Eggs were spawned from the dorsal side of the female genital wing, and dispersed readily in seawater without being trapped in the mucus that enveloped the adult (Fig. 2A). Each female released approximately 20,000 eggs, although this number varied among individuals. Eggs were an opaque light pinkish-brown, about 200 μm in diameter, and had a large germinal vesicle (Fig. 2B). Each egg was enclosed within a transparent membrane that comprised two layers: an outer (thin) layer that was removed soon after spawning, and an inner layer that expanded to about 100 μm in thickness and remained in place until hatching (Fig. 2C). Following artificial insemination, the fertilization envelope was elevated very slightly from the egg surface beneath the inner layer of the egg membrane. Thereafter, the first and second polar bodies were extruded into the fertilization envelope, which was indicative of sperm entry during the first meiosis of egg maturation (Fig. 3A).

Early development

The first cleavage was completed ~100 min after fertilization (Fig. 3B). Subsequent cleavages occurred at ~20-min intervals. The first and second cleavages were meridional, whereas the third was latitudinal (relative to the position of the polar body; Fig. 3C, D). The first three cleavages were generally equal, although slightly unequal cleavages were often observed. Cleavage patterns became more variable after the fourth cleavage, after which the blastomeres divided neither equally nor synchronously (Fig. 3E). After several cleavages, the embryo developed into an opaque blastula.

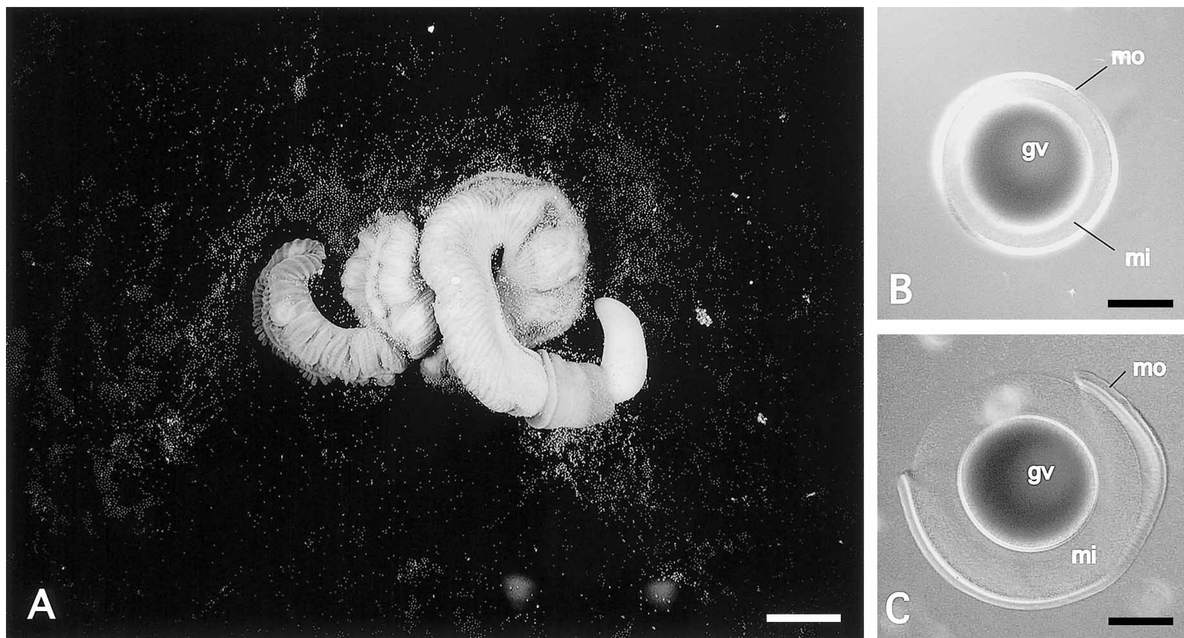

Fig. 2. Eggs of *B. misakiensis*. (**A**) A spawning female. Spawned eggs were dispersed from the mucus that enveloped the adult. (**B**, **C**) Unfertilized eggs with a germinal vesicle (gv). Spawned eggs were enclosed within a two-layered egg membrane. The outer layer (mo) was removed within several minutes of spawning (**B**), while the inner layer (mi) remained and became swollen (**C**). The scale bars represent 1cm in **A** and 100 μm in **B** and **C**.

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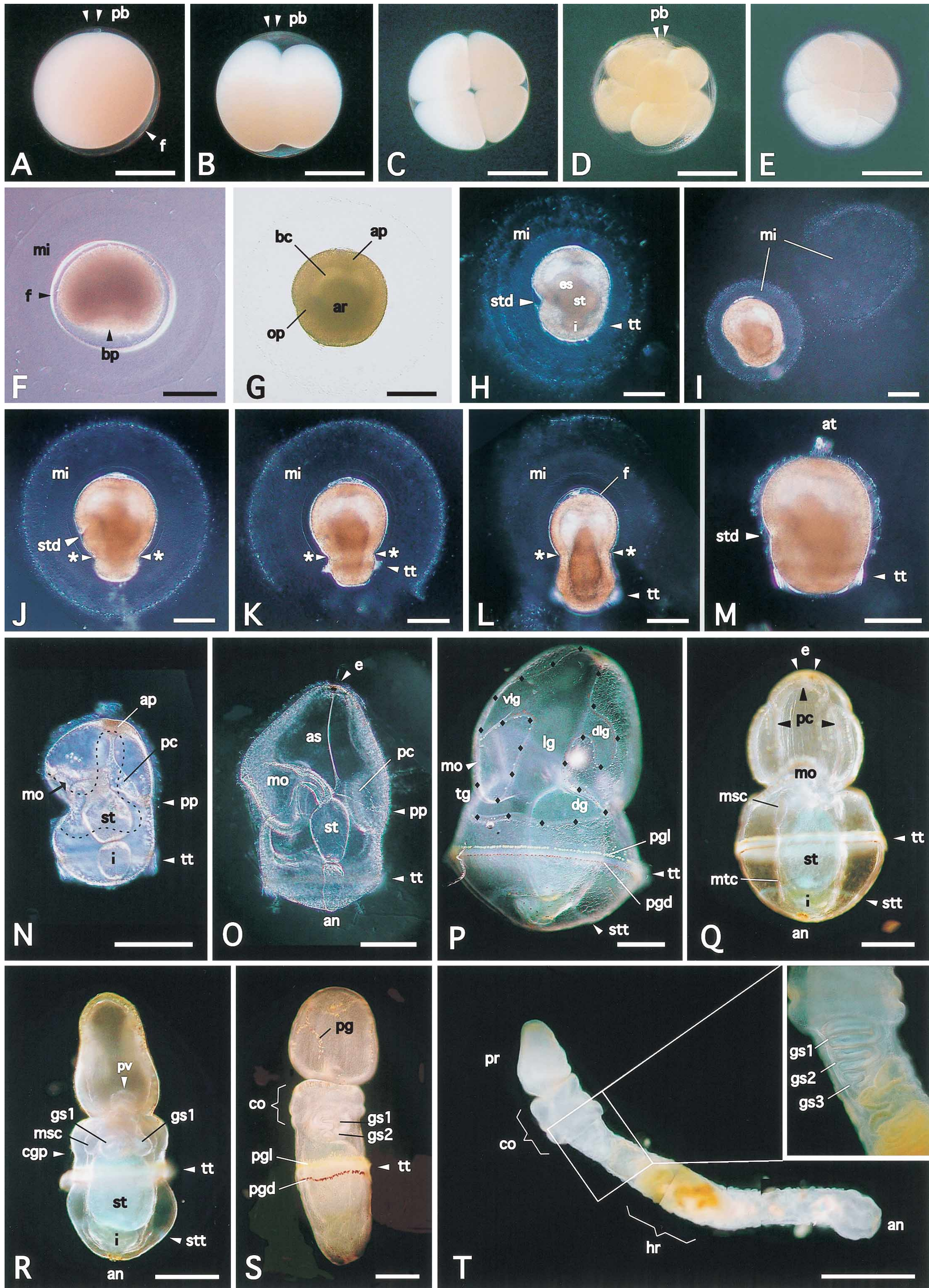

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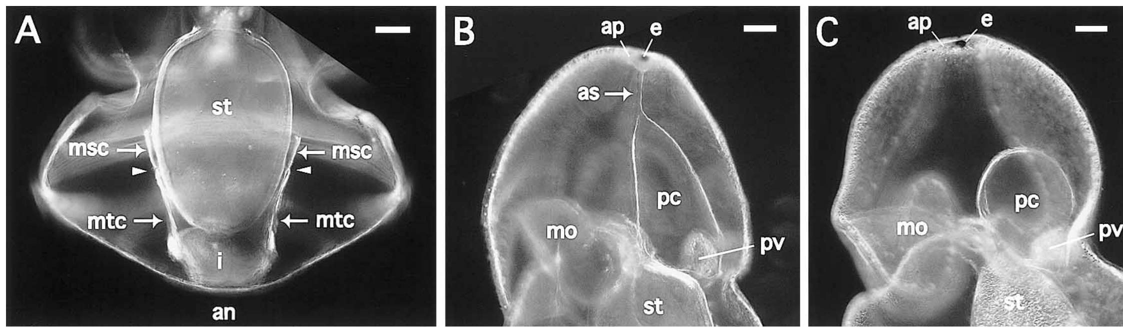

Fig. 4. Formation of adult coeloms in late Metschnikoff-/Spengel-stage larvae. (**A**) Dorsal view of the posterior half. A pair of mesodermal protrusions extended laterally from the intestine, became elongated anteriorly, and were narrowed in the midregion (arrowheads) to form the mesocoel (msc) and metacoel (mtc). (**B, C**) Left lateral view of the anterior half. The apical strand (as) that held the protocoel (pc) to the apical plate (ap) in **B** disappeared (**C**) immediately prior to transformation to the Agassiz stage. Loss of apical strand appeared to allow the protocoel to be reshaped into the proboscis coelom in Agassiz-stage larvae (see Fig. 3**Q**). an, anus; e, eye; i, intestine; mo, mouth; pv, proboscis vesicle; st, stomach. The scale bars represent 100 µm.

Nine hours after fertilization, when the embryo was still opaque, the vegetal side of the blastula became flattened, which indicated that the blastopore had formed (Fig. 3F). The embryo regained a spherical shape during gastrulation, during which the blastopore was closed. At this stage, the embryo began to rotate within the envelope. Nineteen hours after fertilization, the embryo gradually assumed an inverted peach-like shape and became transparent. The blastocoel and archenteron became apparent 21 h after fertilization (Fig. 3G). Portions of the blastowall thickened at the animal pole and the future stomodeum to form the apical and oral plate, respectively. The stomodeum was formed 23–24 h after fertilization, and the archenteron differentiated into a tripartile gut that comprised an esophagus, stomach, and intestine (Fig. 3H). The telotroch was present at this stage, but the longitudinal ciliary band had not yet formed. At this stage, the inner membrane was subdivided into at least four layers. The outermost layers were removed in some (but not all) embryos immediately prior to hatching (Fig. 3H, I).

Hatching

The embryo hatched 1 day after fertilization to become a typical enteropneust larva, *i.e.*, a tornaria (Fig. 3M). Before hatching, embryos were enclosed by the fertilization envelope and by three or four additional outer layers that were derived from the inner egg membrane. A hole in this enclosing structure was formed at the posterior end of the embryo (Fig. 3J, K). The embryo swam spirally in a posterior direction, by means of beating cilia on the telotroch. It then transformed by becoming elongated (stretched), and escaped the enclosing structure via the aforementioned hole (Fig. 3K, L). The hatched larva returned to the same shape that it had been immediately prior to hatching (Fig. 3H), and swam anteriorly (Fig. 3M).

Progressive larval development

One and a half days after fertilization, the larva developed a longitudinal ciliary band in addition to the telotroch (Fig. 3N), which characterizes the Heider stage. The protocoel (future probosis coelom) and a proboscis pore that

Fig. 3. Development of *B. misakiensis* from fertilization to metamorphosis in artificial seawater at 25°C. The scale bars represent 100 µm in **A–M**, 250 µm in **N–S**, and 1 mm in **T**. (**A**) Fertilized egg with two polar bodies (pb) within the fertilization envelope (f). (**B–E**) Two-, 4-, 8-, and 16-cell stage embryos at 100, 120, 140, and 160 min after fertilization, respectively. (**F**) Early gastrula 9 h after fertilization with a blastopore (bp). (**G**) Left lateral view of 21-h-old gastrula with a blastocoel (bc) and archenteron (ar). ap, apical plate; op, oral plate. (**H**, **I**) Embryo prior to hatching with a tripartile gut, esophagus (es), stomach (st), and intestine (i), as well as a stomodeum (std). The telotroch (tt) was present, but the longitudinal ciliary band had not yet formed. The inner egg membrane (mi) that enveloped surrounding embryos was subdivided into several layers (**H**), the outer layers of which were removed before hatching (**I**). (**J–M**) Hatching, 1 day after fertilization. **J**, **K**, and **M** are left lateral views; **L** is a dorsal view. Asterisks indicate an opening of the fertilization envelope. After hatching (**M**), embryos returned to the shape they had been immediately prior to hatching (**H**). at, apical tuft. (**N**) Left lateral view of a Heider-stage larva 2 days after fertilization. The dotted line indicates the longitudinal ciliary band. mo, mouth; pc, protocoel; pp, proboscis pore. (**O**) Left lateral view of an early Metschnikoff-stage larva 3 days after fertilization. an, anus; as, apical strand; e, eye. (**P**) Left lateral view of a late Metschnikoff-/Spengel-stage larva 5 days after fertilization. The longitudinal ciliary band (◆) and grooves that led to the mouth formed primary lobes and saddles. dg, dorsal groove; dlg, dorsolateral groove; lg, lateral groove; tg, transverse groove; vlg, ventrolateral groove. Dark (pgd) and light (pgl) pigment cells were present in a line along the longitudinal ciliary band and telotroch, but not the secondary telotroch (stt). (**Q**) Ventral view of an early Agassiz-stage larva about 7 days after fertilization. Black arrowheads indicate the expansion of the protocoel to form the coelom of the proboscis. A pair of mesocoel (msc) and metacoel (mtc) had been formed. (**R**) Dorsal view of a late Agassiz-stage larva about 7.5 days after fertilization. A posterior collar groove (cgp) became distinguishable. gs1, first gill slit; pv, proboscis vesicle. (**S**) Left lateral view of a juvenile with two gills, approximately 1 day after it had settled into the sand at the bottom of the culture tube. The telotroch was still flanked by dark and light pigment cells, while the longitudinal ciliary band had been lost, although residual pigment cells (pg) were visible. co, collar; gs2, second gill slit. (**T**) Right lateral view of a juvenile with three gills, approximately 8 days after settlement. gs3, third gill slit; hr, hepatic region; pr, proboscis.

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opened on the dorsal midline were also evident. The mouth was open, but the anus was still closed. The anus opened in 1 day after hatching (2 days after fertilization), at which time the larva started to feed.

The larva developed a primary lobe and saddle 3 days after fertilization, and was approximately 950 μm in length and 550 μm wide at this time; this is characteristic of the Metschnikoff stage (Fig. 3O). Subsequently, the larva gradually increased in size without any obvious changes in morphology. Adult structures became evident in the 5-day-old larva (1.5 mm in length and 1.1 mm wide; Fig. 3P), including the mesocoel (future collar coelom), metacoel (future trunk coelom), and proboscis vesicle (future heart), as shown in Fig. 4. The coelom arose from the intestine as a pair of protrusions, which became elongated anteriorly and constricted in the middle to separate into the anterior mesocoel and posterior metacoel (Fig. 4A). The coelom formation in *B. misakiensis* was similar to that in *B. clavigerus* (Stiasny, 1914a) and *Glandiceps stiasnyi* (Rao, 1953). The secondary telotroch was evident by this stage (Fig. 3P). Two types of pigment cells, light and dark, appeared in the ectoderm, and these cells flanked the longitudinal ciliary band as well as the telotroch, but did not the secondary telotroch (Fig. 3P). A few dark pigment cells were also detected in the esophagus and around the anus. The larva proceeded to the Agassiz stage without forming a secondary lobe or a saddle, which are characteristic of the Krohn stage; this suggests that this stage may be regarded as the Spengel stage, not the Metschnikoff stage (see Discussion).

Regressive larval development to metamorphosis

Metamorphosis occurred 7–10 days after fertilization. The putative Spengel-stage larva (see above) lost the apical strand that held the protocoel to the apical plate (Fig. 4B, C). The loss of the apical strand appeared to trigger progression of the larva into the Agassiz stage (Fig. 3Q), which was completed within a few hours. During this transition period, the preoral region decreased in size and was reshaped into a probosis-like structure. The eyes and the longitudinal ciliary band degenerated, while the protocoel expanded into the coelom of the probosis. A pair of rudimentary gills appeared, although a groove between the color and trunk was obscure. The Agassiz-stage larva continued to swim by means of the teletroch, and the larvae often sank to the bottom of the culture tube.

When grains of sand were added to the culture tube, the Agassiz-stage larva transformed into a burrowing juvenile within a few minutes (Fig. 3R). The proboscis-like structure flattened and began to move in a peristaltic manner, while the trunk region decreased in width. The juvenile secreted mucus and burrowed (settled) in the sand. As the trunk became elongated, a groove that separated the collar and trunk became apparent. The telotroch, which was the last remaining larval structure, remained even after the formation of a second gill slit (Fig. 3S). Formation of the adult body was completed within 1 week of settlement. The juvenile was approximately 4 mm long with three pairs of gill slits and a distinctive hepatic region; it swallowed grains of sand within a nest at the bottom of the tube (Fig. 3T).

Development of unfed larvae

To examine the larval potential for facultative feeding, we cultured 55 *B. misakiensis* larvae without feeding them. Although 60% of these unfed larvae lived for more than 2 months, they remained at the early Metschnikoff stage and failed to undergo metamorphosis: no unfed larvae formed any adult structures, including the mesocoel, metacoel and the proboscis vesicle.

DISCUSSION

Appearance and release of eggs

The size and color of the eggs (as well as the breeding season of *B. misakiensis*) were largely consistent with previous descriptions (Kuwano, 1902; Miyashita, 1925). Each egg was enclosed in a two-layered non-mucilaginous membrane, the outer layer of which was removed after spawning. Although the egg membrane resembled that of *B. clavigerus* (Stiasny, 1914a), it was different to that of *S. kowalevskii* (Colwin and Colwin, 1953) and *P. flava* (Tagawa *et al.*, 1998), both of which have a viscous jelly coating. Adult enteropneusts secrete mucus mainly from the anterior portion, and this mucous envelopes the whole body. When spawned, the eggs of *P. flava* are retained within mucus for 20–30 min (Hadfield, 1975), whereas those of *B. misakiensis* were dispersed immediately from the mucus. This feature of *B. misakiensis* egg release is probably due to the nature of the egg membrane, and it allowed us to collect and handle eggs with ease.

Absence of the Müller stage

After hatching, most indirect-developing enteropneusts pass through the Müller developmental stage, which is characterized by the presence of a longitudinal ciliary band and the absence of a telotroch (which develops subsequently). Miyashita (1925), however, has described the swimming larvae of an unidentified enteropneust in which the formation of a telotroch preceded the development of a longitudinal ciliary band. Stiasny (1928) designated such a larval stage as the "Miyashita stage". *B. misakiensis* exhibited the Miyashita stage in its development, and therefore failed to exhibit the characteristics of the Müller stage. However, the Miyashita stage in *B. misakiensis* was observed not in larvae but, rather, in embryos prior to hatching: upon hatching, the larvae utilized the telotroch to escape the egg membrane, as shown in Fig. 3J–L. These observations suggest that the timing of hatching, as well as the formation of the longitudinal band and telotroch, may vary among enteropneust species.

Absence of the Krohn stage

Six sequential larval stages have been described in the

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indirect-developing tornaria, namely, the Müller, Heider, Metchnikoff, Krohn, Spengel, and Agassiz stages. The appearance of young larvae (prior to the Metschnikoff stage) occurs in a similar fashion among tornaria species, whereas larvae in the subsequent Krohn stage exhibit species-specific characters. Differences among species are due largely to the structure of the secondary lobe and the saddle that defines this stage of development. Specifically, the longitudinal ciliary band of *P. flava* elongates into sinuous loops, which ultimately develop into tentacles (Hadfield, 1975), while the longitudinal ciliary band of *B. clavigerus* forms only simple secondary lobes and saddles (Stiasny, 1914b). By contrast, *B. misakiensis* larvae proceeded to the Agassiz stage without forming a secondary lobe or saddle.

In many feeding larvae, the ciliary band functions as a swimming apparatus and a feeding filter by which the animal captures food particles. The transformation of the ciliary band into a long sinuous band in tornaria larvae increases the area of the feeding apparatus and results in the formation of the secondary lobe and saddle. Tornaria, in particular, use the longitudinal ciliary band only for feeding, because these animals have a specialized band of cilia, the telotroch, that is used for locomotion (Strathmann and Bonar 1976). Diversity in the characteristics of the Krohn stage may be associated with differences in the feeding requirements of the various species, because the secondary lobe and saddle of tornaria are the most adaptive features for feeding. This argument is further supported by the fact that larvae in the Metschnikoff and Spengel stages, which flank the Krohn stage, differ in size but otherwise have a similar shape and possess a simple primary lobe. Therefore, the secondary lobe may be a feature that was derived during the evolution of enteropneust larvae.

It has been proposed that transitions within lineages of marine invertebrates, from indirect development with feeding larvae to direct development with non-feeding larvae, evolved frequently and independently (Strathmann, 1978). Whether a species undergoes indirect or direct development is apparently correlated with egg size (Raff *et al*., 1988): species with smaller eggs tend to develop indirectly, whereas those with larger eggs undergo direct development. In sea urchins, the critical egg size is estimated to be 300 μm in diameter. Interestingly, *Clypeaster rosaceus*, which has the largest egg among indirect developers (280 μm in diameter), produces facultatively feeding larvae (Emlet, 1986). In enteropneusts, the eggs of direct developers are 250 μm in diameter or larger (Burdon-Jones, 1952), except for *Harrimania planktophilus*, which produces smaller eggs (75 μm in diameter; Cameron, 2002). The eggs of *B. misakiensis* are the largest that have been described to date for indirect developers (Nishikawa, 1986). As we showed in this paper, *B. misakiensis* larvae are not facultative feeders, as they required feeding to metamorphose. In the case of *P. flava*, which produces eggs that are 100 μm in diameter, it takes about 6 months for larvae to metamorphose, during which the Krohn-stage is estimated to last at least 3 months (Tagawa, personal communication). Therefore, the relatively large egg size of *B. misakiensis* may allow that the Krohn stage is absent from the development of this species, and probably explains the exceptionally short (7–10 day) larval period.

B. misakiensis as a model organism

As mentioned in the Introduction, relatively few species of enteropneust have been investigated, despite their interesting phylogenetic and evolutionary position. Recently, Lowe *et al.* (2003) examined the direct-developing enteropneust, *S. kowalevskii*, and reported the expression pattern of orthologous genes that are involved in anteroposterior patterning of the chordate central nervous system. They showed that the gene expression domains of hemichordates and chordates are surprisingly conserved, an observation that shed new light on the evolution of the central nervous system and the phyletic placement of chordates. In echinoderms, Nakano *et al.* (2003) described the development of the sea lily, *Metacrinus rotundus*, and reported that this most basal echinoderm develops via a dipleurula-type larva. This observation strongly suggests that the latest common ancestor of echinoderms and hemichordates may be an indirect developer with dipleurula-type larvae, and Nakano *et al.* (2003) have suggested that the common ancestor of deuterostomes (including chordates) may be an indirect developer.

Hemichordates have been regarded as a group that are crucial to understanding the evolution of deuterostomes, because their development and adult morphology retain the ancestral features of deuterostomes. *P. flava* has been used as a model to examine the developmental features of indirect-developing hemichordates (Tagawa *et al*., 1998, 2001; Ogasawara *et al*., 1999; Henry *et al*., 2001; Harada *et al*., 2002). These pioneering works have provided new insights on long-lasting debates by the molecular analysis. However, the relatively long larval period of *P. flava* (greater than 6 months) has made it difficult to follow the complete development of this species, particularly the later larval stages and metamorphosis, in the laboratory. By contrast, we believe that the relatively brief larval period of *B. misakiensis* makes this species a potentially valuable model of indirect developers: studies of the development of *B. misakiensis* would further our understanding of the evolution of deuterostomes, and would illuminate the diversification of bilaterians. In addition, the use of *B. misakiensis* as a model organism would promote the preservation of the habitat of this organism.

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