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

Shonan Amemiya,¹,²,³ Taku Hibino,⁴ Hiroaki Nakano,⁵ Masaaki Yamaguchi,⁶ Ritsu Kuraishi³ and Masato Kiyomoto²

Keywords: sea lily, crinoid, isocrinid, echinoderm, Articulata, ciliary band, *Metacrinus rotundus*, dipleurula, doliolaria, scanning electron microscopy

Abstract

Amemiya, S., Hibino, T., Nakano, H., Yamaguchi, M., Kuraishi, R. and Kiyomoto, M. 2015. Development of ciliary bands in larvae of the living isocrinid sea lily *Metacrinus rotundus*. — *Acta Zoologica* (Stockholm) 96: 37–44.

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.

Shonan Amemiya, Marine and Coastal Research Center, Ochomizu University, Ko-yatsu, Tateyama, Chiba 294-0301, Japan. E-mail: shonan@k.u-tokyo.ac.jp

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.

© 2013 The Authors. Acta Zoologica published by John Wiley & Sons Ltd on behalf of Royal Swedish Academy of Sciences. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
during the course of evolution. The other mode found in the larvae of the remaining two classes, the Asteroidea and Echi- 
noidea, involves formation of only the dipleurula-type ciliary 
larvae of the remaining two classes, the Asteroidea and Echi-

Most previous studies of the ciliary bands of doliolaria lar-
vae have focused on holothurians and feather stars. Such stud-
ies 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 rela-
tionship 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 settle-
ment. 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 conse-
quential 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 phy-
genetic 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 trans-
ported 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 agita-
tion. 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) CO2 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 speci-
mens 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 gastru-
late 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 cili-
ated zones is quite clear (Fig. 1C). The cilia-free zone then expands until it covers almost one-third of the entire embryo.
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.

© 2013 The Authors. Acta Zoologica published by John Wiley & Sons Ltd on behalf of Royal Swedish Academy of Sciences.
Larval ciliary bands in sea lily • Amemiya et al.

Acta Zoologica (Stockholm) 96: 37–44 (January 2015)

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 pre-oral 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 auricularia (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 asterooids. We consider that, as
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
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. 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.
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.

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.

Acknowledgements

We are very grateful to Prof. Nicholas D. Holland for encouragement, helpful suggestions and editing the manuscript. This work is dedicated to the late Mr Simpei Amemiya, the elder brother of S.A.

References

Balser, E. J. 2002. Phylum Echinodermata: Crinoidea. In: Young, C. M. (Ed): Atlas of Marine Invertebrate Larvae, pp. 463–486. Academic Press, New York, USA.

Bishop, C. D. and Burke, R. D. 2007. Ontogeny of the holothurian larval nervous system: evolution of larval forms. – Development Genes and Evolution 217: 585–592.

Breimer, A. 1978. Recent crinoids. In: Moore, R. C. and Teichert, C. (Eds): Treatise on Invertebrate Paleontology, part T, Echinodermata 2, Vol. 1, pp. 9–58. Univ. Kansas, Lawrence, KA.

Dan, K. 1968. Echinodermata. In: Kume, M. and Dan, K. (Eds): Invertebrate Embryology, pp. 280–315, 329–331. Prosveta Press, Belgrade, Yugoslavia.

Holland, N. D. 1991. Echinodermata: Crinoidea. In: Giese, A. C., Pearse, J. S. and Pearse, V. B. (Eds): Reproduction of Marine Invertebrates. Echinoderms and Lophophorates, Vol. 4, pp. 247–299. Boxwood Press, Pacific Grove, CA.

Holland, N. D. and Kubota, H. 1975. Correlated scanning and transmission electron microscopy of larvae of the feather star Comanthus japonica. – Transactions of the American Microscopical Society 94: 58–70.

Janies, D. 2001. Phylogenetic relationships of extant echinoderm classes. – Canadian Journal of Zoology 79: 1232–1250.

Lacalli, T. C. 1993. Ciliary bands in echinoderm larvae: evidence for structural homologies and a common plan. – Acta Zoologica 74: 127–133.

Lacalli, T. C. and West, J. E. 1986. Ciliary band formation in the doliolaria larva of Flourometra I. The development of normal epithelial pattern. – Journal of Embryology and Experimental Morphology 96: 303–323.

Lacalli, T. C. and West, J. E. 1987. Ciliary band formation in the doliolaria larva of Flourometra II. Development of anterior and posterior half-embryos and the role of the mesentoderm. – Development 99: 273–284.

Lacalli, T. C. and West, J. E. 2000. The auricularia-to-doliolaria transformation in two aspidochirote holothurians, Holothuria mexicana and Stichopus californicus. – Invertebrate Biology 119: 421–432.

Lacalli, T. C., Gilmour, T. H. J. and West, J. E. 1990. Ciliary band innervation in the bipinnaria larva of Pissaster ochraceus. – Philosophical Transaction: Biological Sciences 330: 371–390.

McEdward, L. R., William, B. J. and Komatsu, M. 2002. Phylum Echinodermata: Asteroidea. In: Young, C. M. (Ed): Atlas of Marine Invertebrate Larvae, pp. 499–512. Academic Press, New York, USA.

Mladenov, P. V. 1985. Development and metamorphosis of the brittle star Ophiocoma violacea: evolutionary and ecological implications. – Biological Bulletin 168: 285–295.
Mladenov, P. V. and Chia, F. S. 1983. Development, settling behaviour, metamorphosis and pentacrinoid feeding and growth of the feather star Florometra serratissima. – Marine Biology 73: 309–323.
Mortensen, T. H. 1921. Studies of the Development and Larval Forms of Echinoderms. G.E.C. Gad, Copenhagen.
Mortensen, T. H. 1937. Contribution to the study of the development and larval forms of Echinoderms III. – Konelige Danske Videnskabernes Selskabs. Skrifter, Naturvidenskabelige Og Mathematisk Afdeling Series 9, 7: 1–65.
Mortensen, T. H. 1938. Contribution to the study of the development and larval forms of Echinoderms IV. – Konelige Danske Videnskabernes Selskabs Skrifter, Naturvidenskabelige Og Mathematisk Afdeling Series 9, 7: 1–59.
Nakano, H., Hibiino, T., Oji, T., Hara, Y. and Amemiya, S. 2003. Larval stages of a living sea lily (stalked crinoid echinoderm). – Nature 421: 158–160.
Nakano, H., Nakajima, Y. and Amemiya, S. 2009. Nervous system development of two crinoid species, the sea lily Metacrinus rotundus and the feather star Oxycomanthus japonicus. – Development Genes and Evolution 21: 565–576.
Nielsen, C., 2012. Animal Evolution. Interrelationships of the Living Phyla 3rd edn. Oxford university Press, New York.
Rouse, G. W., Jermin, L. S., Wilson, N. G., Eeckhaut, I., Lanterbecq, D., Oji, T., et al. 2013. Fixed, free and fixed: the fickle phylogeny of extant Crinoidea (Echinodermata) and their Permian-Triassic origin. – Molecular Phylogenetics and Evolution 66: 161–181.
Sewell, M. A. and McEuen, F. S. 2002. Phylum Echinodermata: Holothuroidea. In: Young, C. M. (Ed): Atlas of Marine Invertebrate Larvae, pp. 513–530. Academic Press, New York, USA.
Smiley, S., McEuen, F. S., Chafee, C. and Krishnan, S. 1991. Echinodermata: Holothuroidea. In: Giese, A. C., Pearse, J. S. and Pearse, V. B. (Eds): Reproduction of Marine Invertebrates. Echinoderms and Lophophorates, Vol. 4, pp. 663–750. Boxwood Press, Pacific Grove, CA.