Metamorphosis Regulation by Retinoic Acid Signaling in Echinoderms: Insights into the Evolution of Animal Life Cycle

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Abstract

The evolution of the biphasic life cycle in marine invertebrates has attracted the interest of many zoologists. A key question is how similar the molecular regulatory mechanisms of metamorphosis are in various taxa. It was recently suggested that retinoic acid (RA) is involved in the regulation of metamorphosis in the starfish. It also plays a role in the life cycle transition of basal echinoderms and jellyfish, a cnidarian; thus, the regulatory machinery of life cycle transitions may be conserved in starfish metamorphosis. However, details of the molecular mechanisms that trigger RA signaling upon settlement during starfish metamorphosis have yet to be elucidated. Furthermore, the function of RA signaling in other animal groups is poorly understood in this context. In this study, I used the starfish *Patiria pectinifera* and revealed the regulatory mechanism of the RA-dependent metamorphosis through the mass spectrometry and genome editing analysis. I also investigated the role of RA signaling during the metamorphosis of other echinoderm species, the starfish *Astropecten latespinosus* and the feather star *Antedon serrata* and determined its evolutionary conserved function in echinoderms. I herein discussed the life cycle evolution of animal from the viewpoint of RA-dependent regulatory mechanism.

General Introduction

Marine invertebrates have evolved various life cycle strategies in different taxa; many show a biphasic life cycle in which floating larvae metamorphose into benthic adult forms (Jagersten, 1972). Many zoologists have proposed evolutionary scenarios underlying this superficial similarity, and some recent studies have posed evolutionary hypotheses based on comparisons of larval morphology or transcriptomes. Arendt *et al*, 2001 and Marlow *et al*., 2018 reported that the molecular patterning mechanism of apical organs is highly conserved among floating larvae including protostomes, deuterostomes, and cnidarians, proposing that the common ancestors of metazoans or bilaterians experience the floating larval stage. In addition, Wang *et al*., 2020 revealed that the transcriptome of the larval phase is comparable among various animal groups including sponges based on the phylostratigraphic approach. However, some researchers have noted that the expression and functions of body patterning genes such as Hox genes vary among the larval bodies of animal taxa (Gonzalez *et al*., 2017; Hejnol and Vellutini, 2017; Sly *et al*., 2003). Although that may support multiple origins of larval forms, the evolutionary history of the life cycle remains controversial. It is important to understand and compare the molecular regulatory mechanisms of metamorphosis and the life cycle among various animal taxa.

Yamakawa *et al.* 2018 recently presented evidence that retinoic acid (RA) signaling is involved in the regulation of metamorphosis in starfish (echinoderms). RA signaling functions in cell-cell communication through secretion of RA synthesized by retinal dehydrogenase (*raldh*) (Gutierrez-Mazariegos *et al.* 2014; Rhinn and Dolle. 2012;

Marlétaz *et al.* 2006). RA signaling is processed through RA binding to the nuclear receptors: RA receptor (*rar*) and retinoid x receptor (*rxr*) (Gutierrez-Mazariegos *et al.* 2014; Rhinn and Dolle. 2012; Marlétaz *et al.* 2006). RA signaling is well known as the regulator of the neural patterning through the regulation of Hox gene expression in vertebrates (Marlétaz *et al.* 2006). Yamakawa *et al.* 2018 previously investigated the role of RA signaling in larvae of starfish (echinoderms), and suggested the involvement of RA signaling machinery on the metamorphosis process after settlement in the starfish *Patiria pectinifera*. Furthermore, RA has also been reported to act in the life cycle transition of cnidarians, the polyp-to-ephyra transition of jellyfish (Fuchs et al., 2014). However, to clarify the evolutionarily conserved role of RA in the life cycle transition, it is important to understand whether RA signaling is involved in neural reception and transduction of environmental cues to commence metamorphosis.

In this study I investigated the mechanism of RA-dependent metamorphosis in starfish (Chapter 1) and revealed its evolutionary conserved role in echinoderms (Chapter 2–3). In chapter 1, the amount of endogenous RA was measured during larval and metamorphic stages, and it is found that endogenous RA is sufficiently high for transduction of RA signaling even before the larvae acquired competence for metamorphosis (6–12 dpf). By demonstrating that the gene knockout of *rar* suppressed the metamorphosis process after settlement, I confirmed that RAR is essential for the commencement of metamorphosis in *P. pectinifera*. Based on these findings, I propose that starfish metamorphosis is regulated not by the increase of RA amount or concentration, but by regulating RA binding to RAR.

In the following Chapter 2–3, I aimed to reveal the evolutionary conservation of the RA signaling function in other echinoderms, which show diverged life cycle evolution. First, I investigated the metamorphosis regulatory mechanism of the starfish having the derived life cycle strategy (Chapter 2). The starfish *Astropecten latespinosus* have the lecithotrophic larvae lacking brachiolar arms, the sensory apparatus for the reception of environmental cues in planktotrophic larvae. Although the metamorphosis of *A. latespinosus* is considered to be independent from external environmental signal, I found that metamorphosis of *A. latespinosus* was stimulated when larvae were cultured with natural sand from their habitat. I also found that RA signaling mediated the metamorphosis process upon environmental stimulation, as in planktotrophic larvae. I examined reagent treatments and gene expression analysis by in situ hybridization. Exogenous RA treatment induced metamorphosis, whereas RA synthesis inhibitor or antagonist for RA receptors suppressed metamorphosis. RA signaling–related genes were expressed in juvenile rudiments. In conclusion, it is proposed that the reception of particular environmental cues and the mediation of RA signaling is required, for the metamorphosis of lecithotrophic larvae.

Although RA signaling is involved in the metamorphosis regulation of starfish, regarding that each group of echinoderms diverged the larval type and settlement style, it was unclear if RA signaling is an ancestral regulator of metamorphosis in echinoderms. Thus, in order to determine the ancestral function of RA signaling in echinoderms, I investigated the role of RA signaling in the metamorphosis of the feather star *Antedon serrata* in Chapter 3. I treated doliolaria larvae of *A. serrata* with exogenous RA, resulting

86 in the induction of cystidean larvae. In contrast, metamorphosis was suppressed by
87 treatment with RA synthesis inhibitor and antagonist for RA receptors. In conclusion, my
88 study suggests that RA signaling functions as a regulator of metamorphosis in the ancestor
89 of echinoderms.
90 Based on my findings about the role of RA signaling in echinoderm
91 metamorphosis, I provide insight into the evolution of the animal life cycle.
92

Chapter 2

Regulation of Metamorphosis by Environmental Cues and Retinoic Acid Signaling in the Lecithotrophic Larvae of the Starfish *Astropecten latespinosus*

Abstract

Common ancestors of starfish (echinoderms) are believed to have planktotrophic larvae, although some species shows lecithotrophic larvae, which do not feed before metamorphosis. Furthermore, some lecithotrophic paxillosidan larvae, such as those of *Astropecten latespinosus*, lack brachiolar arms, the sensory apparatus for the reception of environmental cues in planktotrophic larvae. In this study, I found that metamorphosis of *A. latespinosus* was stimulated when larvae were cultured with natural sand from their habitat. I also found that RA signaling mediated the metamorphosis process upon environmental stimulation, as in planktotrophic larvae. I examined reagent treatments and gene expression analysis by *in situ* hybridization. Exogenous RA treatment induced metamorphosis, whereas RA synthesis inhibitor or antagonist for RA receptors suppressed metamorphosis. RA signaling–related genes were expressed in juvenile rudiments. In conclusion, I propose that the reception of particular environmental cues is required, for the metamorphosis of lecithotrophic larvae.

Introduction

Many marine invertebrates have biphasic life cycles, with planktonic larval and sessile adult phases (Jägersten 1972). Because sessile adults have restricted motility, the settling of larvae in suitable environments during metamorphosis is of critical importance (Morse 1990). Therefore, planktonic larvae usually possess sensory apparatus to respond to specific environmental cues (Morse 1990).

The ancestors of starfish (echinoderms) are believed to have planktotrophic larvae (Oguro 1989; McEdward *et al.* 2001; Byrne 2006). Planktotrophic larvae need to be fed to commence metamorphosis; thus, their development proceeds depending on the larval nutritional state (McEdward 1997). Some species changed their strategies and develop through lecithotrophic larvae (Oguro 1989; McEdward *et al.* 2001; Byrne 2006). In contrast to that of planktotrophic larvae, the development of lecithotrophic larvae proceeds in a cascade-like manner because they do not feed before metamorphosis (Oguro 1989; Byrne 2006). However, whether these lecithotrophic larvae sense environmental cues to commence metamorphosis is not clear.

After lecithotrophy, some starfish species retain their sensory apparatus, brachiolar arms (McEdward *et al.* 2001). Many planktotrophic starfish larvae use their brachiolar arms to sense environmental cues about where to settle (Murabe *et al.* 2007). This suggests that these species have continued to sense environmental cues, even after their transition to lecithotrophy. Some lecithotrophic paxillosidan species, such as *Astropecten latespinosus*, however, do not possess brachiolar arms (Komatsu 1975; Komatsu 1982; Komatsu and Nojima 1985).

In this study, I found that metamorphosis of *A. latespinosus* was stimulated when larvae were cultured with natural sand from their habitat. I also found that RA signaling mediated the metamorphosis process upon environmental stimulation, as in planktotrophic larvae. I examined reagent treatments and gene expression analysis by *in situ* hybridization. Exogenous RA treatment induced metamorphosis, whereas RA synthesis inhibitor or antagonist for RA receptors suppressed metamorphosis. RA signaling–related genes were expressed in juvenile rudiments. In conclusion, it is proposed that the reception of particular environmental cues is required, for the metamorphosis of lecithotrophic larvae.

Material and Methods

1. Sampling and Culture of Larvae

I collected adult specimens of *A. latespinosus* from Notojima Island, Ishikawa Prefecture, Japan, and obtained fertilized eggs as described previously (Komatsu 1975). I cultured the larvae in artificial sea water at 22℃.

2. Reagent Treatments

I prepared 100 mM stock of *all-trans* RA (Sigma-Aldrich, St Louis, CAS number: 302-79-4), 1 M stock of N, N-diethylaminobenzaldehyde (DEAB, Tokyo Chemical Industry, Tokyo, Japan, CAS number: 120-21-8) and 50 mM stock of RO41-5253 (RO, Focus Biomolecules, Plymouth Meeting, PA, USA, CAS number: 144092-31-9) in dimethyl sulfoxide (DMSO). I incubated the larvae in 2 mL of artificial seawater containing 2 μL of reagents or DMSO in 12-well plates at 22 °C. For the experiment with a substrate, 10 larvae were incubated in one well. Natural sands from Notojima Island were used for the experiments conducted to induce metamorphosis. For cases in which reagent treatment continued for more than two days, I changed the seawater with the same concentration of reagents every other day.

I judged whether the larvae were metamorphosed by the enlargement of juvenile rudiment and absorption of larval body. From these observations, the numbers of individuals who metamorphosed were counted. The rates of metamorphosis were calculated by dividing by the number of treated larvae. I carried out experiments using

three batches of larvae from different adults. In particular, experiments were conducted once to several times in each batch.

4. Statistical Analysis

As in described in the Method section of Chapter 1, I examined the statistical analyses to evaluate differences in the effects of the treatments of substrate or reagents on settlement or metamorphosis.

5. Construction of the Phylogenic Trees

To gather sequences of RA signaling–related genes, I used the transcriptome data which was previously *de novo* assembled and deposited in the DDBJ Sequence Reads Archives (DRA008444) to recovere the nucleotide sequences coding for *raldha*, *raldhb*, *raldhc*, *rar*, and *rxr* of *A. latespinosus*. In addition to the previously used datasets (Yamakawa *et al.* 2018), a sequence alignment was performed using MAFFT (default value in the online version) and the phylogenetic sequence was filtered using trimAL with a gap threshold of 0.8 (Katoh *et al.* 2017; Capella-Gutiérrez *et al.* 2009). The estimate of the amino acid substitution model and preparation of the maximum likelihood tree were carried out using RAxML (Stamatakis 2014). Confidence values were calculated after 1000 bootstrap runs. Accession numbers for each gene are shown in Table 2-2. Abbreviations for species are as shown following. Hs (human); *Homo sapiens*, Xt (frog); *Xenopus tropicalis*, Dr (zebrafish); *Danio rerio*, Bf (amphioxus); *Branchiostoma floridae*, Ci (tunicate); *Ciona intestinalis*, Al (starfish); *Astropecten latespinosus*, Pp (starfish); *Patiria pectinifera*, Sp

(sea urchin); *Strongylocentrotus purpuratus*; Sk (acorn worm); *Saccoglossus kowalevskii*). Mm (mouse); *Mus musculus*, Bl (amphioxus); *Branchiostoma lanceolatum*, Pm (tunicate); *Polyandrocarpa misakiensis*, Dm (fly); *Drosophila melanogaster*, Rc (snail); *Reishia clavigera*, Ls (snail); *Lymnaea stagnalis*, and Tc (jellyfish); *Tripedalia cystophora*.

6. Whole-mount *in situ* hybridization

I prepared the DIG-labeling antisense probes for *raldha–c*, *rar* and *rxr* using the primers shown in Table 2-1 and conducted *in situ* hybridization as previously de scribed (Morino *et al.* 2012; Yamakawa *et al.* 2018).

612 **Results**

613

614 **1. Larvae of *A. latespinosus* respond to the environmental cues for metamorphosis**

615 First, I investigated whether lecithotrophic larvae could sense environmental cues by
616 testing whether larvae of *A. latespinosus* commenced metamorphosis in seawater
617 containing sand from the habitat of adult specimens. During this experiment, I also
618 investigated when larvae became competent for metamorphosis. Previously, Komatsu
619 1975 stated that larvae began to develop juvenile rudiments at around 30 hpf, after the
620 gastrula elongated along archenteron (Fig. 2-1b). Juvenile rudiment development then
621 proceeds until approximately 72–96 hpf (Fig. 2-1c, d) (Komatsu 1975). She reported that
622 larvae metamorphosed to juveniles after 75 hpf (Fig. 2-1e, f), although she did not
623 experimentally examine the time to acquisition of competency (Komatsu 1975).

624 I introduced the habitat sand to the wells of the 24-hpf larvae, corresponding to
625 gastrula (n=50 from three batches; Fig. 2-1a). Then, I cultured larvae and counted the
626 number of larvae that completed metamorphosis every day for 1 week. In the seawater
627 without substrate, larvae did not metamorphose before 72 hpf (Fig. 2-2, Table 2-3). Small
628 numbers of larvae (5 of 50 larvae) metamorphosed into juveniles after 96 hpf (Fig. 2-2,
629 Table 2-3), although the metamorphosis ratio was less than 50% (21 of 50 larvae; Fig. 2-
630 2, Table 2-3). On the other hand, when substrates were added to the seawater, small
631 numbers of larvae (7 of 50 larvae) were induced to metamorphose even at 72 hpf (Fig. 2-
632 2, Table 2-3). More than 70% of larvae (36 of 50 larvae) metamorphosed after 96 hpf (Fig.
633 2-2, Table 2-3). At 192 hpf, significant differences in the metamorphosis ratios were

observed between treatments (P=0.009, T-TEST). These results indicate that *A. latespinosus* can sense environmental cues, such as natural sand, to commence metamorphosis. I also found that most of the larvae metamorphosed at 72–96 hpf (Fig. 2-2), suggesting that they became competent around 72 hpf.

2. RA signaling also involves in the metamorphosis regulation of *A. latespinosus*

In planktotrophic larvae of starfish, Murabe *et al.* 2007 found that brachiolar arms perform critical roles to receive environmental cues for metamorphosis. Recently, Yamakawa *et al.* 2018 suggested that retinoic acid (RA) signaling mediated the commencement of metamorphosis process after settlement, through RA synthesis by retinal dehydrogenase (RALDH) and binding to retinoic acid receptor (RAR) and retinoid x receptor (RXR) (Rhinn and Dolle 2012). As shown above, I found that paxillosidan larvae also received environmental cues to commence metamorphosis, though they use different apparatus from brachiolar arms for reception, thus it is unclear if RA signaling involves the metamorphosis regulation in this group.

Here, I examined whether the commencement of metamorphosis was also mediated by RA signaling in *A. latespinosus*. Firstly, I investigated the effect of exogenous RA treatment of competent larvae (n=40 from three batches). As more than half of 72-hpf larvae treated with habitat sand completed metamorphosis in 24 h (Fig. 2-2), I tested the effect of exogenous RA on 72-hpf larvae. I found that exogenous RA (1 μM) treatment induced metamorphosis (32 of 40 larvae; Fig. 2-3a, c, Table 2-4). The larvae commenced metamorphosis immediately after treatment and completed their

transitions to juveniles in 24 h. On the other hand, only one out of 40 DMSO-treated larvae metamorphosed (Fig. 2-3b, c, Table 2-4). I observed that the presence of RA significantly affected the metamorphosis ratio ($P<0.001$, T-TEST). These results suggest that RA mediates the internal signaling to commence the metamorphosis of *A. latespinosus*.

Additionally, I investigated the effect of exogenous RA treatment of larvae of various ages on metamorphosis to test whether RA also affected the timing of larval competence to respond to cues for metamorphosis. I treated 24- and 48-hpf larvae with RA (1 μM) and counted the number of metamorphosed larvae every 24 h until 96 hpf (n=30 and 40 from three batches, respectively). I observed that metamorphosis was induced only after 72 hpf in both cases (3 of 30 and 2 of 40 with 24- and 48-hpf initiations, respectively; Fig. 2-3d, e, Tables 2-5, 2-6). Thus, regardless of when the larvae were treated with RA, they responded and metamorphosed at 72 hpf, which is comparable to the stage at which larvae acquire competence to metamorphose during normal development (Figs. 2-2, 2-3d, e). Furthermore, at 96 hpf, almost half of the larvae metamorphosed (15 of 30 and 19 of 40 from three batches with 24-hpf and 48-hpf initiation, respectively; Fig. 2-3d, e, Tables 2-5, 2-6). I found significant differences at 96 hpf in the batches with 24- and 48-hpf initiations ($P=0.034$ and $P=0.019$, respectively, T-TEST). These timelines are similar to those induced by a substrate (Fig. 1). These results suggest that RA does not affect the development of competence for metamorphosis, but rather functions as an internal mediator of the signaling to commence metamorphosis when added to competent larvae.

Next, I investigated whether endogenous RA synthesis is required for metamorphosis. To investigate the effect of treatment with DEAB, an RA synthesis inhibitor, on metamorphosis, I treated 72-hpf larvae with DEAB (300 μM) and natural sand in the experiments described above, and counted the larvae that had completed metamorphosis 24 h after treatment (n=40 from three batches). As a control, I treated 72-hpf larvae with DMSO and natural sand. More than half of the DMSO-treated larvae transitioned to juveniles (26 of 40 larvae; Fig. 2-4b, c, Table 2-7). In contrast, DEAB treatment decreased the number of metamorphosed larvae (6 of 40 larvae; Fig. 2-4a, c, Table 2-7). The metamorphosis ratio was significantly suppressed by DEAB treatment (P=0.022, T-TEST). I observed particular larval behavior prior to metamorphosis, such as attachment to the substrate with rudiments under the DEAB treatment. Thus, larvae were likely to sense the environmental cue, but did not commence metamorphosis. These findings suggest that endogenous RA synthesis is required for the commencement of metamorphosis.

RA binding to RAR is required for RA signaling activation (Rhinn and Dolle 2012). Thus, I investigated the effect of RAR antagonist treatment on metamorphosis to test the hypothesis that RA signaling pathways mediate the metamorphosis process. I treated 72-hpf larvae (n=40 from three batches) with RO41-5253 (RO; 1 μM), RAR antagonist, and the natural sand used above, and counted the number of metamorphosed larvae after 24 h. As a control, I treated 72-hpf larvae with DMSO and natural sand. Under the DMSO treatment, 67.5% of larvae (27 of 40 larvae) transitioned to juveniles (Fig. 2-4e, f, Table 2-8). In contrast, no larva metamorphosed under the RO treatment (Fig. 2-4d,

f, Table 2-8). The metamorphosis ratio was significantly repressed by RO treatment (P=0.008, T-TEST). As I observed with DEAB treatment, larvae also stopped floating and attached to the substrate with rudiments following RO treatment.

As shown previously, exogenous RA treatment induces metamorphosis in 72-hpf larvae (Fig. 2-3). To support the idea that RA binding to RAR is required for metamorphosis, I examined whether RO treatment blocked metamorphosis induced by RA treatment. I treated 72-hpf larvae (n=40 from three batches) with RA (1 μM) or RA (1 μM) plus RO (1 μM). Under the case RA-only treatment, 77.5% of larvae (31 of 40 larvae) metamorphosed (Fig. 2-4g–i, Table 2-9). On the other hand, the RA (1μM) plus RO (1 μM) treatment induced metamorphosis in only 12.5% of larvae (5 of 40 larvae; Fig. 2-4h, k, Table 2-9). RO significantly repressed the metamorphosis ratio (P<0.001, T-TEST). These data suggest that RA signaling activation through RA binding to RAR is required for the commencement of metamorphosis.

I examined the expression patterns of genes involved in RA signaling. I confirmed their orthologies by constructing phylogenic trees (Figs. 2-5, 2-6). As conclusion, from de novo transcriptome, I identified three *raldh*s (*raldha*, *raldhb*, and *raldhc*), single *rar*, and single *rxr*. I also investigated the spatial expression patterns of the three *raldh*s, *rar*, and *rxr* by whole-mount *in situ* hybridization of 72-hpf larvae (Fig. 2-7). I identified the expression of two types of receptor, *rar* and *rxr*, in the juvenile rudiment (Fig. 2-7j–l and m–o, respectively), as well as that of *raldha*, *raldhb*, and *raldhc* (Fig. 2-7a–c; d–f; and g–i, respectively). Especially, in juvenile rudiment, all genes were expressed in epidermis region with different expression pattern; patchwise expression of

722 *raldh*s, broad expression of *rar* and *rxr* (Fig. 2-7c, f, i, l and o). I also found that all genes
723 except *raldhc* were expressed in hydrolobes (primordium of primary podia and tube feet;
724 Fig. 2-7c, f, l and o). These expression patterns were consistent with the idea which
725 hydrolobes are used for sensory of environmental cues in paxillosida (Byrne 2013; Pernet
726 *et al.* 2017). These data support the conclusion that RA signaling mediates the
727 metamorphosis process in *A. latespinosus*.
728
729

730 **Discussion**

731 Here, I provided the evidence that metamorphosis is triggered by environmental cues in
732 *A. latespinosus* larvae. When I introduced natural sand from the *A. latespinosus* habitat,
733 the larvae stopped floating, became attached to the substrate, and commenced
734 metamorphosis (Fig. 2-2). Furthermore, my data suggest that RA signaling mediated the
735 commencement of metamorphosis upon environmental cue reception. Exogenous RA
736 treatment of competent larvae induced metamorphosis (Fig. 2-3), and metamorphosis was
737 suppressed by the inhibition of two distinct RA signaling pathways (Fig. 2-4): RA
738 synthesis (Fig. 2-4a–c) and RA binding to RAR (Fig. 2-4d–i). The spatial expression
739 pattern of RA signaling–related genes is consistent with the results described above (Fig.
740 2-7). Particularly, overlapping expression of two kinds of receptor (*rar* and *rxr*) was
741 observed in juvenile rudiments of competent larvae (Fig. 2-7j–l and m–o, respectively).
742 It should be noted that I did not examine the gene function analysis in this study. To
743 strengthen my hypothesis, future study should focus on the function of each regulatory
744 component.

745 Planktonic starfish larvae sense environmental cues for metamorphosis with
746 brachiolar arms (Murabe *et al.* 2007), but paxillosidan larvae, even those that are
747 planktonic, lack brachiolar arms (McEdward and Miner 2001; Pernet *et al.* 2017). This
748 absence is regarded as a secondary loss due to the transition to a sandy habitat
749 (Linchangco *et al.* 2017). In this study, I found that metamorphosis of *A. latespinosus* is
750 induced by culture with natural sand from their habitat, suggesting that paxillosidan
751 larvae also respond to environmental cues for metamorphosis. Despite my findings, how

752 paxillosidan larvae sense environmental cues remains unclear.

753 Previously, several researchers suggested that tube feet (primary podia) are
754 used as the sensory apparatus for the reception of environmental cues in this group (Byrne
755 2013; Pernet *et al.* 2017). On the other hand, Komatsu 1975 and Oguro *et al.* 1976 stated
756 that tube feet did not appear before metamorphosis was mostly completed in *A.*
757 *latespinosus* and A. scoparius (Komatsu 1975; Oguro *et al.* 1976). Whether larvae sense
758 environmental cues with this structure is difficult to judge based on hydrolobe
759 morphology. I suggest that researchers' attention be broadened to juvenile structures in
760 efforts to identify the sensory apparatus for environmental cue detection in paxillosidan
761 species.

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763

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Chapter 3: Retinoic Acid Signaling Regulates the Metamorphosis of Feather Stars (Crinoidea, Echinodermata)

Abstract

Many marine invertebrates have a life cycle with planktonic larvae, although the evolution of this type of life cycle remains enigmatic. It is recently proposed that the regulatory mechanism of life cycle transition is conserved between jellyfish (Cnidaria) and starfish (Echinoderm); retinoic acid (RA) signaling regulates strobilation and metamorphosis, respectively. However, the function of RA signaling in other animal groups is poorly understood in this context. Here, to determine the ancestral function of RA signaling in echinoderms, I investigated the role of RA signaling during the metamorphosis of the feather star, *Antedon serrata* (Crinoidea, Echinodermata). Although feather stars have different larval forms from starfish, I found that exogenous RA treatment on doliolaria larvae induced metamorphosis, like in starfish. Furthermore, blocking RA synthesis or binding to the RA receptor suppressed metamorphosis. These results suggested that RA signaling functions as a regulator of metamorphosis in the ancestor of echinoderms. My data provides insight into the evolution of the animal life cycle from the viewpoint of RA signaling.

Introduction

The life cycle of many marine invertebrates includes a shift from swimming as a planktonic larva with cilia to a benthic adult (Jägersten 1972). Various larval forms exist in animals, including sponges, cnidarians, and various bilaterians; this has attracted the interest of many zoologists to the origin of the larvae and evolution of the life cycle (Jägersten 1972; Jackson *et al.* 2002; Degnan and Degnan 2010). The patterning mechanism of the larval body is conserved in various animal groups, including Protostomes, Deuterostomes, and Cnidaria, suggesting an older evolutionary origin of planktonic larvae (Marlow *et al.* 2014; Darras *et al.* 2011; Marlow *et al.* 2013; Range *et al.* 2013). Nevertheless, as Raff 2008 hypothesized that larval forms evolved multiple times over the course of evolution, the evolution of the life cycle in the animal kingdom is still controversial. Therefore, in addition to the morphological aspects, it is important to understand the evolution of the regulatory mechanisms underlying the life cycles of marine invertebrates.

The life cycle transition in jellyfish (Cnidaria) and starfish (Echinoderm) is regulated by the conserved machinery of retinoic acid (RA) signaling (Fuchs *et al.* 2014; Yamakawa *et al.* 2018). Planktonic larvae of many marine invertebrates settle on an external substrate (settlement) and subsequently transit to a benthic adult phase (metamorphosis) (Jackson *et al.* 2002). In jellyfish, the planula larvae settle on the seafloor and commence the polyp stage; subsequently, environmental signals, including cold temperatures, stimulate strobilation and the transition to ephyra stage (Fuchs *et al.* 2014). Fuchs *et al.* 2014 suggested that endogenous RA mediates the regulation of

807 strobilation after environmental signals are received. On the other hand, when the
808 competent starfish larvae settle on the external substrate using brachiolar arms, they
809 transition to the juvenile stage through metamorphic processes such as enlargement of the
810 juvenile rudiment (Murabe *et al.* 2007; Yamakawa *et al.* 2018). Yamakawa *et al.* 2018
811 suggested that, like in jellyfish, RA signaling mediates the regulation of metamorphosis
812 in starfish larvae after environmental cues are received. Although different types of
813 receptors for RA are used in each lineage (Fuchs *et al.* 2014; Yamakawa *et al.* 2018),
814 these findings suggest that the RA functions widely in the life cycles of marine
815 invertebrates. To demonstrate this idea, it is necessary to clarify the function of RA
816 signaling in various animal groups. Notably, RA signaling might not function in the
817 metamorphosis of marine annelids. Handberg-Thorsager *et al.* 2018 showed that RA
818 receptor functions as a low-affinity sensor triggering neural differentiation but did not
819 report a metamorphosis-regulating function in a study of trochophore and early
820 nectochaete larvae.

821 In the present study, I made an attempt to determine the ancestral function of
822 RA signaling in echinoderms. Echinoderms comprise five classes: the most basal
823 Crinoidea and their sister group, Eleutherozoa, consisting of Echinozoa (Echinoid and
824 Holothuria) and Asterozoa (Asteroid and Ophiuroid) (Telford *et al.* 2014). Notably, the
825 larval morphology and the machinery for settlement vary among echinoderm taxa (Hart
826 2002; Hyman 1955; McEdward 2001; Raff 2006); for example, planktotrophic pluteus
827 larvae of sea urchins and brittle stars settle to the sea bottom using tube feet, while in
828 crinoids, lecithotrophic doliolaria larvae settle using adhesive tufts. Furthermore, it

829 should be noted that the regulation of metamorphosis in sea urchins has been clarified in
830 relatively great detail (Heyland *et al.* 2018; Sutherby *et al.* 2012; Chino *et al.* 1994);
831 thyroid hormone and histamine signaling modulate larval growth and the acquisition of
832 competency. Although previous studies have suggested that nitric oxide signaling
833 negatively controls the post-settlement process and that the receipt of environmental cues
834 decreases nitric oxide synthesis to commence metamorphosis (Bishop and Brandhorst
835 2001; 2007), it has not been reported that RA signaling is involved in the regulation of
836 metamorphosis in sea urchins. Therefore, it is unclear whether metamorphosis in
837 echinoderm ancestors is regulated by RA signaling as in starfish.
838 Here, I investigated whether RA signaling regulates metamorphosis in the
839 feather star (Crinoidea), Antedon serrata. I treated doliolaria larvae of *A. serrata* with
840 exogenous RA, resulting in the induction of cystidean larvae. In contrast, metamorphosis
841 was suppressed by treatment with RA synthesis inhibitor and antagonist for RA receptors.
842 In conclusion, my study suggests that RA signaling functions as a regulator of
843 metamorphosis in the ancestor of echinoderms, providing insight into the evolution of the
844 animal life cycle from the viewpoint of RA signaling.
845

Materials and Methods

1. Sampling and Culture of Larvae

The adult specimens of *A. serrata* with fertilized eggs or embryos in their pinnular surface were collected and provided by Dr. Hisanori Kohtsuka (Misaki Marine Biological Station, School of Science, University of Tokyo) from Misaki (Miura, Kanagawa Prefecture, Japan) and Onahama (Iwaki, Fukushima Prefecture, Japan). I incubated the adult specimens in artificial sea water at 14 °C. For experiments, I used doliolaria larvae that hatched from the pinnular surface of adults.

2. Immunohistochemistry

I fixed the larvae in 4% PFA in MOPS buffer and washed them with phosphate-buffered saline (PBS) with 0.1% Tween 20 buffer (PBST). The fixed embryos were then labeled with anti-acetylated tubulin antibody (Sigma, St. Louis, MO, USA) in a solution containing 0.5% blocking reagent (Roche, Basel, Switzerland), followed by Alexa Fluor 555 goat anti-mouse IgG antibody (Thermo Fisher Scientific, Waltham, MA, USA). Stained embryos were washed with PBST and then observed under a fluorescence microscope.

3. Reagent Treatments

As described in the Method section of Chapter 2, I prepared the reagent *all-trans* RA (Sigma-Aldrich, St Louis, CAS number: 302-79-4), N, N-diethylaminobenzaldehyde

(DEAB, Tokyo Chemical Industry, Tokyo, Japan, CAS number: 120-21-8) and RO41-5253 (RO, Focus Biomolecules, Plymouth Meeting, PA, USA, CAS number: 144092-31-9) in dimethyl sulfoxide (DMSO). I incubated the larvae in 2 mL of artificial seawater containing 2 μL of reagents or DMSO in 12-well plates at 14 °C. For the experiment without a substrate, 10 larvae were incubated in one well. Natural sands from Misaki (Miura, Kanagawa Prefecture, Japan) were used for the experiments conducted to induce metamorphosis. In these experiments, a single larva was cultured in one well to identify individuals. For cases in which reagent treatment continued for more than two days, I changed the seawater with the same concentration of reagents every other day.

I evaluated the attachment of larvae to the external substrates by an adhesive tuft as settlement and judged whether the larvae were metamorphosed by clear formation of the calyx, stalk and adhesive plate. From these observations, the numbers of individuals who settled and metamorphosed were counted. The rates of settlement and metamorphosis were calculated by dividing by the number of treated larvae and the number of settled larvae. I carried out experiments using two batches of larvae hatched from different adults. In particular, experiments were conducted once to several times in each batch.

4. Statistical Analysis

I examined the statistical analyses to evaluate differences in the effects of the treatments of substrate or reagents on settlement or metamorphosis as described in the Method section of Chapter 1.

890

891 **5. Construction of the Phylogenic Trees**

892 I obtained RA signaling-related genes (*raldh*, *rar*, and *rxr*) from the transcriptome data
893 assembled previously for the crinoid *Metacrinus rotundus* (Koga *et al.* 2016).
894 Reconstruction of phylogenic tree was conducted using same dataset which was described
895 in Chapter 2. The sequences for RA signaling-related genes of *M. rotundus* are shown in
896 Table 3-1.

897

898

899

Results

1. Incubation with Natural Substrates Stimulated the Metamorphosis of *A. serrata*

Doliolaria larvae of *A. serrata* hatch from the pinnular surface of adult specimens and swim in the water column using ciliary bands (Fig. 3-1A). As described in the same genus species, *A. mediterranea* (Barbaglio *et al.* 2012), doliolaria larvae of *A. serrata*, have five ciliary bands and an apical tuft that can be labeled by anti-acetylated tubulin antibody (Fig. 3-1B,C). Within a few days after hatching, larval development reaches a plateau, and larvae become competent for metamorphosis. Then, doliolaria larvae attach to a substrate with adhesive tufts and transition to cystidean larvae through development of calyx and adhesive plates, the elongation of stalks and the disappearance of ciliary bands (Fig. 3-1A–I). This process begins immediately after settlement, but it takes approximately two days for the stalk and other structures to be clearly observed. After metamorphosis completion, cystidean larvae transit to pentacrinoid larvae by the formation of tube feet, opening of the mouth and finally discard the stalk to become juveniles and start free-swimming life.

Although the metamorphosis process of several species of crinoid is described in detail (Barbaglio *et al.* 2012; Amemiya *et al.* 2016; Haig and Rouse 2008; Mladenov and Chia 1983), how the larvae of crinoids determine the proper site for settlement is still debated. Previously, it was reported that the larvae of feather star aggregate and settle to the bottom of dishes in the laboratory (Mladenov and Chia 1983). On the other hand, just as larvae of several species of feather stars respond to natural substrates such as fragments of shell and coral (Pearce 1997), the reception of environmental cues would be required

for their settlement. Here, I examined whether larvae of *A. serrata* can respond to environmental cues for metamorphosis by incubation of its doliolaria larvae with natural sands from the habitat of adult specimens (Miura, Kanagawa Prefecture, Japan).

I reared competent doliolaria larvae of *A. serrata* with or without substrates for six days and counted the number of individuals that metamorphosed during this period. I found that approximately 30% of larvae metamorphosed to cystidean larvae in the absence of substrates (17 of 60 larvae from two batches, Fig. 3-2A,C, Table 3-2). On the other hand, the number of metamorphosed larvae doubled in the presence of substrate (34 of 60 larvae from two batches, Fig. 3-2B,C, Table 3-2). Larvae settled to the bottom of the plates or substrates and normally metamorphosed to cystidean larvae through the development of calyx, stalk and adhesive plates (Fig. 3-2A,B). Significant differences in the metamorphosis ratios were observed between treatments ($P = 0.0423$, T-TEST). The response to the substrate did not differ between the Misaki and Onahama samples, as the metamorphosis ratio values were similar (Misaki: 13 of 20 larvae vs. Onahama: 21 of 40 larvae; Table 3-2), although a statistical analysis was not possible due to the small number of samples. These data suggest that the presence of environmental cues stimulated the commencement of metamorphosis.

2. Exogenous RA Treatment Induced the Metamorphosis of *A. serrata*

Next, I investigated the role of RA signaling in the metamorphosis of *A. serrata*. RA signaling plays a variety of developmental roles in chordates (Rhinn and Dolle 2012), through the synthesis of RA by RALDH (retinal dehydrogenase) and its binding to

receptors such as RAR (retinoic acid receptor) and RXR (retinoid x receptor) to regulate downstream gene expression (Rhinn and Dolle 2012; Marlétaz *et al.* 2006). Although I could not conduct a genomic survey of *A. serrata* due to poor genomic information on the species, I identified the RA signaling components in transcriptome data from the sea lily *M. rotundus* (single genes: *raldh*, *rar*, and *rxr*; Figs 3-3, 3-4), suggesting that the RA signaling machinery is conserved in the crinoid lineage.

First, I treated competent doliolaria larvae of *A. serrata* for four days, with exogenous *all-trans* RA (0.1 or 1 μM) without substrates (Fig. 3-1). I judged whether the larvae were metamorphosed by clear formation of the calyx, stalk and adhesive plate. In the control experiments (DMSO treatment), almost no larvae metamorphosed within four days after treatment (3 of 60 larvae from two batches, Fig. 3-5A, Table 3-3), whereas exogenous RA treatments induced the metamorphosis process, including the development of calyx, stalk and adhesive plates (0.1, 1 μM; 57, 59 of 60 larvae from two batches, respectively; Fig. 3-5D,G, Table 3-3). Metamorphosis was induced within 24 h after treatment and continued to proceed until 72–96 h after treatment so that the calyx and stalk were gradually more clearly observed (Fig. 3-6). Spicules were observed in the calyx and stalk of individuals in which metamorphosis was induced (Fig. 3-5D,E,G,H). The time scales of development and morphogenesis after the induction of metamorphosis by RA are similar to those of the transition of doliolaria larvae to cystidean larvae after settlement during normal development (Fig. 3-1 and Fig. 3-5). Therefore, the metamorphosis induced by RA without substrates was similar in structure and time scale to the metamorphosis in normal development, suggesting that RA is an endogenous

966 regulator of metamorphosis.

967

968 **3. Endogenous RA Synthesis is Required for the Metamorphosis of *A. serrata***

969 To test whether the endogenous synthesis of RA is necessary for the metamorphosis
970 process in *A. serrata*, I examined RALDH inhibitor (DEAB) treatment and its effects on
971 metamorphosis. As shown above, natural sand from the habitat of adult specimens
972 stimulated metamorphosis (Fig. 3-2). Thus, I treated larvae with DEAB (300 μM) in
973 seawater containing natural sand for six days and investigated its effect on settlement and
974 metamorphosis for up to six days after treatment. I evaluated attachment of larvae to the
975 external substrate by an adhesive tuft as settlement and judged whether the larvae were
976 metamorphosed by clear formation of the calyx, stalk, and adhesive plate.

977 In both the control (DMSO) and DEAB 300 μM treatment, doliolaria larvae
978 showed specific behaviors before metamorphosis, such as crawling around the substrate.
979 Then, up to six days after treatment, I found that most of the larvae normally settled to
980 substrates (DMSO; 29 of 36 larvae from two batches, DEAB; 31 of 36 larvae from two
981 batches, Fig. 3-7A,B,D, Table 3-4). I did not detect any significant differences in effect
982 on the settlement between treatments ($P = 0.45$, T-TEST). However, although 62% of the
983 larvae metamorphosed into cystidean larvae in the DMSO control (18 of 29 larvae from
984 two batches, Fig. 3-7D, Table 3-4), only a few larvae metamorphosed in the presence of
985 DEAB treatment (2 of 31 larvae from two batches, Fig. 3-7D, Table 3-4). DEAB inhibited
986 metamorphosis significantly ($P < 0.001$, T-TEST). These data suggest that endogenous
987 RA synthesis did not affect settlement but was required to commence metamorphosis.

988

989 **4. RA Binding with** RAR **is Required for the Metamorphosis of *A. serrata***

990 In a typical RA signaling pathway, the reception of RA by RAR has been shown to be
991 essential for signal transduction (Rhinn and Dolle 2012; Gutierrez-Mazariegos *et al.*
992 2014). Therefore, I examined whether the reception of RA by RAR is necessary to
993 commence metamorphosis. During the above experiment, I also treated larvae for six
994 days with the RARα antagonist, RO41-5253 (RO), which was used in the previous work
995 with starfish (Yamakawa *et al.* 2018). As in the DMSO or DEAB treatment, larvae treated
996 with RO 1 μM also showed specific behavior before metamorphosis, and most of them
997 settled on the substrate (28 of 36 larvae from two batches, Fig. 3-7C,D, Table 3-4). There
998 was no statistically significant difference in the effects on settlements between RO
999 treatment and control ($P = 0.308$, T-TEST). However, in the RO treatment, only a very
1000 small number of settled larvae were able to metamorphose (6 of 28 larvae from two
1001 batches, Fig. 3-7C, D, Table 3-4). Significant differences in the metamorphosis ratios
1002 were observed between treatments ($P < 0.001$, T-TEST).

1003 I also investigated whether RO treatment suppressed the induction of
1004 metamorphosis by RA treatment. As shown above, I found that treatment with 0.1 μM
1005 exogenous RA induced the metamorphosis of doliolaria larvae 72 h after treatment (16 of
1006 16 larvae from two batches, Fig. 3-8A, Table 3-5). Conversely, treatment of larvae with
1007 0.1 μM RA plus 1 μM RO did not induce metamorphosis in most larvae (2 of 16 larvae
1008 from two batches, Fig. 3-8B, Table 3-5). Although a statistical analysis was not possible
1009 due to the small number of samples in this experiment, the presence of RO suppressed

1010 metamorphosis. The effect of exogenous RA treatment on metamorphosis was examined
1011 96 h after the treatment (Fig. 3-5). Although the effects of exogenous RA, DEAB and RO
1012 treatments on metamorphosis were examined up to 96 h after the treatment, the RA + RO
1013 treatment had a fatal effect on larvae at 96 h after treatment, as the larval body swelled.
1014 Thus, the effect on metamorphosis was examined 72 h after RA + RO treatment.
1015 Nonetheless, my experimental data suggest that RA binding to RAR is required for the
1016 metamorphosis of *A. serrata*.
1017

Discussion

1. Metamorphosis Regulation by RA Signaling in the Ancestor of Living Echinoderms

In this study, I hypothesized that RA signaling mediates the metamorphosis process, including development of stalk and calyx, once environmental cues are received in feather stars (Fig. 3-9). Although my idea is supported by interfering with RA signaling at the levels of RA synthesis and RAR-activation (Rhinn and Dolle 2012), I recognize that my conclusion will become more robust after future studies, including testing if all trans-retinaldehyde, the RA precursor molecule, or other forms of RA, are able to promote metamorphosis. I also should determine if RA signaling is activated after settlement through a quantitative polymerase chain reaction analysis of downstream genes.

In addition, the disappearance of ciliary bands was independent of RA signaling, as the ciliary bands did not disappear in larvae in which metamorphosis was induced by exogenous RA treatment (Fig. 3-5). Therefore, other regulatory components must be investigated to understand the comprehensive regulatory mechanism of the metamorphosis of feather stars.

My findings support that metamorphosis was RA-dependent in the ancestors of extant echinoderms. Crinoids (feather star and stalked sea lily) are the most basal group of extant echinoderms, forming a sister group with Eleutherozoa including other echinoderm taxa (Telford *et al.* 2014). Both the feather star and stalked sea lily develop doliolaria-type larvae before settlement (Amemiya *et al.* 2015; Nakano *et al.* 2003), although it should be noted that the stalked sea lily develops semidoliolaria stages but not

1040 a full doliolaria stage (Amemiya *et al.* 2015). Thus, it is hypothesized that the ancestors
1041 of crinoids had a life cycle in which the doliolaria-type larvae metamorphosed into the
1042 cystidean larvae (Nakano *et al.* 2003). Namely, as shown in feather stars, it is suggested
1043 that metamorphosis is regulated by RA in the ancestor of crinoids. In addition, among the
1044 lineages of Eleutherozoa, I previously reported that the metamorphosis of starfish is
1045 regulated by RA signaling (Yamakawa *et al.* 2018). In both feather stars and starfish, RA
1046 signaling mediates the process of metamorphosis after receiving an environmental signal
1047 at settlement, suggesting that the developmental role of RA signaling is evolutionarily
1048 conserved. These findings support an ancient origin of RA-dependent metamorphosis
1049 during echinoderm evolution.

1050 Although echinoderms have evolved various larval morphologies in each
1051 lineage (McEdward 2001), the metamorphosis regulatory mechanisms might be
1052 evolutionarily conserved, as in feather star and starfish. In this context, I should especially
1053 focus on metamorphosis regulation in sea urchins, which acquired larval skeletons and
1054 evolved a pluteus larval form (McEdward 2001). The metamorphosis regulation in the
1055 sea urchin has been clarified in comparatively high detail (Heyland *et al.* 2018; Sutherby
1056 *et al.* 2012). Generally, thyroid hormone and histamine signaling modulate larval growth
1057 and competency acquisition, and nitric oxide signaling negatively controls the
1058 postsettlement process (Heyland *et al.* 2018; Sutherby *et al.* 2012). Despite the above
1059 findings, it has not been reported that RA signaling is involved in the metamorphosis
1060 regulation of sea urchin. In parallel with the investigations of metamorphosis regulaton,
1061 genomic survey revealed that the typical RALDH (Aldh1a family) genes are absent in the

genome of sea urchin (Cañestro *et al.* 2006).

Note that the above information does not necessarily indicate that the metamorphosis of sea urchin is independent of RA signaling. Rather, RA signaling is expected to be functional even in sea urchin because other RA signaling components such as RAR and RXR were identified (Cañestro *et al.* 2006). Furthermore, Aldh8 gene, which has the potential to synthesize RA, was also found in the genomic data of sea urchin, suggesting that RA signaling works in sea urchin without typical RALDH genes (Albalat 2009). Therefore, to deepen our understanding of the evolution of metamorphosis regulation in echinoderms, I suggest that it is important to investigate the role of RA signaling in the metamorphosis of sea urchins.

Finally, it would be interesting to know if RA signaling regulates the metamorphosis in sea cucumbers, which show gradual metamorphosis and a secondary bilateral axis (Smirnov 2014), as well as in sea urchins. By studying the function of RA signaling in various echinoderms, we can better understand the evolution of the echinoderm life cycle.

2. Life Cycle Evolution from the Viewpoint of RA Signaling

The evolution of planktonic larvae in marine invertebrates has attracted great interest from many zoologists (Jägersten 1972; Degnan and Degnan 2010). It has been hypothesized that the common ancestor of cnidarians and bilaterians had planktonic larvae based on the formation mechanism of an apical organ and body patterning (Marlow *et al.* 2014; Darras *et al.* 2011; Marlow *et al.* 2013; Range *et al.* 2013). Furthermore,

endogenous RA is reported to mediate strobilation and metamorphosis in jellyfish and starfish, respectively, once environmental cues are received (Fuchs *et al.* 2014; Yamakawa *et al.* 2018). My study also suggests that metamorphosis is regulated by RA signaling in echinoderm ancestors. Based on these findings, I hypothesized that RA has the function of transiting the life cycle in the common ancestor of cnidarians and bilaterians, suggesting that such functions have been co-opted to regulate strobilation and metamorphosis in cnidarians and echinoderms, respectively.

Further studies are required to reveal which processes RA regulates in the life cycle of the common ancestor. Although the life cycle evolution of cnidarians remains controversial, recent molecular phylogenic analyses support the polyp-first hypothesis, suggesting that the jellyfish stage is a derived feature in the lineage of cnidarians (Kayal *et al.* 2018). Thus, it is important to learn the ancestral function of RA signaling in cnidarians. In particular, it is of interest to investigate whether RA regulates the transition process of planktonic planula larva to sessile polyps in the ancestor of cnidarians, as in echinoderms. Previous studies with exogenous RA treatment provided insights into such functions. For example, Pennati *et al.* 2013 examined RA treatment in the planula larvae of the hydrozoan *Clava multicornis* and reported influence on the anterior-posterior positioning of peptidergic neurons but not on the induction of polyp. Nevertheless, the RA signaling machinery is lacking in the anthozoan and several lineages of hydrozoans, as no RXR genes have been identified in their genomic data (Fuchs *et al.* 2014). Because it is unclear whether *C. multicornis* has the RXR gene due to limited genetic information, it is difficult to reveal the ancestral function of RA in cnidarians through investigations

without genomic surveys. I suggest that future studies should re-examine the function of RA with species with the RXR gene.

3. Insight into the Ancestral Function of RA Signaling

Although my data illuminate the ancestral function of RA signaling in echinoderms as a regulator of life cycle transition, its validity throughout the animal kingdom still requires further assessments. In particular, the following two points should be noted. The first is a study by Handberg-Thorsager *et al.* 2018 using the marine annelid *Platynereis dumerilii*. This study clarified the detailed biochemical features of RA signaling and its developmental role in neurogenesis in *P. dumerilii*, suggesting that RAR ancestrally functions as a low-affinity sensor triggering neural differentiation (Handberg-Thorsager *et al.* 2018). This work reported no function in life cycle transition, although such a function might not be captured in their framework, which focused on the neurogenesis of embryos and the early nectochaete larval stage (Handberg-Thorsager *et al.* 2018). Namely, in *P. dumerilii*, it is reported that the late nectochaete larvae settle on external substrates and commence "settlement metamorphosis" to transition to the errant juvenile stage (Fischer *et al.* 2010). Therefore, I suggest that future studies should focus on the function of RA signaling in later stages, such as the late nectochaete larval stage or phase after settlement.

Second, in invertebrates of deuterostomes other than echinoderms, the regulation of metamorphosis by RA has not been reported. In particular, ascidians have a life cycle similar to that of many marine invertebrates, in which swimming larvae settle

to the bottom and begin sessile life (Cloney 1982). Furthermore, their metamorphosis regulatory mechanism has been clarified in detail (Karaiskou *et al.* 2015), although there are no reports that RA signaling functions as a regulator of metamorphosis control. Instead, it has been suggested that RA signaling functions conservatively with other chordates, such as in the regulation of Hox gene expression (Marlétaz *et al.* 2006). In this context, it is important to determine whether metamorphosis was regulated by RA in the ancestor of the deuterostomes. In particular, we should investigate the role of RA signaling in hemichordates, a sister group of echinoderms. Although the life cycle of hemichordates is similar to that of echinoderms, where planktonic tornaria larvae metamorphose to juveniles after settlement (Röttinger and Lowe 2012), it is unclear whether their metamorphosis is regulated by RA signaling.

As described above, my study showed that we can approach the origin of larvae and the life cycle evolution from the viewpoint of life cycle regulation. Further research on various animal groups should lead to a comprehensive understanding of life cycle evolution.

General Discussion

My findings shed the light into the evolution of regulatory mechanism for life cycle transition in animal kingdom. Molecular mechanisms for metamorphosis in amphibians and insects have been investigated in detail (Laudet 2011; Buszczak and Segraves 1998; Buszczak and Segraves 2000l Thummel 1995; Brown and Cai 2007). In both taxa, metamorphosis is regulated by hormones received by receptors (TH for amphibians and an ecdysone receptor for insects), which make a heterodimer with RXR. A recent study by Fuchs 2014 indicated that RXR is also involved in cnidarian life-cycle transition. Exogenous treatment with RA was shown to induce the metamorphic process of strobilation (Fuchs *et al.* 2014). Here, I present evidence that RA signaling is also involved in starfish and feather star metamorphosis. This provides additional evidence that components of RA signaling are conserved in the metamorphosis. However, amphibians and insects use different hormones for signaling, and RXR makes heterodimers with different counterparts accordingly (Laudet 2011; Buszczak and Segraves 1998; Buszczak and Segraves 2000l Thummel 1995; Brown and Cai 2007). Even in cnidarians, RXR is considered to make a heterodimer with different counterparts from amphibians, insects and echinoderms (Fuchs *et al.* 2014). Therefore, it is suggested that the gene function of RXR for life cycle regulation is highly conserved while its partner and ligand changed during evolutionary process. This hypothesis will be tested by further biochemical investigation of RA signaling machinery in echinoderms. For example, it needs to be revealed if RAR/RXR heterodimer is required for the metamorphosis. Moreover, this hypothesis should be tested by the investigation of

1166 metamorphosis in various taxa, such as sea urchins, annelids and molluscs.

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Tables

Table 2-1 Accession numbers for the genes used for phylogenic analysis

Species	gene	accession number	gene	accession number
Hs; *Homo sapiens*	*aldh1a1*	P00352.2	*thra*	P10827
	aldh1a2	O94788	*thrb*	P10828
	aldh1a3	P47895	*rara*	P10276
	aldh1b1	P30837	*rarb*	P10826
	aldh2	P05091	*rarg*	P13631
	aldh3a1	P30838	*rxra*	P19793
	aldh3a2	P51648	*rxrb*	P28702
	aldh3b1	P43353	*rxrg*	P48443
	aldh3b2	P48448		
	aldh4a1	P30038		
	aldh5a1	P51649		
	aldh6a1	Q02252		
	aldh7a1	P49419		
	aldh8a1	Q9H2A2		
	aldh9a1	P49189		
Mm; *Mus musculus*			*thra*	P63058
			thrb	P37242
			rara	P11416
			rarb	P22605
			rarg	P18911
			rxra	P28700
			rxrb	P28704
			rxrg	P37238

Xt; *Xenopus tropicalis*	*aldh1a1*	Q4VBE1		
	aldh1a2	Q9DEX5		
	aldh1a3	F7BV06		
	aldh1b1	F7DQF8		
	aldh2	Q6DJ49		
	aldh3a2	B1WBI3		
	aldh3b2	F6X8Y6		
	aldh4a1	A4QNJ0		
	aldh5a1	F6QFQ2		
	aldh6a1	F6SRL8		
	aldh7a1	F7BQF6		
	aldh8a1	F6UH88		
	aldh9a1	F6VC33		
Dr; *Danio rerio*	*aldh1a2*	Q90XS8	*thraa*	Q98867
	aldh1a3	Q0H2G3	*thrab*	U3JAT9
	aldh2a	Q8QGQ2	*thrb*	Q9PVE4
	aldh2b	Q6TH48	*raraa*	Q90271
	aldh3a1	X1WBM4	*rarab*	Q7ZTI3
	aldh3a2a	A0A2R8PW97		
	aldh3a2b	E9QH31	*rarga*	Q91392
	aldh3b1	Q90ZZ7	*rargb*	A2T928
	aldh4a1	Q7SY23		
	aldh5a1	A0A0R4IIB7		
	aldh6a1	Q6DHT4		
	aldh7a1	Q803R9		
	aldh8a1	Q66I21		

	aldh9a1a1	Q7ZVB2		
	aldh9a1a2	B0S7W5		
	aldh9a1b	Q802W2		
Bf; *Branchiostoma floridae*	*aldh1a_1*	C3ZGK4	*rxr*	Q8MX78
	aldh1a_2	C3ZG63		
Bl; *Branchiostoma lanceolatum*			*rar*	O18608
Ci; *Ciona intestinalis*	*aldh1a_1*	A0A1W2WB51	*rar*	Q4H2W1
	aldh1a_2	A0A1W5BCT1	*rxr*	Q4H2U9
	aldh1a_3	A0A1W2WDC1		
	aldh2	A0A1W5B7N8		
Pm; *Polyandrocarpa misakiensis*			*rxr*	K7ZLP3
Sk; *Saccoglossus kowalevskii*	*aldh1a_1*	XP_006823779.1	*rar*	XP_002742241.1
	aldh1a_2	XP_006822197.1	*rxr*	D2XNK4
	aldh1a_3	XP_002736989.1		
	aldh1a_4	XP_002731204.1		
	aldh1a_5	XP_006824634.1		
	aldh2	XP_006816163.1		
Sp; *Strongylocentrotus purpuratus*	*aldh2_1*	SPU_007284	*thr*	SPU_025239
	aldh2_2	SPU_023801	*rar*	SPU_016523

	aldh5a1_1	SPU_007492.1	*rxr*	SPU_028422
	aldh5a1_2	SPU_016767.1		
	aldh6a1	SPU_026493.1		
	aldh7	SPU_024895.3a		
	aldh8a1_1	SPU_017403.1		
	aldh8a_2	SPU_000522.1		
	aldh9	SPU_002901.3a		
Pp; *Patiria pectinifera*	*raldha*	LC379260	*rar*	LC379258
	raldhb	LC379261	*rxr*	LC379259
	raldhc	LC379262	*thr*	*
	aldh2	*		
Al; *Astropecten latespinosus*	*raldha*	LC485972	*rar*	LC485975
	raldhb	LC485973	*rxr*	LC485976
	raldhc	LC485974		
Dm; *Drosophila melanogaster*			*usp*	P20153
Rc; *Reishia clavigera*			*rar*	T2HRZ4
			rxr	E9RHD8
Ls; *Lymnaea stagnalis*			*rar*	D5LIR6
			rxr	Q5I7G2
Tc; *Tripedalia cystophora*			*rxr*	O96562

Table 2-2 Sequences of primer for amplification of *raldha*, *raldhb*, *raldhc*, *rar*, and *rxr*

We used 40-bp reverse primers including a 20-bp T3 promoter sequence to synthesize Dig-labelled RNA probes for *in situ* hybridization. Capital characters mean consensus sequence for T3 promoter.

	Forward Primer (5'→3')	Reverse Primer including T3 promoter region (5'→3')
raldha	gcaaccgatcgtcttcagaaggcacacatt	ATTAACCCTCACTAAAGGGAgcagaaaccacgtcttgtat
raldhb	accatcaatccggcaactggggagaagata	ATTAACCCTCACTAAAGGGAatattcataaaacgcacaca
raldhc	gacggtgatttcttctgctactcccgctac	ATTAACCCTCACTAAAGGGAtagttgttgacccagatcac
rar	gcgttacaccaaggtcccaacaacatgtcc	ATTAACCCTCACTAAAGGGAtacatggcatgaagtgttga
rxr	gtaaaggtcggcattctcctgaccagtgct	ATTAACCCTCACTAAAGGGAatcagcttgaagaagaagag

Table 2-3 Number of metamorphosed/treated larvae of each batch under seawater with and without substrate

number of metamorphosed / treated larvae

	Substrate (+)					Substrate (-)				
age	batch 1		batch 2		batch 3	batch 1		batch 2		batch 3
well	1	2	1	2	1	1	2	1	2	1
48hpf	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
72hpf	2/10	3/10	0/10	1/10	1/10	0/10	0/10	0/10	0/10	0/10
96hpf	8/10	8/10	7/10	8/10	4/10	0/10	1/10	2/10	1/10	1/10
120hpf	8/10	8/10	7/10	8/10	4/10	2/10	2/10	2/10	3/10	2/10
144hpf	8/10	8/10	8/10	8/10	4/10	3/10	2/10	6/10	4/10	4/10
168hpf	8/10	8/10	8/10	8/10	4/10	3/10	4/10	6/10	4/10	4/10
192hpf	8/10	8/10	8/10	8/10	4/10	3/10	4/10	6/10	4/10	4/10

Table 2-4 Number of metamorphosed larvae/treated larvae of each batch in retinoic acid (RA) or dimethyl sulfoxide (DMSO) treatment

number of metamorphosed / treated larvae				
treatment	batch 1		batch 2	batch 3
well	1	2	1	1
RA	8/10	8/10	7/10	9/10
DMSO	1/10	0/10	0/10	0/10

Table 2-5 Number of metamorphosed larvae/treated larvae of each batch in retinoic acid (RA) or dimethyl sulfoxide (DMSO) treatment in the case that treatment was commenced at 24 hours postfertilization (hpf)

number of metamorphosed / treated larvae

treatment	RA			DMSO		
age	batch 1	batch 2	batch 3	batch 1	batch 2	batch 3
well	1	1	1	1	1	1
48hpf	0/10	0/10	0/10	0/10	0/10	0/10
72hpf	0/10	1/10	2/10	0/10	0/10	0/10
96hpf	3/10	6/10	6/10	0/10	0/10	1/10

Table 2-6 Number of metamorphosed larvae/treated larvae of each batch in retinoic acid (RA) or dimethyl sulfoxide (DMSO) treatment in the case that treatment was commenced at 48 hours post-fertilization (hpf)

number of metamorphosed / treated larvae

age	RA				DMSO			
treatment	batch 1		batch 2	batch 3	batch 1		batch 2	batch 3
well	1	2	1	1	1	2	1	1
72hpf	1/10	0/10	0/10	1/10	0/10	0/10	0/10	0/10
96hpf	7/10	2/10	5/10	5/10	0/10	0/10	0/10	0/10

Table 2-7 Number of metamorphosed larvae/treated larvae of each batch in DEAB or dimethyl sulfoxide (DMSO) treatment

number of metamorphosed / treated larvae				
treatment	batch 1		batch 2	batch 3
well	1	2	1	1
DEAB	0/10	4/10	2/10	0/10
DMSO	6/10	9/10	8/10	3/10

Table 2-8 Number of metamorphosed larvae/treated larvae of each batch in RO41-5253 (RO) or dimethyl sulfoxide (DMSO) treatment

number of metamorphosed / treated larvae				
treatment	batch 1		batch 2	batch 3
well	1	2	1	1
RO	0/10	0/10	0/10	0/10
DMSO	8/10	8/10	9/10	2/10

Table 2-9 Number of metamorphosed larvae/treated larvae of each batch in retinoic acid (RA) or retinoic acid plus RO41-5253 (RA1RO) treatment

number of metamorphosed / treated larvae				
treatment	batch 1		batch 2	batch 3
well	1	2	1	1
RA	8/10	8/10	9/10	6/10
RA+RO	1/10	2/10	1/10	1/10

Table 3-1 The sequences of *raldha*, *rar* and *rxr* retrieved from the sea lily *Metacrinus rotundus* for phylogenetic analysis

Mr	MNLQLVFSRPFLLSVNRGFFVHSYSMSQLNSAPEVKFTQLFINNEFVNSVSGKTFPTLNPCTGEKICDVQEGDKA
R	DVDLAVKAAREAFKLGSPWRRLDPTKRAKHMTKLAELLEQNKDQLSALETLDNGMPYFESQMWVDSFVNTLT
AL	YFAGWCDKVHGKTIPIDGDYFCYTKHEPVGVCGAIIPWNYPMDMLGWKVGPALACGNTMVIKPAEQTPLTALH
D	IASLIKEAGFPPGVINIIPGYGPTAGAAISEHMDVDKVAFTGSTEVGRLIQQAAGKSNLKRVALELGGKSPNIVFAD
H	SDLDFAVDEAHEAVMCNEGQCCSAGSRTFVQEGIYDEFVKKSIEMAKARVIGDPYVEGTQSGPQIDEEQFTKVL
A	EKIKSGKNEGATLGCGGSRHGDKGFFLESTVFSDVSDEMSIAQEEIFGPVQVILKFKTIEEVIERAHKTHYGLAGA
	VFTKDIDTAMTVAHSLSAGTVWVNCYNVGGPQTPFGGYKQSGVGRDLGEDSLKEYYEVKTVIIKVPQKNS
M	FTNGLTLSHEQLKSGGFGALLDTIFQFAGALSKMKIDETEVSLLGAICLISSDRSGLKDPIKIEKMQEPLLEGLRYY
rR	VRKRRPLEPHIFAKILMKITD
A	
R	
M	IAAFSHRSIAVTDGILLATGLHVHRNSAHTAGVGTIFDRVLTELVAKMREMRMDKTELGCLRAIVLFNPDAKNLT
rR	SVQKVEELREKVYASLEEYCRTQYPEESGRFAKLLLRLPALRSIGLKCLEHLFFFKLIGDTPIDTFLMEMLEAPNN
X	S
R	

Table 3-2 Number of metamorphosed/treated larvae of each batch in substrate treatment experiment

	Substrate (+)		Substrate (-)	
	number	metamorphosis	number	metamorphosis
batch 1-1	10	8	10	1
batch 1-2	10	5	10	0
batch 2-1	10	8	10	2
batch 2-2	10	4	10	4
batch 2-3	10	4	10	4
batch 2-4	10	5	10	6
Total	60	34	60	17

Table 3-3: Number of metamorphosed/treated larvae of each batch in RA treatment experiment

	DMSO		RA 0.1 μM		RA 1 μM	
	number	metamorphosis	number	metamorphosis	number	metamorphosis
batch 1	20	2	20	20	20	20
batch 2-1	10	0	10	9	10	10
batch 2-2	10	0	10	8	10	10
batch 2-3	10	0	10	10	10	9
batch 2-4	10	1	10	10	10	10
Total	60	3	60	57	60	59

Table 3-4: Number of settled or metamorphosed/treated larvae of each batch in DEAB or RO treatment experiment

	DMSO			DEAB			RO		
	treatment	settlement	metamorphosis	treatment	settlement	metamorphosis	treatment	settlement	metamorphosis
Batch 1	12	8	5	12	12	1	12	9	2
Batch 2-1	12	11	7	12	9	1	12	10	4
Batch 2-2	12	10	6	12	10	0	12	9	0
Total	36	29	18	36	31	2	36	28	6

Table 3-5: Number of metamorphosed/treated larvae of each batch in RA+RO treatment experiment

	RA 0.1 µM		RA 0.1 µM + RO 1 µM	
	number	metamorphosis	number	metamorphosis
batch 1	10	10	10	0
batch 2	6	6	6	2
Total	16	16	16	2

Figures

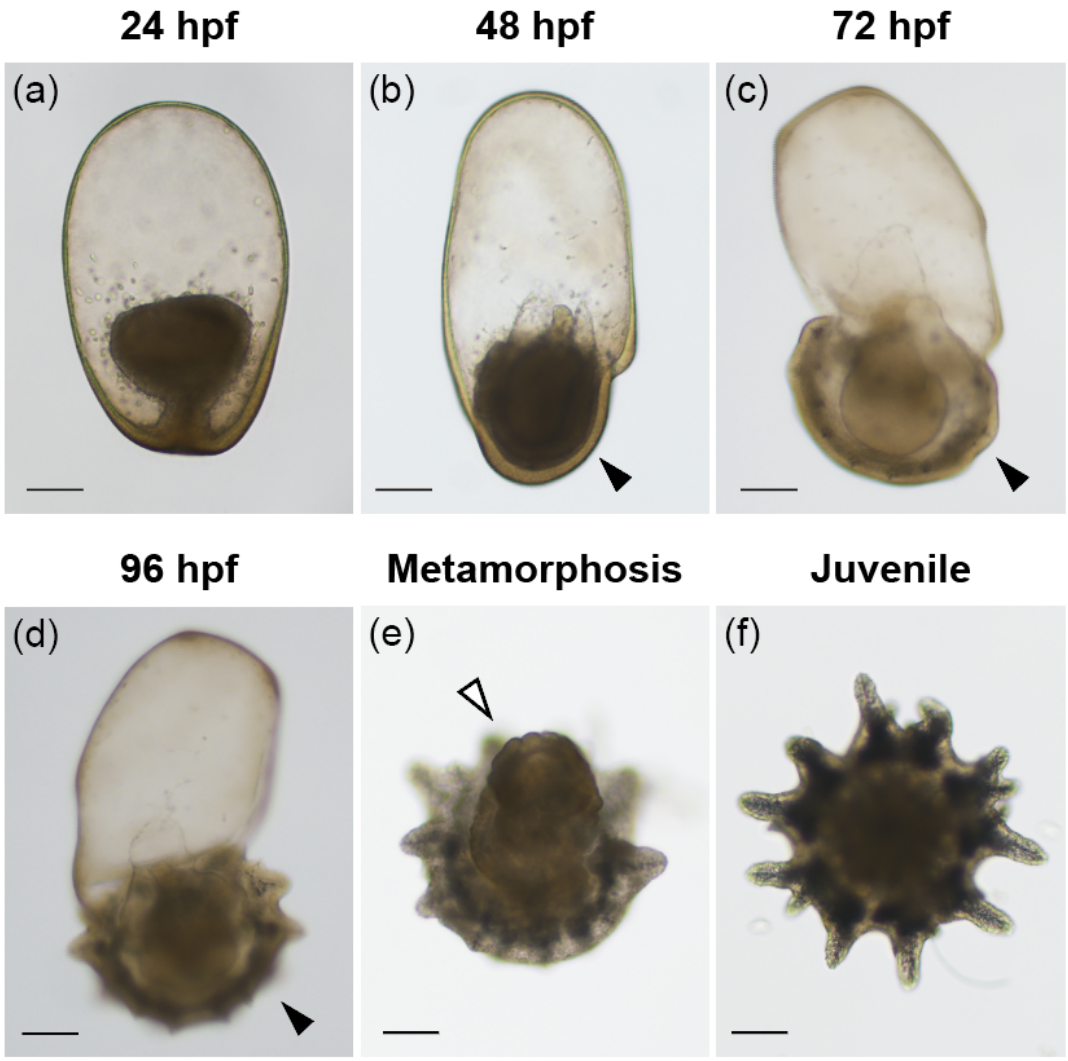

Fig. 2-1 Developmental process of *Astropecten latespinosus*

(a–d) Larvae at 24, 48, 72, and 96 hours postfertilization (hpf), respectively. After the larvae commence metamorphosis, the juvenile rudiments develop; the larval bodies are absorbed, as in (e); and the larvae finally transition to juveniles (f). The black arrowheads indicate the juvenile rudiment, and the white arrowhead indicates the absorbed larval body. Scale bars: 100 mm.

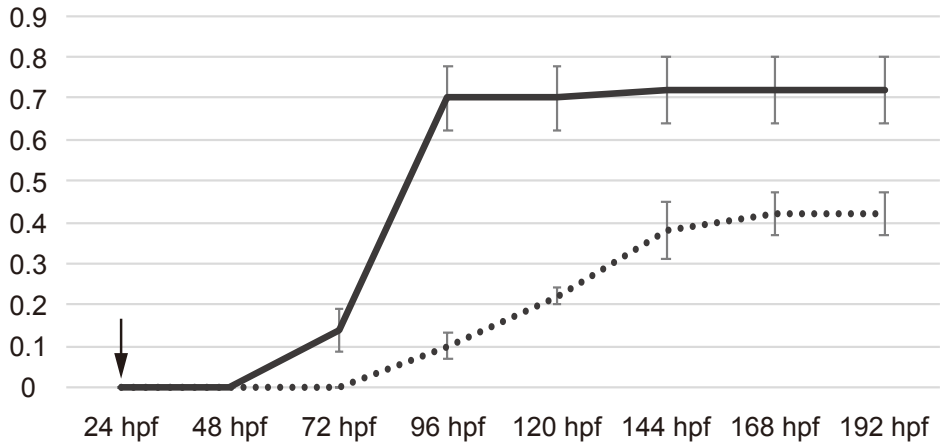

Fig. 2-2 Effect of culture with natural sand from habitat on metamorphosis

Ten *Astropecten latespinosus* larvae were incubated with natural sand in 2 mL of artificial seawater (Marin-Tech, Aichi, Japan) in 12-well plates at 22 ℃. Metamorphosis ratios were recorded every 24 hours after treatment. The solid and dotted lines indicate the ratios under incubation of larvae with and without natural sand from their habitat, respectively. The arrow indicates the time of treatment commencement. hpf, hours post-fertilization.

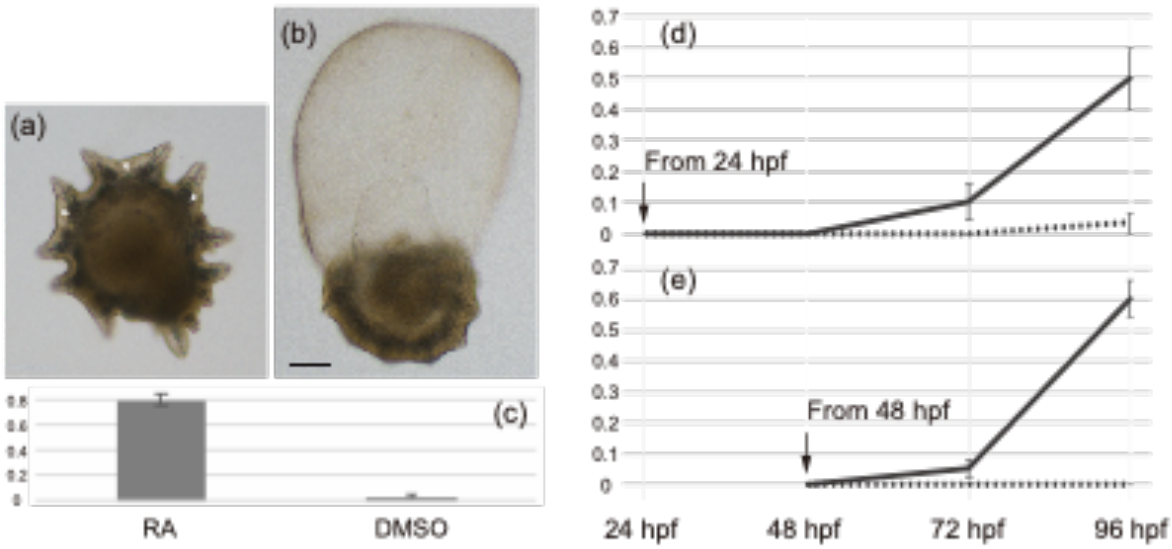

Fig. 2-3 Metamorphosis induction by exogenous retinoic acid (RA) treatment in *Astropecten latespinosus*

Reagent treatment experiments were conducted as in our previous work (11). (a) Metamorphosis induced by RA. (b) Control larvae treated with dimethyl sulfoxide (DMSO). (c) Metamorphosis induction ratios under the RA and DMSO treatments obtained 24 hours after treatment. The scale bars indicate 100 mm, and the arrows indicate the primary podia. (d, e) Metamorphosis induction ratios under the RA (solid lines) and DMSO (dotted lines) treatments obtained every 24 hours after the commencement of treatment of 24- and 48-hours post-fertilization (hpf) larvae, respectively.

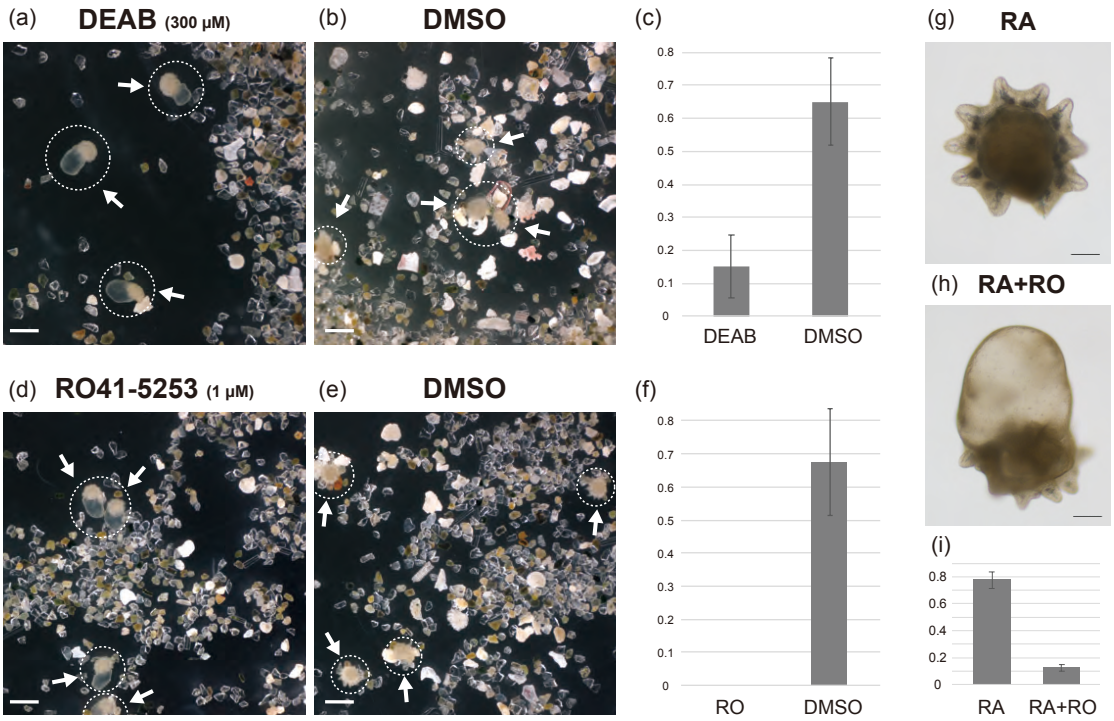

Fig. 2-4 Effect of inhibition of retinoic acid (RA) synthesis or RA binding to retinoic acid receptor (RAR) on metamorphosis.

(a) *Astropecten latespinosus* larvae in which metamorphosis was inhibited by N,Ndiethylaminobenzaldehyde (DEAB) (300 mmol /L) treatment. Under the dimethyl sulfoxide (DMSO) treatment, larvae correctly completed metamorphosis (b). (c) Metamorphosis ratios under the DEAB (300 mmol /L) and DMSO treatments. (d, e) Metamorphosis-inhibited larvae and completed juveniles under the RO41-5253 (RO; 1 mmol /L) and DMSO treatments, respectively. (f) Metamorphosis ratios under the RO (1 mmol /L) and DMSO treatments. Larvae treated with RA (1 mmol /L) (g) and RA (1

mmol /L) plus RO (1 mmol /L) (h). (i) Metamorphosis ratios. The dotted circles and arrows indicate larvae and juveniles, respectively. The scale bars in (a–e) and (g, h) indicate 500 and 100 mm, respectively

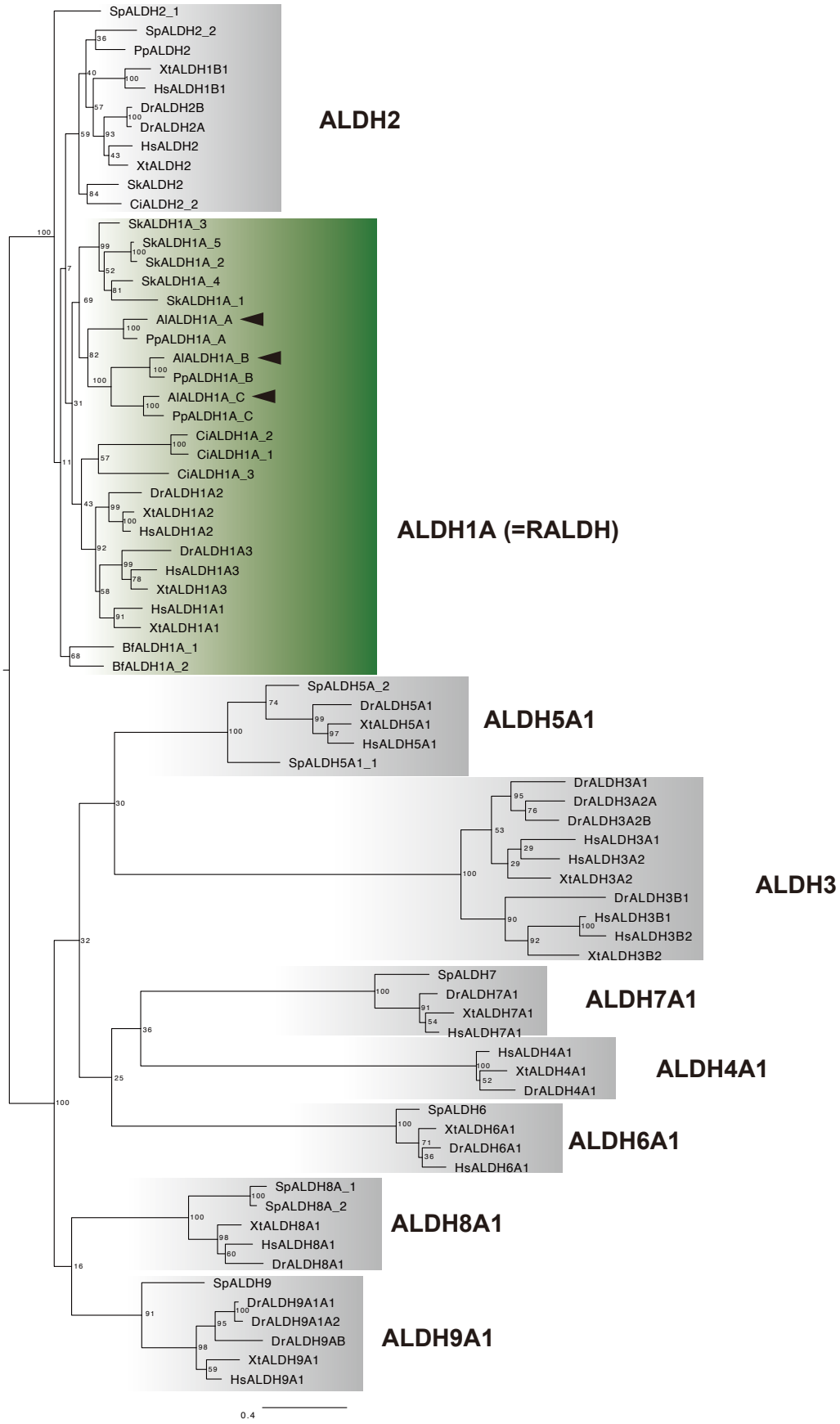
SpALDH2_1
SpALDH2_2
PpALDH2
XtALDH1B1
HsALDH1B1
DrALDH2B
DrALDH2A
HsALDH2
XtALDH2
SkALDH2
CiALDH2_2
ALDH2
SkALDH1A_3
SkALDH1A_5
SkALDH1A_2
SkALDH1A_4
SkALDH1A_1
AlALDH1A_A
PpALDH1A_A
AlALDH1A_B
PpALDH1A_B
AlALDH1A_C
PpALDH1A_C
CiALDH1A_2
CiALDH1A_1
CiALDH1A_3
DrALDH1A2
XtALDH1A2
HsALDH1A2
DrALDH1A3
HsALDH1A3
XtALDH1A3
HsALDH1A1
XtALDH1A1
BfALDH1A_1
BfALDH1A_2
ALDH1A (=RALDH)
SpALDH5A_2
DrALDH5A1
XtALDH5A1
HsALDH5A1
SpALDH5A1_1
ALDH5A1
DrALDH3A1
DrALDH3A2A
DrALDH3A2B
HsALDH3A1
HsALDH3A2
XtALDH3A2
DrALDH3B1
HsALDH3B1
HsALDH3B2
XtALDH3B2
ALDH3
SpALDH7
DrALDH7A1
XtALDH7A1
HsALDH7A1
ALDH7A1
HsALDH4A1
XtALDH4A1
DrALDH4A1
ALDH4A1
SpALDH6
XtALDH6A1
DrALDH6A1
HsALDH6A1
ALDH6A1
SpALDH8A_1
SpALDH8A_2
XtALDH8A1
HsALDH8A1
DrALDH8A1
ALDH8A1
SpALDH9
DrALDH9A1A1
DrALDH9A1A2
DrALDH9AB
XtALDH9A1
HsALDH9A1
ALDH9A1
0.4

Fig. 2-5 Maximum likelihood tree of the aldehyde dehydrogenase (ALDH) family

We constructed the tree of the aldehyde dehydrogenase (aldh) gene family to identify *raldh* (aldh1a) genes in *Astropecten latespinosus*. In these trees, the phylogeny was not clearly dissolved in the clade including *raldh*, aldh1b, and aldh2, although we found that *raldh* genes of starfish (*A. latespinosus* and *Patiria pectinifera*) made a clade with *raldh* genes of a hemicordate previously identified with comparatively high support value.

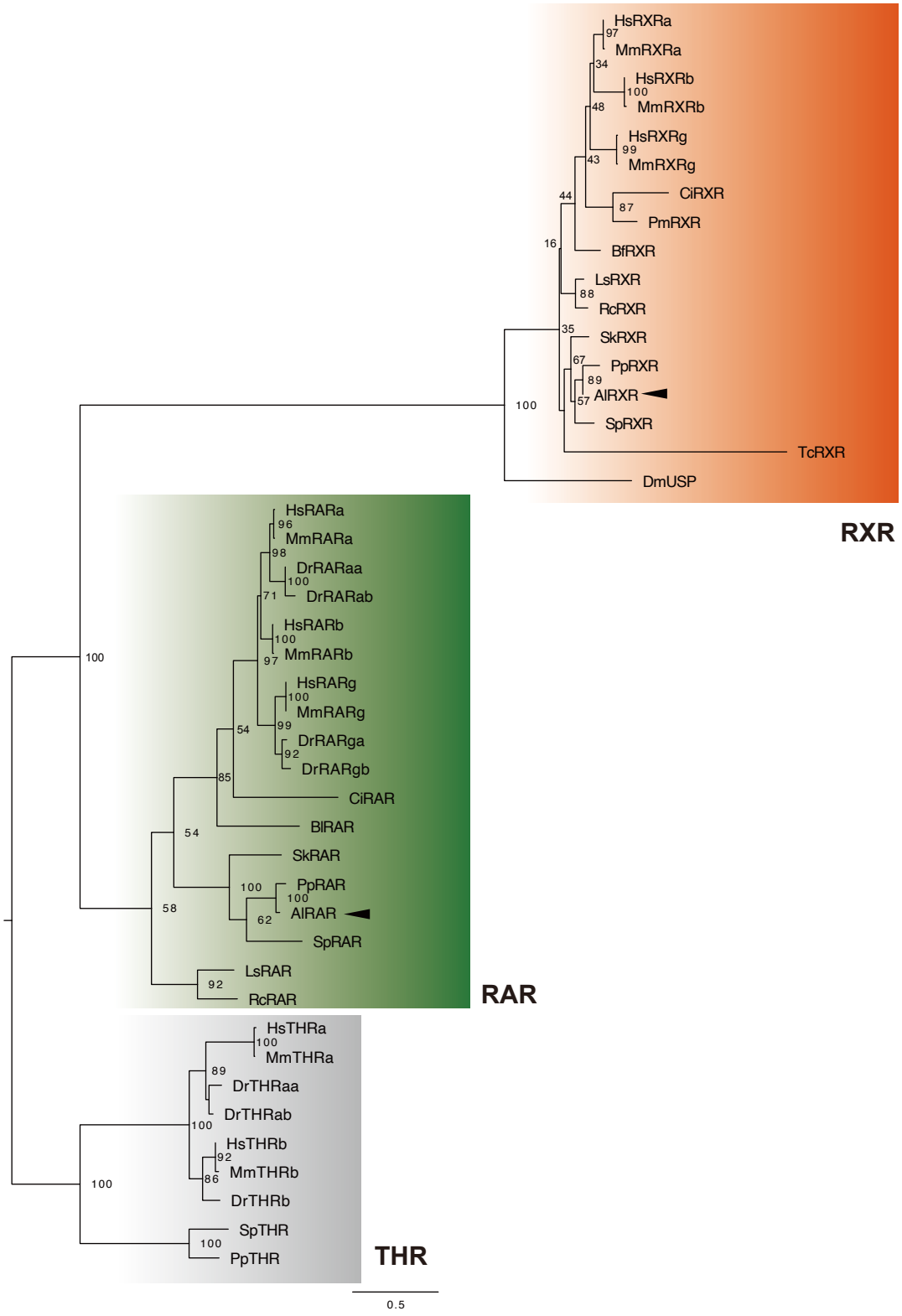
HsRXRa
MmRXRa
HsRXRb
MmRXRb
HsRXRg
MmRXRg
CiRXR
PmRXR
BfRXR
LsRXR
RcRXR
SkRXR
PpRXR
AlRXR
SpRXR
TcRXR
DmUSP
RXR
HsRARa
MmRARa
DrRARaa
DrRARab
HsRARb
MmRARb
HsRARg
MmRARg
DrRARga
DrRARgb
CiRAR
BlRAR
SkRAR
PpRAR
AlRAR
SpRAR
LsRAR
RcRAR
RAR
HsTHRa
MmTHRa
DrTHRaa
DrTHRab
HsTHRb
MmTHRb
DrTHRb
SpTHR
PpTHR
THR
0.5

Fig. 2-6 Maximum likelihood tree of retinoic acid receptor (RAR), retinoid X receptor (RXR), and thyroid hormone receptor (THR)

In phylogenic trees of *rar*, *rxr*, and outgroup thr, we found that *rar* and *rxr* of *Astropecten latespinosus* were respectively positioned in the clade of *rar* and *rxr*.

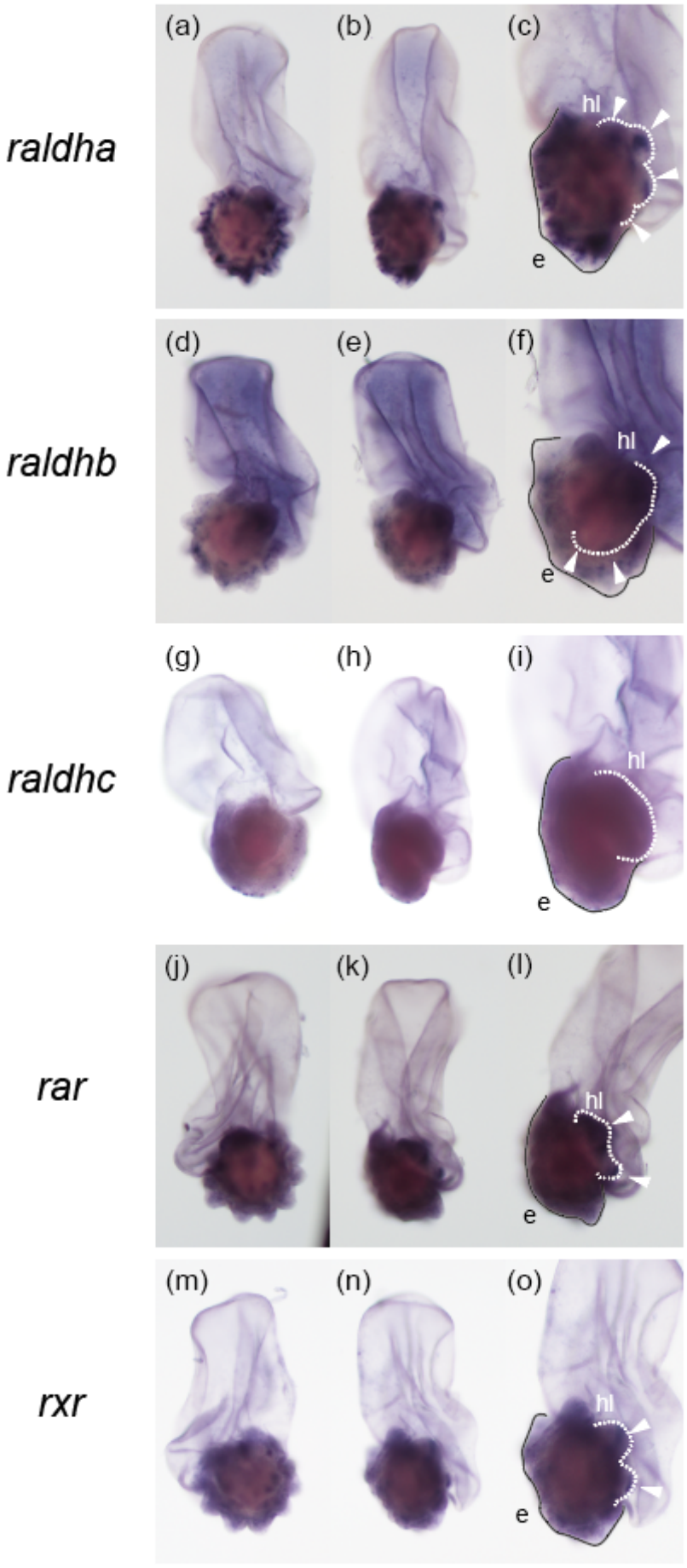
raldha
raldhb
raldhc
rar
rxr
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)
(n)
(o)
hl
e

Fig. 2-7 Spatial expression pattern of retinoic acid (RA) signaling components.

(a–c), (d–f), (g–i), (j–l), and (m–o) show the expression patterns of *raldha*, *raldhb*, *raldhc*, *rar*, and *rxr*, respectively, in 72-hours postfertilization (hpf) larvae. In (c), (f), (i), (l), and (o), white dotted lines and black solid lines indicate hydrolobes (hl) and epidermis of juvenile rudiment (e), respectively. Arrowheads point toward the expression in hydrolobes.

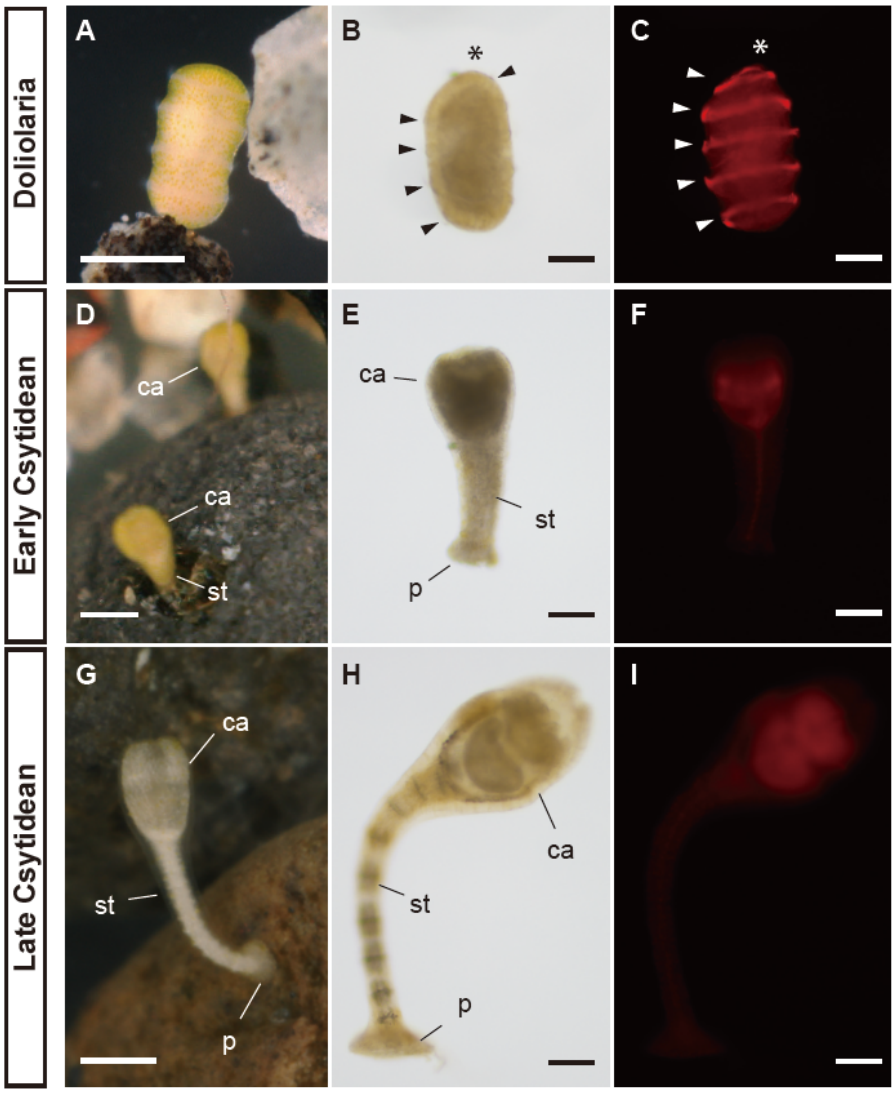

Fig. 3-1 Development process and localization of ciliary bands in the feather star *A. serrata*

(A,D,G) show the living specimens of *A. serrata* larvae. Competent doliolaria larvae settle to the substrate with the apical tuft (A), then commence the metamorphosis process to transit to the csytidean larval phase (D, G; approximately two, four days after settlement, respectively). (D) shows the early cystidean larvae just after metamorphosis

commenced. Calyx (ca), stalk (st) and adhesive plate (p) can be clearly observed in a few days after metamorphosis (G). (B,C,E,F,H,I) indicates the fixed embryos labeled with anti-acetylated tubulin antibody in doliolaria, early cystidean and late cystidean larvae, respectively (light field; B, E and H, observation of fluorescence; C, F and I). The specific fluorescence in ciliary bands (arrow heads) and apical tuft (asterisk) were observed in doliolaria larvae (C), whereas no specific fluorescence was observed in cystidean larvae (F,I). Scale bars: 250 μm (A,D,G), 100 μm (B,C,E,F,H,I).

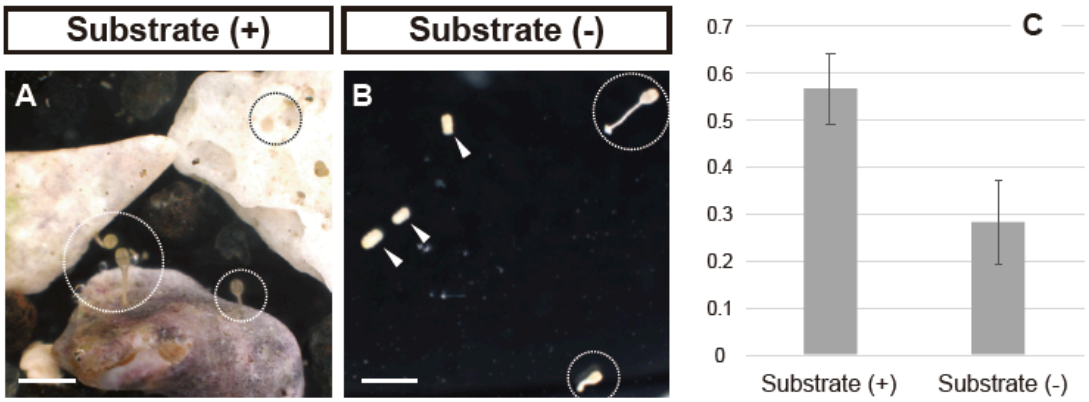

Fig. 3-2 Effects of the presence or absence of substrates on settlement and metamorphosis

(A,B) indicate the doliolaria (arrowheads) or cystidean (dotted line circles) larvae incubated for six days with or without substrate, respectively. Scale bars: 1 mm. (C) shows the metamorphosis ratio for each treatment.

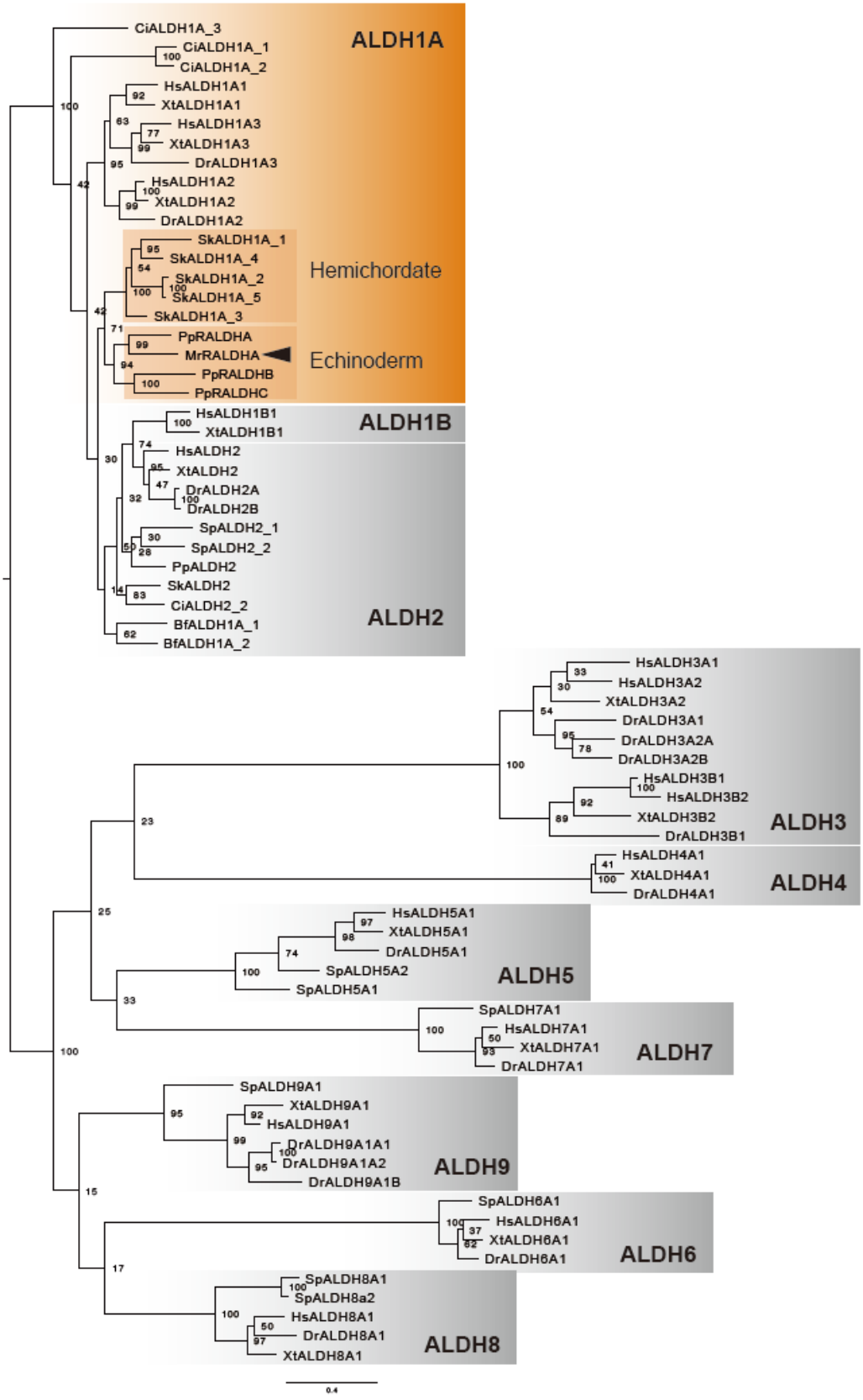

Fig. 3-3 The phylogenic tree of ALDH gene family

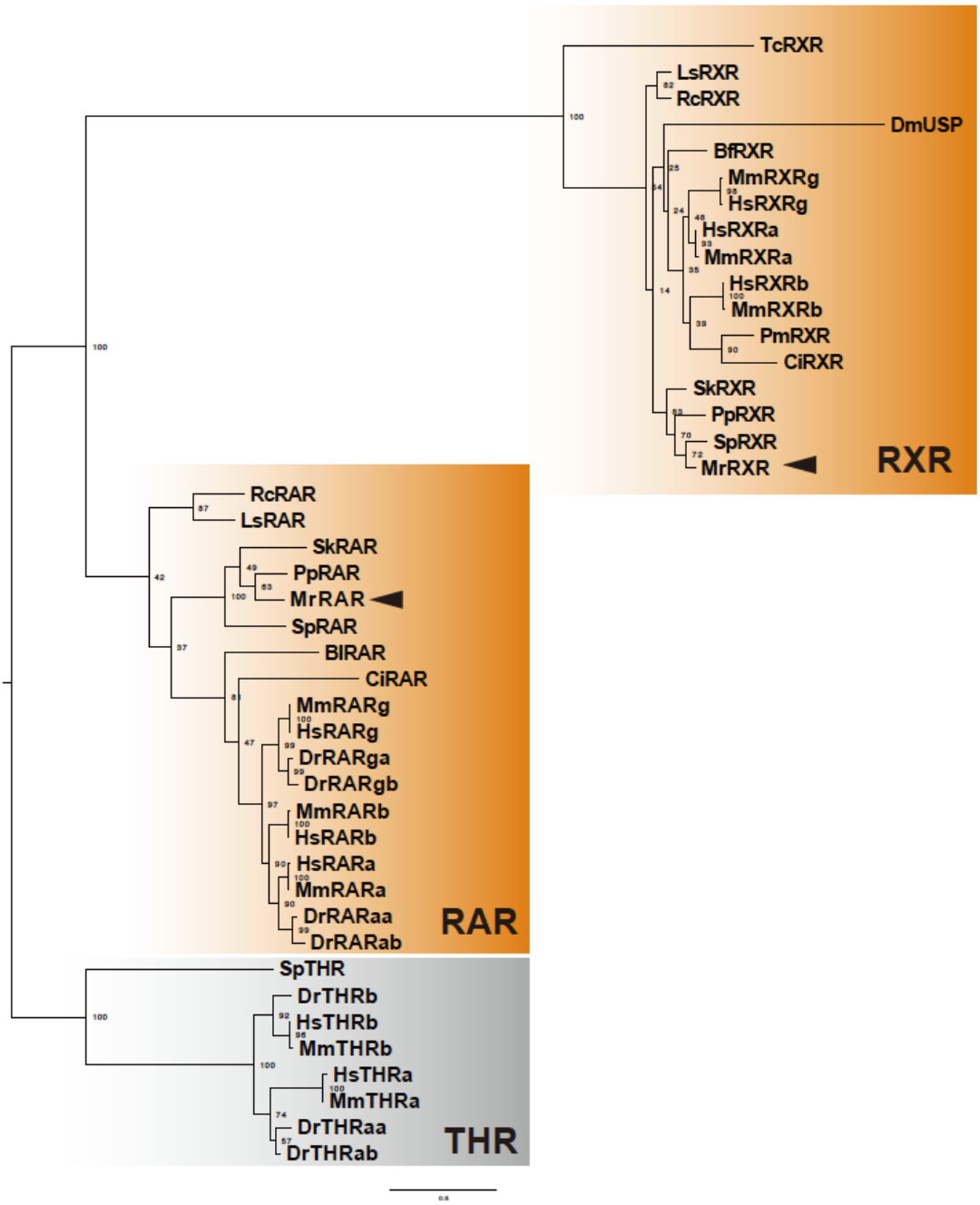

Fig. 3-4 The phylogenic tree of RAR, RXR and THR (Thyroid hormone receptor)

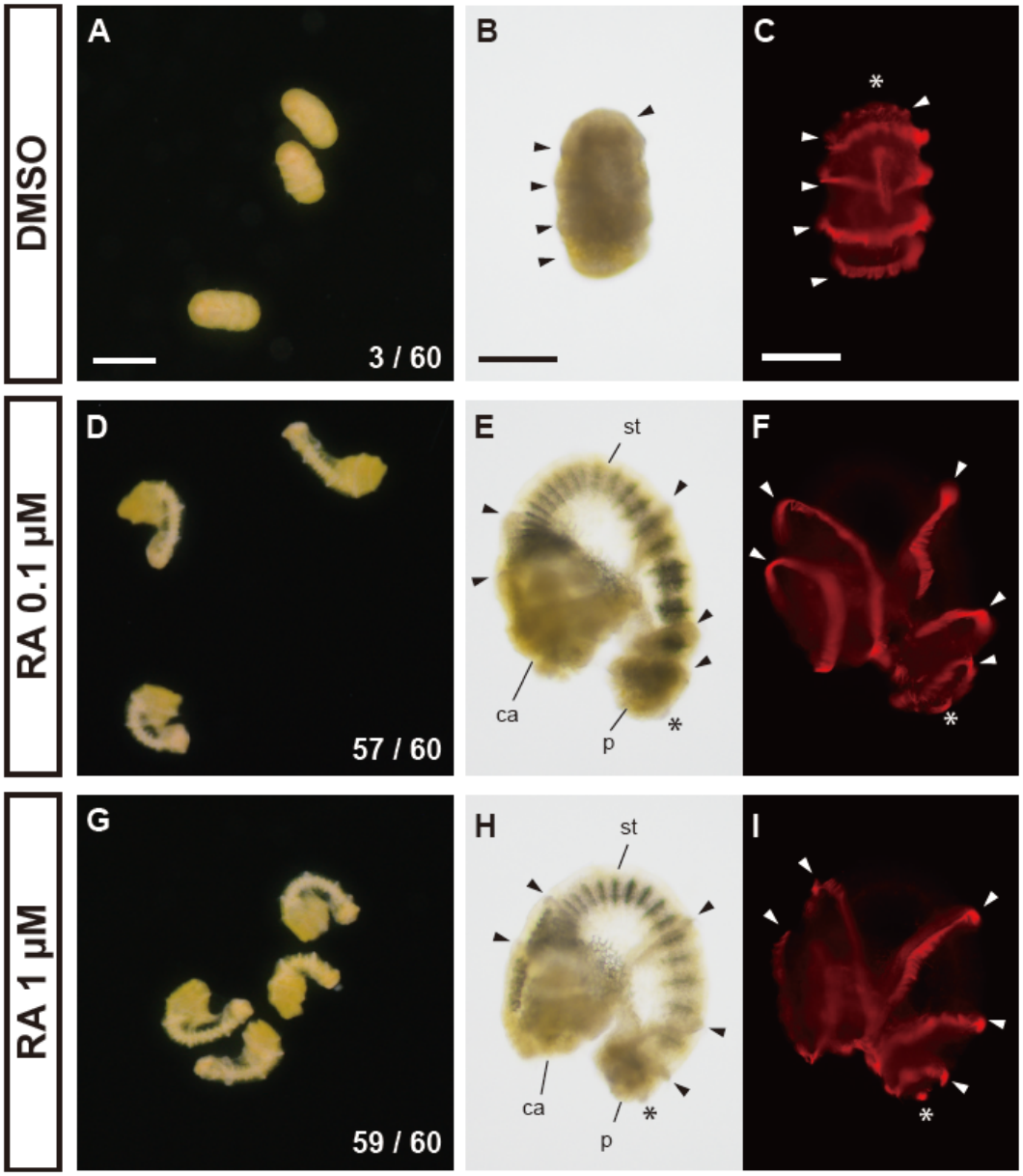

Fig. 3-5 Induction of metamorphosis by exogenous RA treatment

(A–I) respectively show the larvae treated for 96 h with DMSO, RA 0.1 µM and RA 1 µM. While almost all doliolaria larvae did not metamorphose to cystidean larvae (A), metamorphosis was induced by the treatment of RA 0.1 µM and RA 1 µM (D and G, respectively). The numbers in (A,D,G) refer to “the number of metamorphosed larvae” /

“the number of treated larvae”. (B,C,E,F,H,I) indicate the fixed larvae labeled with anti-acetylated tubulin antibody after DMSO, RA 0.1 μM and RA 1 μM treatment, respectively (light field; B, E and H, observation of fluorescence; C, F and I). In RA treatment, metamorphosis was induced as the calyx (ca), stalk (st) and adhesive plate (p) can be clearly observed, whereas ciliary bands (arrowheads) and apical tuft (asterisk) did not disappear (E,F,H,I) like in doliolaria larvae with DMSO treatment (C). Scale bars: 250 μm (A), 125 μm (B,C).

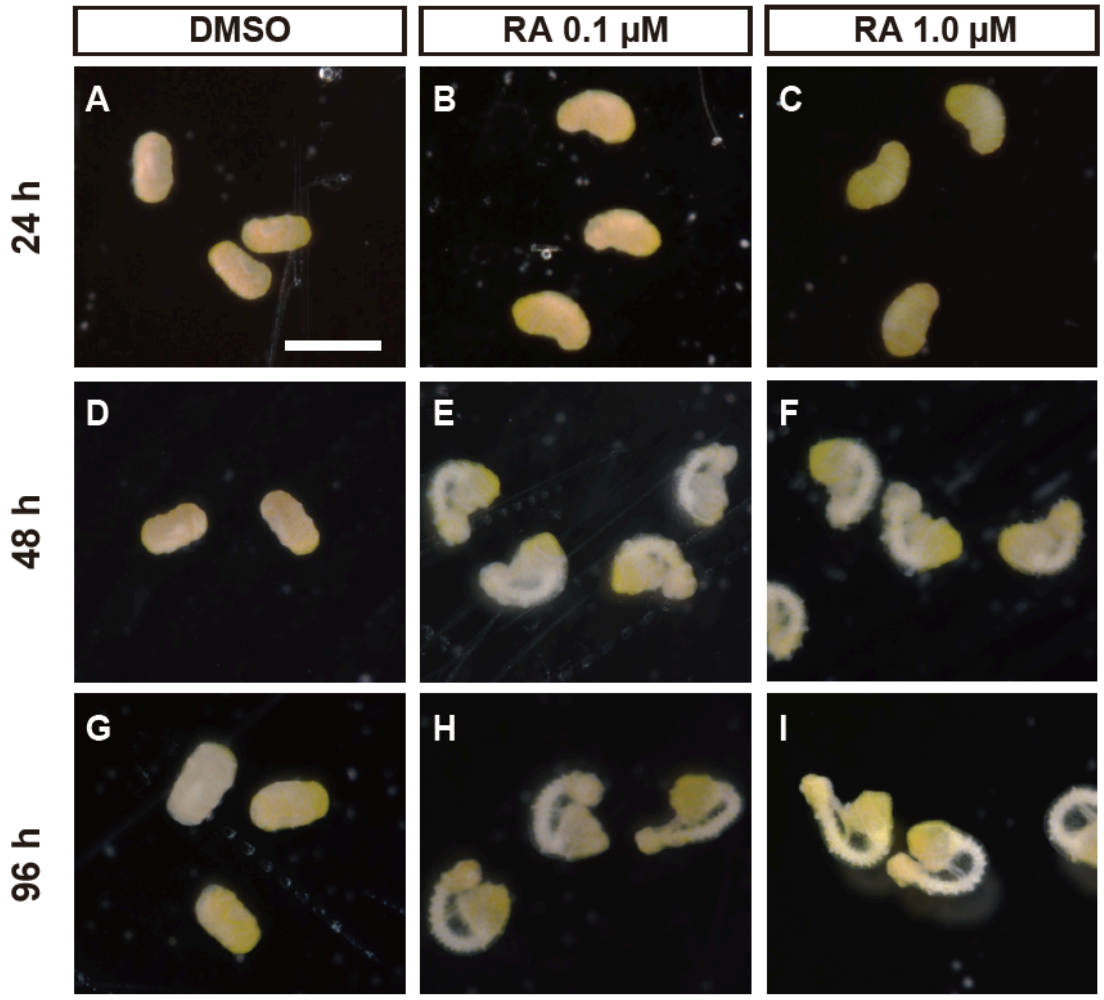

Fig. 3-6 Temporal change of larvae in metamorphosis induction by exogenous RA treatment

(A, D, G), (B, E,H) and (C,F,I) respectively show the DMSO-treated, RA 0.1μM-treated and RA 1μM-treated larvae. In the RA treatment, larval forms slightly bent at 24 h after treatment (B, C) and the structures like stalk became gradually visible at 48–72 h after treatment (E–F, H–I).

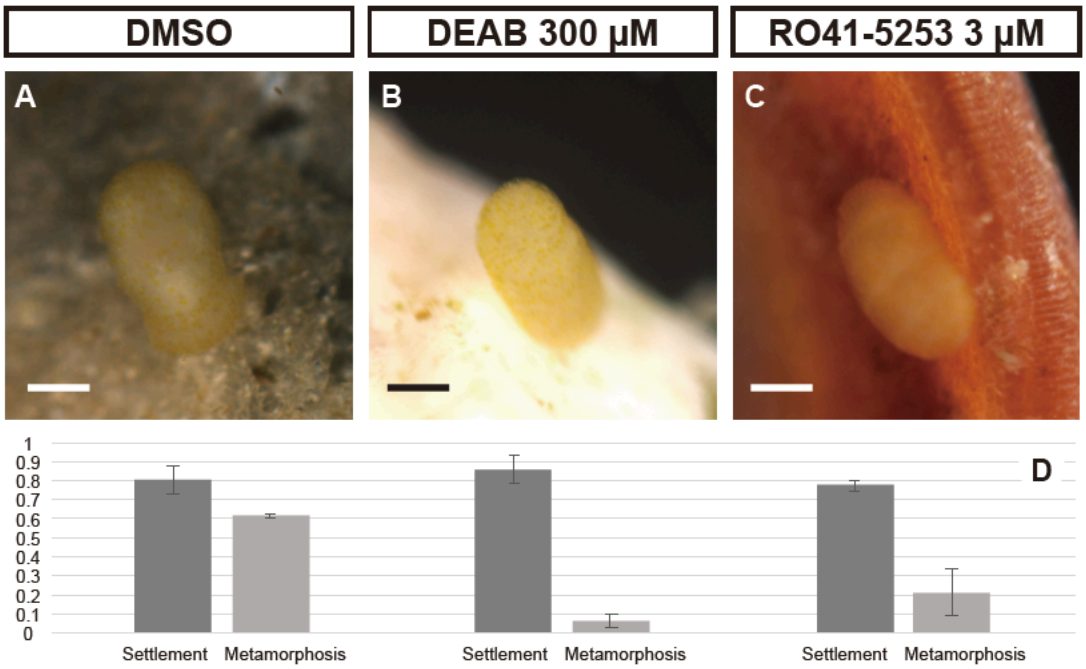

Fig. 3-7 Effects of DEAB or RO treatment on settlement and metamorphosis

We treated doliolaria larvae with DMSO (control), DEAB 300 µM or RO 3 µM and examined the effects on settlement and metamorphosis. (A–C) show the settled larvae on substrates (natural sands from their adult habitat) in DMSO, DEAB and RO treatments. The ratio of settlement and metamorphosis is shown in (D) (dark gray; settlement ratio, light gray; metamorphosis ratio). Scale bars: 125 µm.

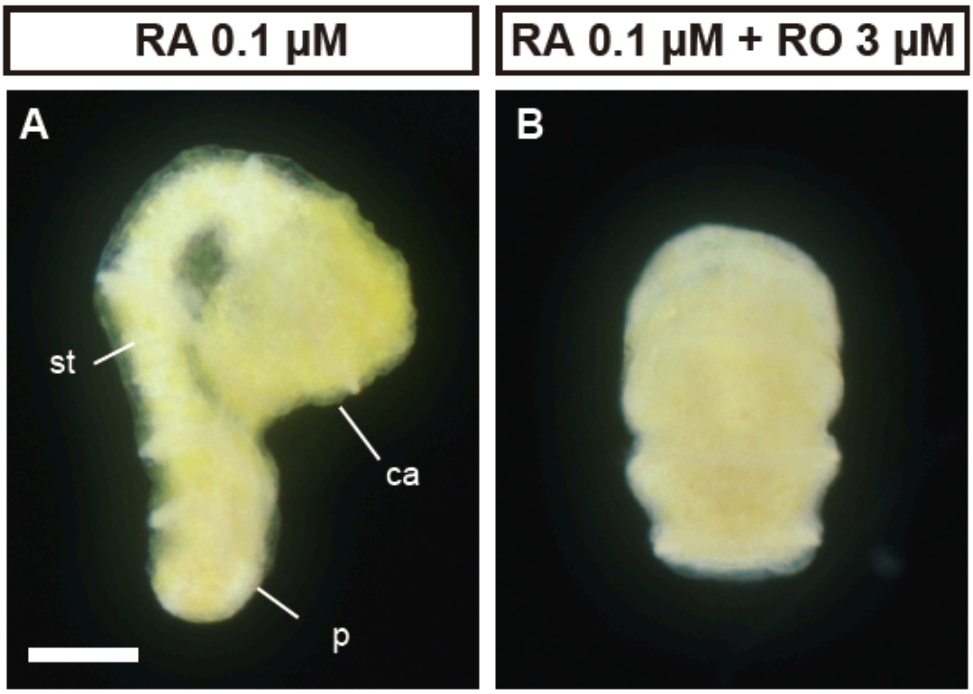

Fig. 3-8 RO treatment suppressed the induction of metamorphosis by RA

RA 0.1 μM treatment with doliolaria larvae induced the metamorphosis (A), while this induction was suppressed by adding RO 3 μM (B). ca; calyx, st; stalk and p; adhesive plate. Scale bar: 125 μm.

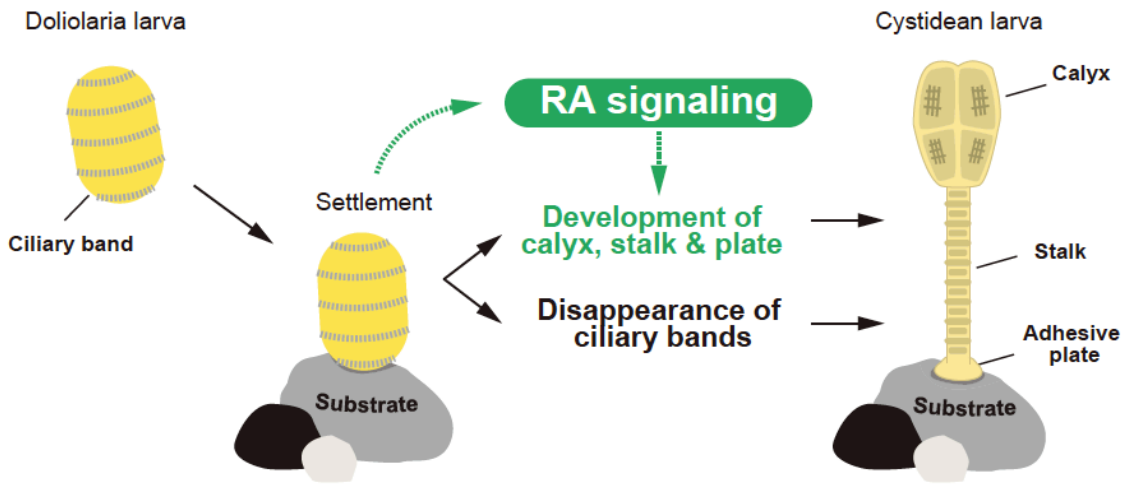

Fig. 3-9 Hypothetical regulatory mechanism for metamorphosis of feather stars

Black arrows indicate the developmental process of *A. serrata*. Competent doliolaria larvae settle to external substrate with reception of environmental cues to commence metamorphosis process. Finally, transition to sessile cystidean larvae is completed through the disappearance of ciliary bands and the development of calyx, stalk and adhesive plate. We hypothesized that RA signaling mediates the metamorphosis process such as the development of calyx upon the reception of environmental signals (shown in green).