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Development of ciliary bands in larvae of the living isocrinid sea lily *Metacrinus rotundus*

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Abstract

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Embryos and larvae of an isocrinid sea lily, *Metacrinus rotundus*, are described by scanning electron microscopy. Around hatching (35 h after fertilization), the outer surface of the gastrula becomes ubiquitously covered with short cilia. At 40 h, the hatched swimming embryo develops a cilia-free zone of ectoderm on the ventral side. By 3 days, the very early dipleurula larva develops a cilia-free zone ventrally, densely ciliated regions laterally, and a sparsely ciliated region dorsally. At this stage, the posterior and anterior ciliary bands first appear: the former runs along a low ridge separating the densely from the sparsely ciliated epidermal regions, while the latter is visible, at first discontinuously, along the boundary between the densely ciliated lateral regions and the cilia-free ventral zone. In the late dipleurula larva (5 days after fertilization), the anterior and posterior loops of ciliary bands are well defined. The transition from the dipleurula to the semidoliolaria larva occurs at 6 days as the posterior loop becomes rearranged to form incompletely circumferential ciliary bands. The larva becomes competent to settle at this stage. The arrangement of the ciliary bands on the semidoliolaria is maintained during the second week of development, while the larva retains its competence to settle. The larval ciliary patterns described here are compared with those of stalkless crinoids and eleutherozoan echinoderms. The closest morphological similarities are between *M. rotundus* and the basal eleutherozoan class Asteroidea.

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Introduction

The larvae of marine invertebrates generally have ciliary band (s) comprising densely packed cilia. Echinoderms have two typical larval forms, the dipleurula and doliolaria types, each of which has characteristic ciliary band patterns. The dipleurula-type larva has a set of two ciliary bands: an anterior loop encircling the frontal field and a posterior loop surrounding dorsal area and the anal field (Dan 1968; Nakano *et al.* 2003). Occasionally, two bands form a single, continuous band (Sewell and McEuen 2002). The doliolaria-type larva has 3–5 circumferential rings of ciliary bands (Sewell and McEuen 2002). The number of these rings depends on the species.

These two types of ciliary bands in echinoderm larvae show two modes of development, which vary in each of the five echinoderm classes. In the larvae of three of these classes, the Crinoidea, Ophiuroidea and Holothuroidea, dipleurula-type ciliary bands are formed first, followed by the doliolaria-type bands (Mortensen 1921, 1937, 1938; Mladenov 1985; Sewell and McEuen 2002; Nakano *et al.* 2003). The larvae of feather stars, the stalkless crinoids, seem to have only doliolaria-type ciliary bands, probably due to loss of most (Lacalli and West 1986) or all stages involving dipleurula-type bands during the course of evolution. The other mode found in the larvae of the remaining two classes, the Asteroidea and Echinoidea, involves formation of only the dipleurula-type ciliary band (Dan 1968). The former mode is considered to be the ancestral one (Nakano *et al.* 2003).

Most previous studies of the ciliary bands of doliolaria larvae have focused on holothurians and feather stars. Such studies have concentrated mainly on the process of transition of ciliary bands from the dipleurula to the doliolaria type (Lacalli and West 1986, 2000; Lacalli 1993; Nielsen 2012), the relationship between the ciliary bands and neurogenesis (Lacalli *et al.* 1990; Bishop and Burke 2007; Nakano *et al.* 2009) and the effects of the endo-mesoderm on formation of the ciliary band patterns (Lacalli and West 1987). However, the process of ciliary band formation from the early ciliated stage to the doliolaria stage has not yet been described in detail.

The importance of clarifying the ontogeny of sea lilies, the most basal group of the living echinoderms (Janies 2001; Rouse *et al.* 2013), has long been recognized (Breimer 1978; Holland 1991; Nielsen 2012). Nakano *et al.* (2003) were the first to describe the development of a species of living isocrinid sea lily, *Metacrinus rotundus,* from fertilization to larval settlement. That work, although accurate, determined the position of the ciliary bands mainly from pigment patterns on the larval surface. In addition, only the ciliary bands after the dipleurula stage were described, and thus the preceding stages, such as the beginning of band formation, remain to be examined. Because the developmental anatomy of sea lilies is so consequential for evolutionary discussions, our purpose here is to describe the entire process of ciliary band development from the prehatching stage to the doliolaria stage based on detailed scanning electron microscopy (SEM) and to discuss the phylogenetic relationships among the five echinoderm classes based on the ciliary band patterns.

Materials and Methods

Laboratory culture of embryos and larvae

Adult males and females of *Metacrinus rotundus* Carpenter were collected using a gill net from a depth of 130 m in Sagami Sea (more specifically, the southeastern area of Uraga Channel), Japan, from late September to mid October. Sexes could be distinguished from the color of the ripe gonads visible through the body wall of the genital pinnules: Females had light orange ovaries, and males had whitish testes. After transport to the nearby Misaki Marine Biological Station or Marine and Coastal Research Center of Ochanomizu University, the sea lilies were maintained in sea water in the dark at 14 °C or, when unavoidable, briefly under dim light conditions. On the day of collection, a fragment of an arm was dissected from each animal, and the pinnules in the arm were examined under a dissection microscope to check gonad maturation. Animals with mature ovaries were placed individually in large beakers. If the ripe females spawned in the laboratory, they invariably did so spontaneously in the evening of the date of collection. Some of the females that failed to spawn that day were kept in an aquarium and fed ground commercial fish food. About 1 month later, each female was removed from the aquarium and transferred to a small container which was then transported by road on a cart for approximately 500 m at a slow speed to gently agitate the water for 10 min. Some of the females spawned fertilizable eggs about 10 h after the agitation. Between 2006 and 2009, we collected a total of 33 females, among which 16 spawned fertilizable eggs. The embryos that were derived from four of these 16 females were used in this study. Sperm that had been dissected from the ripe testes of males were suspended in 5 mL of Millipore-filtered sea water maintained at 14 °C in 10-mL test tubes and used to fertilize the eggs in 10-L plastic beakers. Nonfertilizing sperm were washed away from the fertilized eggs by thorough changes of the filtered seawater. The cultures were then maintained in a dark incubator at 14 °C. To prevent bacterial infection, which was a problem between hatching and settlement, we cleaned the cultures by replacing the filtered seawater every few days.

Preparation of embryos and larvae for SEM observation

Embryos and larvae were fixed overnight in seawater-Bouin's fluid, dehydrated in a graded ethanol series, and critical point-dried in a CP-5A (Topcon, Tokyo, Japan) or JCPD-5 (JEOL, Tokyo, Japan) CO_2 dryer. The dried specimens were attached to stubs via double-sided adhesive tape. Occasionally, the specimens of embryos before hatching were rolled on the tape with a needle to remove the fertilization envelope. The specimens were coated with gold in an E-101 ion sputter coater (Hitachi, Tokyo, Japan) and observed with an S-3000N (Hitachi, Tokyo, Japan) or JSM-6510VL (JEOL, Tokyo, Japan) SEM. The combination of Bouin's fixation, critical point drying and SEM clearly showed the arrangement of the larval ciliated bands.

Results

Early stages of ciliary band formation

It has been reported that *Metacrinus rotundus* embryos gastrulate in the fertilization envelope and begin hatching at about 38 h after fertilization (Nakano *et al.* 2003). Embryos that are spherical in shape with a blastopore at the posterior end begin to rotate just before hatching. The embryos are almost entirely ciliated, although the cilia are still short and sparse (Fig. 1A). After hatching, embryos elongate along the anterior-posterior axis to develop into the oval-shaped predipleurula stage (Fig. 1B). The embryos are not uniformly ciliated, and a cilia-free zone is present in a limited area. The outer surface of the embryo, except for this cilia-free zone, is covered with well-developed cilia. The boundary between the cilia-free and ciliated zones is quite clear (Fig. 1C). The cilia-free zone then expands until it covers almost one-third of the entire embryo

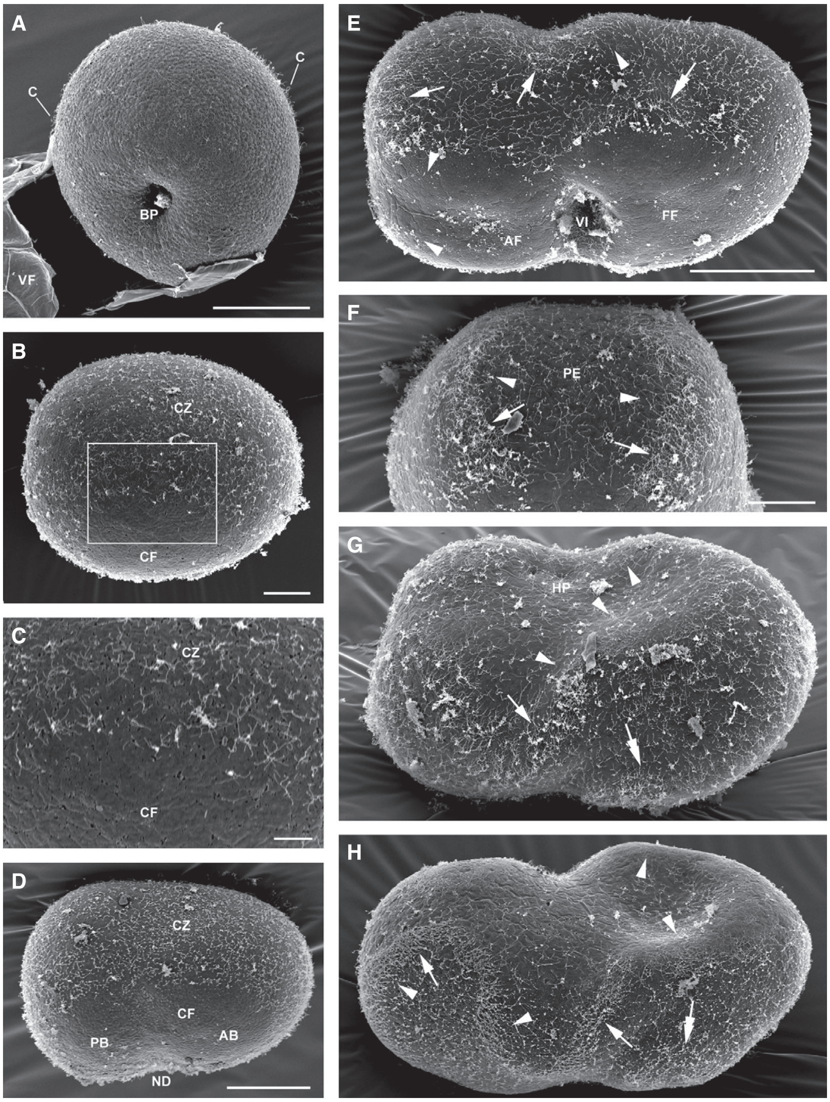

Fig. 1—Scanning electron microscopy of the early development stages of the ciliary bands in the embryos and larvae of *M. rotundus*. —**A**. An embryo 35 h after fertilization just before hatching. The fertilization envelope has been removed as described in Materials and Methods. The vestigial fertilization envelope remains in the bottom left-hand corner of the picture. —**B**. An embryo at the predipleurula swimming stage 40 h after fertilization soon after hatching. —**C**. Enlargement of the rectangle in **B**, showing the boundary between the ciliated zone and cilia-free zone. —**D**. An embryo at the predipleurula swimming stage 2 days after fertilization. —**E**. A larva at the very early dipleurula stage 3 days after fertilization viewed from the right-ventral side. Arrows and arrowheads in **E**–**H** show the ciliary bands and a continuous low ridge, respectively. Tandem arrows in **E**, **G,** and **H** show fragment of ciliary band formed at the boundary between the densely ciliated lateral areas and the cilia-free zone. —**F**. A part around the posterior end of the same larva shown in **E** viewed from the posterior-ventral side. —**G**. A larva 3 days after fertilization viewed from the right-dorsal side. This specimen is a different individual from that in **E**. —**H**. A larva at the early dipleurula stage 3.5 days after fertilization viewed from the right-dorsal side. Scale line in **A**, **D,** and **E** (applicable also to **G** and **H**) = 100 μm. Scale line in **B** and **F** = 50 μm. Scale line in **C** = 20 μm. AB, anterior bulge; AF, anal field; BP, blastopore; C, cilia; CF, cilia-free zone; CZ, ciliated zone; FF, frontal field; HP, hydropore; ND, narrow dent; PB, posterior bulge; VF, vestigial fertilization envelope; PE, posterior end; VI, vestibular invagination.

surface about 2 days after fertilization (Fig. 1D). Two bulges appear in this zone and are separated by a narrow dent running sideways. One bulge occupies the anterior half and the other the posterior half of the embryo. These bulges seem to be the first sign of the frontal and anal fields on the ventral side. The timing of blastopore closure is variable. In embryos just before hatching, the opening of the blastopore is evident, as shown in Fig. 1A. The blastopore begins to close soon after hatching, but the pore is visible in some embryos as late as 10 h after hatching. In the very early dipleurula larva about 3 days after fertilization, a vestibular invagination appears in the median region along the narrow dent in the cilia-free zone

(Fig. 1E). The dorsal area is sparsely ciliated relative to the lateral areas (Fig. 1G). The first signs of ciliary bands and a continuous low ridge become recognizable at this stage (Fig. 1E–G). The low ridge is formed along the boundary between the densely ciliated lateral areas and the sparsely ciliated dorsal area, and extends to the anal fields on the ventral side. The ciliary bands are associated with the low ridge, although the bands have not yet appeared in the anal field. Fragments of the ciliary band are formed in the anterior half of the larva at the boundary between the densely ciliated lateral areas and the cilia-free zone (Fig. 1E, G, H). The hydropore opens on the left dorsal surface, and scattered darkly pigmented cells appear in the epidermis by this stage. The ciliary bands formed along the low ridge become more conspicuous in the larva about 3.5 days after fertilization (Fig. 1H).

Typical dipleurula larval stage

As the swimming larvae get older, their epidermal pigment cells progressively darken to a deep black and become distributed more densely in the posterior than in the anterior half. By 5 days, the larva has reached the later dipleurula stage (Fig. 2A–I), which resembles the later bipinnaria larva of a starfish described by Lacalli *et al.* (1990). There are two separate ciliary bands on the surface of the sea lily larva at this stage—a relatively small anterior loop and a larger posterior loop (Fig. 2A, B, AL and PL, respectively). The clover leaf-shaped anterior loop surrounds the frontal field on the ventral side, and the posterior loop surrounds the posterior three-fourths of the dorsal area and the anal field on the ventral side. Five pairs of projecting ridges develop along the ciliary bands (Fig. 2A–F) which have initially appeared along the low ridges at the very early dipleurula stage (Fig. 1E–G). These are the ventral anterior (*va*), dorsal anterior (*da*), dorsal posterior (*dp*), lateral posterior (*lp*) and ventral posterior (*vp*) ridges (Fig. 2B). Elsewhere on the surface of the larva, cilia are sparsely distributed, except in three regions of relatively dense ciliation (namely, the ciliary tuft, the adhesive concavity, and the vestibular invagination). The hydropore opens to the outside, the blastopore has closed, and the vestibular invagination remains shallow. In the dipleurula larva, the adhesive concavity and adhesive pit are conspicuous, although the larva is not competent yet for settlement. The dipleurula larva of *M. rotundus* does not feed.

Semidoliolaria larval stage

By 6 days, the larva has become a semidoliolaria (Fig. 3A–I), which also does not feed. The arrangement of the anterior loop of the ciliary band is similar to that at the preceding dipleurula stage, but the posterior loop has become rearranged into segments that partially or completely encircle the body. Thus, the dorsal and ventral views of the larva show doliolaria characteristics, with appearance of transverse ciliary bands. However, in lateral view, the ciliary bands retain some features of the dipleurula-type stage, as shown for some feather star species (Holland and Kubota 1975; Lacalli and West 1986).

The hydropore remains open to the exterior, the position of the closed blastopore is still recognizable as a small ciliated area, the ciliary tuft has become indistinct, and the vestibular invagination, which is now more heavily ciliated, is still relatively shallow. The low ridges that were conspicuous in the dipleurula larvae are no longer clear. By this stage, scattered mucus cells (illustrated by Nakano *et al.* 2009) can be detected in the epidermis, and the larva becomes competent for settlement. If settlement was not induced, however, the semidoliolaria larvae continued swimming for another week—their overall dimensions slightly decreased. The ciliary band pattern does not change during this period, essentially the same semidoliolaria-like pattern being maintained.

Discussion

The asteroid bipinnaria has two separated ciliary bands (Lacalli *et al.* 1990; McEdward *et al.* 2002). One is the preoral band encircling the pre-oral lobe and the frontal field in a clover leaflike shape. The other is the postoral band surrounding most of the dorsal area and the anal field (Fig. 4). Five pairs of conspicuous ridges (arms) are evident in the bipinnaria along the ciliary bands. These are the anterior-dorsal (*ad*), posterior-dorsal (*pd*), posterior-lateral (*pl*), postoral (*po*) and pre-oral (*pr*) ridges (Lacalli 1993; McEdward *et al.* 2002), which appear to correspond to the dorsal anterior (*da*), dorsal posterior (*dp*), lateral posterior (*lp*), ventral posterior (*vp*) and ventral anterior (*va*) ridges in the *M. rotundus* larva (Fig. 2B), respectively. The holothurian auricularia is similar to the asteroid bipinnaria in external morphology, but has some distinctive features (Fig. 4). The most remarkable morphological difference is that the ciliary band in the auricularia is single and continuous (Dan 1968; Smiley *et al.* 1991; Sewell and McEuen 2002). The auricularia has six pairs of ridges, the *ad*, *md* (mid-dorsal), *pd*, *pl*, *po*, and *pr*, along the ciliary band, in which the bipinnaria lacks the pair of *md* ridges (Lacalli 1993; McEdward *et al.* 2002; Sewell and McEuen 2002). The plutei of echinoids and ophiuroids share these two external characteristics – a continuous ciliary band and six pairs of ridges – with the holothurian auriculariae (Dan 1968; Sewell and McEuen 2002).

The dipleurula-type larva of the isocrinid sea lily *M. rotundus* has two separated ciliary bands, the relatively small anterior loop with clover leaf shape and the larger posterior loop, thus resembling an asteroid bipinnaria. Moreover, only five, not six, pairs of ridges are identified along the ciliary bands of the *M. rotundus* larva, which also supports their similarity to those of asteroids.

Based on these findings, we speculate that two separated ciliary bands, the anterior and posterior loops, and five pairs of ridges along the ciliary bands are the ancestral condition for the echinoderm larval form and are inherited by the dipleurula-type larvae of crinoids and asteroids. We consider that, as

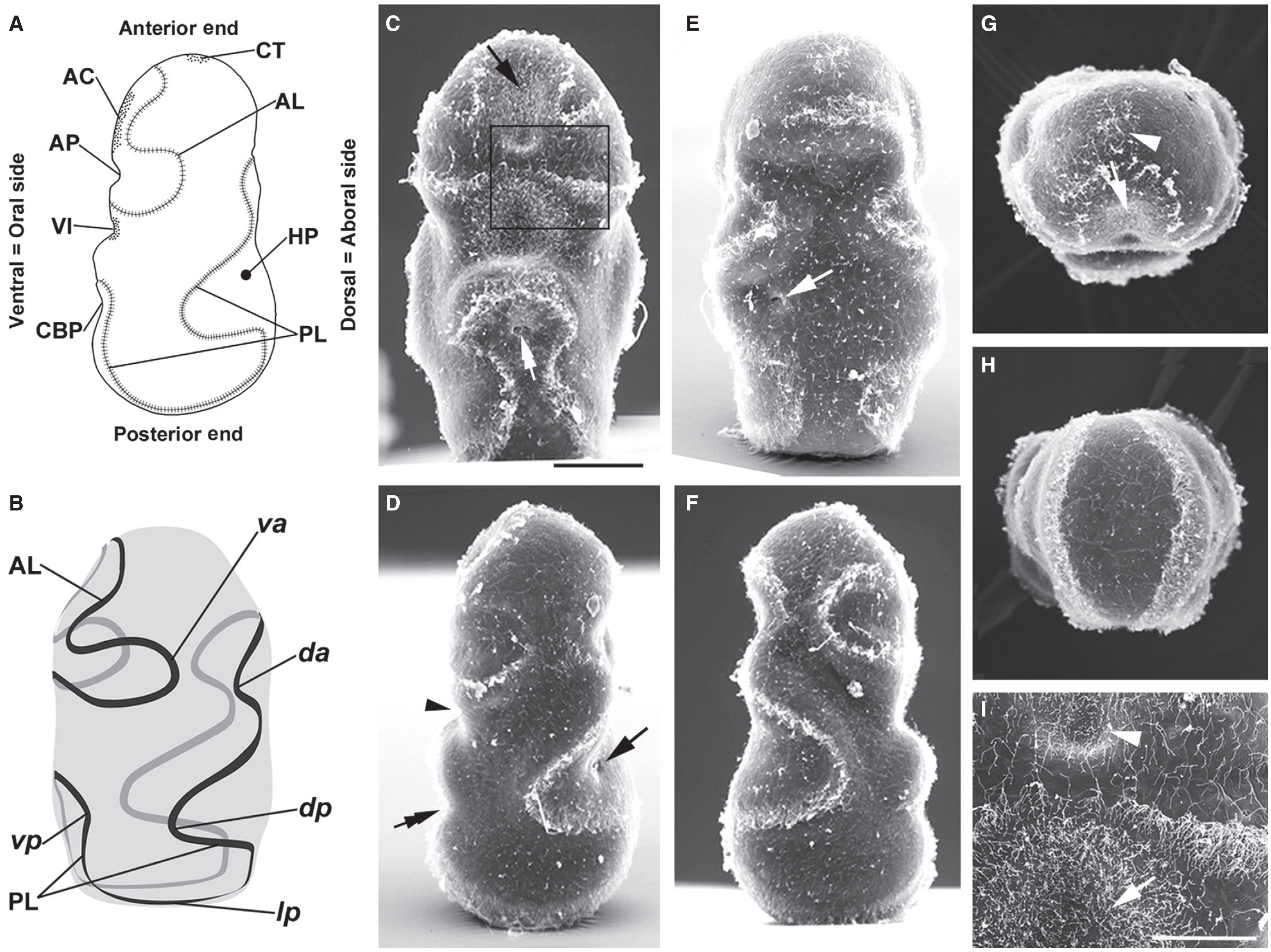

Fig. 2—Dipleurula larva of *M. rotundus* 5 days after fertilization. —**A**. Diagram of the larva from the left side (ciliary bands shown by hatching and other heavily ciliated regions by stippling). The diagram indicates the anterior-posterior axis and oral-aboral (ventral-dorsal) axis. Abbreviations, in clockwise order from the top: CT, ciliary tuft; AL, anterior loop of ciliary band; HP, hydropore; PL, posterior loop of ciliary band; CBP, closed blastopore; VI, vestibular invagination; AP, adhesive pit; AC, adhesive concavity. —**B**. A perspective view showing the total pattern of two ciliary bands formed along the low ridges and five pairs of projecting ridges along the bands in the larva. The figure is viewed from the left ventral side of the larva with the anterior end at the top. Abbreviations, in clockwise order from the top: *va*, ventral anterior ridge; *da*, dorsal anterior ridge; *dp*, dorsal posterior ridge; *lp*, lateral posterior ridge; PL, posterior loop of the ciliary band; *vp*, ventral posterior ridge; AL, anterior loop of the ciliary band. —**C**. SEM showing the ventral side, including the adhesive concavity (single arrow) and closed blastopore (tandem arrow); boxed region enlarged in **I**. —**D**. SEM showing the left side; vestibular invagination, closed blastopore, and hydropore indicated, respectively, by an arrowhead, tandem arrow, and single arrow. —**E**. SEM showing the dorsal side; hydropore is indicated by a single arrow. —**F**. SEM showing the right side. —**G**. SEM showing the animal pole with an ciliary tuft (arrowhead) and adhesive concavity (single arrow). —**H**. SEM showing the posterior end. —**I**. Enlargement of the rectangle in **C**, showing part of the adhesive pit (arrowhead) and vestibular invagination (single arrow). Scale line in **C** (applicable also to **D**–**H**) = 100 μm. Scale line in **I** = 50 μm.

far as these characteristics are concerned, the dipleurula-type larvae of crinoids and asteroids constitute one group, whereas those of ophiuroids, echinoids, and holothurians constitute another group in the echinoderms.

In feather stars, the doliolaria stage follows the uniformly ciliated swimming stage (Holland 1991). The *M. rotundus* embryo is almost entirely ciliated at the gastrula stage before hatching, although the cilia are still short and sparse. Soon after hatching, a cilia-free zone is detectable on the surface of the oval-shaped predipleurula embryo, and this zone expands with embryo development. Thus, it is difficult to specify the uniformly ciliated swimming stage during the development of *M. rotundus*. The zone develops into the frontal and anal fields on the ventral side of the dipleurula larva. Therefore, the dorsal-ventral axis of the embryo is specified soon after hatching at the latest.

The cilia-free zone found in hatched embryos is a characteristic specific to *M. rotundus* in the echinoderms. No such

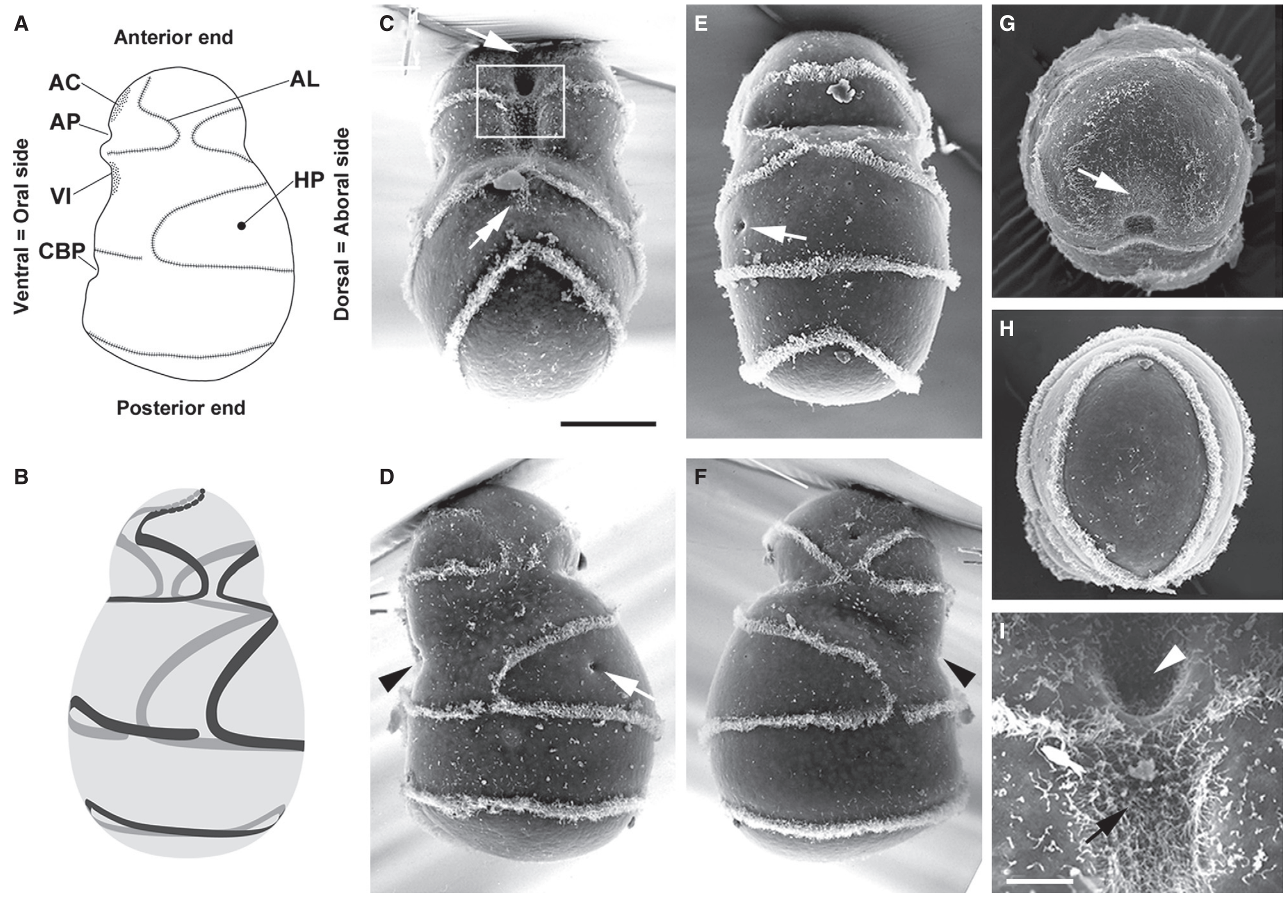

Fig. 3—Semidoliolaria larva of *M. rotundus* 6 days after fertilization. —**A**. Diagram of the larva from the left side (ciliary bands shown by hatching and other heavily ciliated regions by stippling). Abbreviations, in clockwise order from the top: AL, anterior loop of ciliary band; HP, hydropore; CBP, closed blastopore; VI, vestibular invagination; AP, adhesive pit; AC, adhesive concavity. The posterior ciliary band has become rearranged into segments that partially or completely encircle the body. —**B**. A perspective view showing the total ciliary band pattern in the larva. The larva is viewed from the left ventral side with the anterior end at the top. —**C**. SEM showing the ventral side, including the adhesive concavity (single arrow) and closed blastopore (tandem arrow); boxed region enlarged in **I**. —**D**. SEM showing the left side; vestibular invagination and hydropore indicated, respectively, by an arrowhead and a single arrow. —**E**. SEM showing the dorsal side; hydropore indicated by a single arrow. —**F**. SEM showing the right side; vestibular invagination indicated by an arrowhead. —**G**. SEM showing the animal pole with the adhesive concavity (single arrow). —**H**. SEM showing the posterior end. —**I**. Enlargement of the rectangle in **C**, showing part of the adhesive pit (arrowhead) and vestibular invagination (single arrow). Scale line in **C** (applicable also to **D**–**H**) = 100 μm. Scale line in **I** = 20 μm.

zone has been reported for the embryos of any other echinoderms before developing into the dipleurula-type larvae. Isocrinid sea lilies constitute the most basal group of living echinoderms (Rouse *et al.* 2013), and therefore, it is possible that the cilia-free zone is the ancestral trait. However, as embryos of all other extant echinoderm species, including feather stars, lack this trait, this possibility seems unlikely. We are unable to draw any firm conclusion as to whether the stage of the embryo with the cilia-free zone was evolutionarily lost in most echinoderm lineages except the isocrinid sea lilies, or whether this stage was introduced only into the isocrinid lineage. An alternative explanation is that the cilia-free zone was overlooked in embryos by previous researchers. It will be necessary to investigate this issue in more detail using different species at higher SEM resolution before any conclusion can be reached.

In ciliary band reorganization during the auricularia-to-doliolaria transition in holothurians, the band breaks up into many segments, and the circumferential bands are formed by fusion of these segments (Lacalli and West 2000). In *M. rotundus*, the ciliary bands do not break up, but are continuously rearranged into the circumferential bands (Fig. 5).

The doliolaria larvae of feather stars (Mladenov and Chia 1983; Lacalli and West 1986; Balser 2002) and holothurians (Sewell and McEuen 2002) form complete circumferential rings of ciliary bands. The *M. rotundus* larva becomes competent for settlement at the semidoliolaria stage at 6 days after fertilization. At this stage, the ciliary bands are not complete

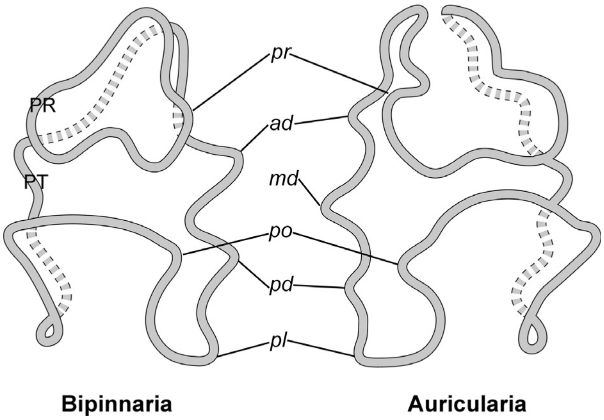

Fig. 4—Perspective views showing the total pattern of ciliary bands and five (left) or six (right) pairs of projecting ridges along the bands in the asteroid bipinnaria (left) and holothurian auricularia (right) larvae (based on Lacalli 1993). The bipinnaria is viewed from the left ventral side, and the auricularia is viewed from the right-ventral side with the anterior end at the top. Abbreviations, in clockwise order from the top: *pr*, pre-oral ridge; *ad*, anterior-dorsal ridge; *md*, mid-dorsal ridge; *po*, postoral ridge; *pd*, posterior-dorsal ridge; *pl*, posterior-lateral ridge; PT, postoral band; PR, pre-oral band.

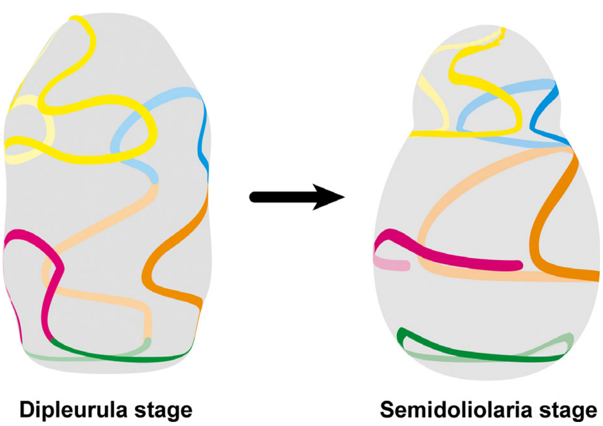

Fig. 5—Perspective views showing the transition of ciliary band patterns in *M. rotundus* from the dipleurula to the semidoliolaria type. Each colored region in the dipleurula ciliary bands forms the region of the corresponding color in the semidoliolaria bands after transition.

rings, but retain some features of the dipleurula-type bands. The band pattern remains for another week of larval development, and we have not yet succeeded in obtaining any *M. rotundus* larvae with complete circumferential rings of ciliary bands. The incomplete circumferential ciliary bands probably represent the final pattern of development of the *M. rotundus* larva because the larva is competent for settlement, although there is also a possibility that our technique still has some inadequacies for raising fully grown doliolaria larvae. The nervous system continues to develop and forms the basiepithelial nerve net (Nakano *et al.* 2009) during the semidoliolaria stage with incomplete circumferential ciliary bands, suggesting that the differentiation of the nervous system is, at least to some extent, dissociable from the development of the ciliary band pattern.

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