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Endogenous thyroid hormone synthesis in facultative planktotrophic larvae of the sand dollar *Clypeaster rosaceus*: implications for the evolutionary loss of larval feeding

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SUMMARY

Critical roles of hormones in metamorphic life history transitions are well documented in amphibians, lampreys, insects, and many plant species. Recent evidence suggests that thyroid hormones (TH) or TH-like compounds can regulate development to metamorphosis in echinoids (sea urchins, sand dollars, and their relatives). Moreover, previous research has provided evidence for endogenous hormone synthesis in both feeding and nonfeeding echinoderm larvae. However, the mechanisms for endogenous synthesis remain largely unknown. Here, we show that facultatively planktotrophic larvae (larvae that reach metamorphosis in the absence of food but have the ability to feed) from the subtropical sea biscuit *Clypeaster rosaceus* can synthesize thyroxine endogenously from incorporated iodine (I^{125}). When treated with the goitrogen thiourea (a peroxidase inhibitor), iodine incorporation, thyroxine synthesis, and metamorphosis are all blocked in a dose-dependent manner. The inhibitory effect on metamorphosis can be rescued by administration of exogenous thyroxine. Finally, we demonstrate that thiourea induces morphological changes in feeding structures comparable to the phenotypic plastic response of larval structures to low food conditions, further supporting a signaling role of thyroxine in regulating larval morphogenesis and phenotypic plasticity. We conclude that upregulation of endogenous hormone synthesis might have been associated with the evolution of nonfeeding development, subsequently leading to morphological changes characteristic of nonfeeding development.

INTRODUCTION

Development in diverse organisms such as cnidarians, echinoderms, insects, and amphibians is frequently accompanied by a dramatic transition between distinct life history stages involving changes in morphology, physiology, and habitat (Heyland et al. 2005; Bishop et

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al. 2006; Heyland and Moroz 2006). This transition, known in animals (Metazoa) as metamorphosis, appears to have evolved many times independently (Wray 1995; Hadfield 2000). Although several studies began to elucidate the signaling mechanisms involved in a few marine invertebrate phyla (Degnan and Morse 1995; Degnan et al. 1997; Bishop and Brandhorst 2001; Bishop et al. 2001; Woods et al. 2004; Amador-Cano et al. 2006), our knowledge about the signaling mechanisms involved in the coordination of such complex life histories originates primarily from representatives of two phyla, namely arthropods and chordates, which use steroid and thyroid hormones (TH), respectively, to coordinate postembryonic development and metamorphosis (reviewed in Nijhout 1994; Denver et al. 2002).

Despite the predominant focus on arthropods and chordates, a plethora of research has shown that hormones have dramatic effects on development and morphogenesis in other invertebrates (reviewed in Heyland et al. 2005). One such group is the echinoderms. Consistent with evidence from chordates, some echinoderms are also responsive to TH during development (Chino et al. 1994; Suyemitsu et al. 1997; Johnson 1998; Saito et al. 1998; Hodin et al. 2001; Heyland et al. 2004, 2005; Heyland and Hodin 2004). The thyroid hormone thyroxine (T4) accelerates development to metamorphosis in sea urchins (Chino et al. 1994; Johnson 1998), sand dollars (Suyemitsu et al. 1997; Saito et al. 1998; Hodin et al. 2001; Heyland et al. 2004; Heyland and Hodin 2004), and one starfish (Johnson and Cartwright 1996). In addition, Chino et al. (1994) showed that T4 is approximately 10 times more potent than T3 in feeding sea urchin larvae. Note that this effect is in contrast to the role of TH in vertebrates, where T3 is considered the active hormone due to its higher-binding affinity to the thyroid hormone receptor (TR). However, in another echinoderm species, the sand dollar *Peronella japonica*, T3 and T4 have comparable effects on development (Saito et al. 1998).

Marine invertebrates show a remarkable diversity of life history strategies, making them ideal organisms to study questions of life history evolution. Two fundamental strategies can be distinguished based on feeding mode: feeding larvae (larvae need to feed in order to reach metamorphosis) and nonfeeding larvae (larvae do not need to feed in order to reach metamorphosis). Nonfeeding larvae have evolved many times independently from feeding larvae in several marine invertebrate groups such as annelids (i.e., Pernet 2003) and echinoids (sand dollars, sea urchins, and sea biscuits) (i.e., Strathmann 1985; Wray 1995). Ecological considerations such as trade offs between egg size and egg number, differences in survival in the plankton, limitations to dispersal, as well as differences in juvenile growth and mortality have been discussed as important factors shaping the evolution of nonfeeding development (reviewed in Hart 2002). Moreover, dramatic differences in morphogenesis between closely related species developing from small and large eggs have been identified (Raff andWray 1989; Wray and Raff 1991a, b; Raff et al. 1999).

The alteration of ontogenetic (i.e., developmental) mechanisms could be involved in or may be a product of the evolution of nonfeeding development from feeding development (Wray and McClay 1989; Raff et al. 1999; Wray and Lowe 2000). Because of their pleiotropic action, hormones have the potential to alter dramatically development and, more broadly, the entire life history of an organism, as is well known for insects, amphibians, and plants

(Nijhout 1994; Shi et al. 1996; Flatt et al. 2005; Buchholz et al. 2006; Heyland and Moroz 2006). Moreover, changes in the synthesis and metabolism of these same hormones have been discussed as driving forces for the evolution of alternative life history strategies (Frieden 1981; Yaoita and Brown 1990; Jennings and Hanken 1998; Hodin and Riddiford 2000; Hodin et al. 2001; Truman and Riddiford 2002; Heyland et al. 2004, 2005; Heyland and Hodin 2004).

THS are likely candidates for such mechanisms in echinoid postembryonic development (Hodin et al. 2001; Heyland et al. 2004, 2005). By inducing a phenotypically plastic response of larval and juvenile structures in feeding larvae (as shown by Strathmann et al. 1992), TH treatment simulates heterochronic developmental shifts similar to those hypothesized to have been involved in the evolution of nonfeeding development (Heyland and Hodin 2004). In feeding larvae, the plasticity signal (thyroxine) seems to originate from unicellular algae that larvae feed on (Chino et al. 1994; Heyland and Moroz 2005). If the only source of THS is through exogenous feeding, then nonfeeding larvae lost this hormone source when they lost the ability to feed. If TH signaling is still a requirement for development in nonfeeding larvae, then these larvae must either have evolved the ability to synthesize hormones endogenously or receive it maternally. Saito et al. (1998) presented evidence for endogenous TH synthesis (rather than maternal loading) in the nonfeeding larvae of the sand dollar *P. japonica*. Nevertheless, the mechanisms of TH synthesis for *P. japonica* or any other echinoderm have not been characterized.

In this study, we investigated mechanisms of TH synthesis and its involvement in metamorphosis in the sea biscuit *Clypeaster rosaceus*. This species has an uncommon life history in that it produces facultatively planktotrophic larvae that have the ability to feed but do not need to feed in order to reach metamorphosis (Emlet 1986). Facultatively planktotrophic larvae have been hypothesized to represent a transitional strategy in the generally unidirectional evolution of nonfeeding development from feeding development. If TH is necessary for development to metamorphosis, we predict that these larvae will have the ability to synthesize endogenously sufficient TH to reach metamorphic competence, indicating independence from exogenous hormone sources. Here, we present evidence that *C. rosaceus* larvae do synthesize thyroxine (T4, a thyroid hormone) from incorporated iodine. Whereas thiourea slows development to metamorphic competence and leads to an increased investment into larval structures relative to juvenile structures, exogenously applied TH has opposite effects in that it leads to a decrease in growth of larval structures relative to juvenile structures and an acceleration of development to metamorphic competence. We also show that these inhibitory effects of TH on larval development and juvenile morphogenesis are rescued by adding exogenous thyroxine. Our previously published data on the related sand dollar *Dendraster excentricus* (Heyland and Hodin 2004) show that thiourea effects on metamorphic competence and the plasticity of larval feeding structures relative to juvenile structures are much stronger in *C. rosaceus*. Moreover, data from the current study provide evidence that juvenile size and energy content are less plastic in *C. rosaceus* development when compared with obligatorily feeding larvae. Comparatively, these data provide an interesting insight into the evolutionary loss of larval feeding within the Clypeasteroids. We interpret these new data as evidence that upregulation

of endogenous TH synthesis was an important developmental mechanism in the evolutionary transition from feeding to nonfeeding development, leading to the reduction of larval structures and an acceleration of larval development to metamorphic competence among echinoids.

MATERIALS AND METHODS

Larval culturing

Adult *C. rosaceus* were collected at a depth of 3–7m during September 2000, October 2001, and September 2003 at Long Key Channel, Florida, and used for the following experiments: morphometrics experiments (October 23–November 3, 2000); metamorphosis experiment (December 4–December 13, 2001); and iodine experiment (October 2–13, 2003). Adults were maintained in aquaria with recirculating seawater (18–20°C) at the University of Florida in Gainesville. On October 23, 2000, December 4, 2001, and October 2, 2003 we spawned one female and one male by shaking the adult animal until gametes were released (different individuals in different years). Eggs were fertilized with a dilute suspension of sperm (1:10,000) in a 2-l glass beaker. Hatching of blastulae occurred within 12 h. Larvae were then diluted to 1 larva/ 5mlMFSW(millipore-filtered seawater; 0.2 μm) and cultured in 1-l glass jars (filled with 800 ml MFSW) for the morphometrics and metamorphosis experiment, and in gallon jars (filled with 3.8 l MFSW) for the iodine experiment. No food was provided in any of the experiments. Water was changed on days 1, 3, 5, and 7 for both the morphometrics and metamorphosis experiment and every 3 days for the iodine experiment. The culturing temperature for all experiments was 26°C.

Experimental designs

Morphometrics and metamorphosis experiment

Experimental treatments were set up after embryo hatching and each treatment was replicated four times. Experimental treatments were as follows: morphometrics experiment [Thyroxine (10^{-9} M thyroxine), High Inhibitor (10^{-3} M thiourea), Low Inhibitor (10^{-5} M thiourea), Rescue (10^{-9} M thyroxine+10^{-3} M thiourea), Control (MFSW)], and metamorphosis experiment [Thyroxine (10^{-9} M thyroxine), Rescue (10^{-9} M thyroxine+10^{-3} M thiourea), Thiourea 10^{-3} (10^{-3} M thiourea), Thiourea 10^{-4} (10^{-4} M thiourea), Thiourea 10^{-5} (10^{-5} M thiourea), Thiourea 10^{-6} (10^{-6} M thiourea), Thiourea 10^{-7} (10^{-7} M thiourea), Control (MFSW)]. Larvae were exposed to the chemical treatments immediately from hatching until the end of the experiment. In the morphometrics experiment, 10 larvae per replicate were removed from cultures for morphometrics analysis at 36, 72, 100, and 140 h. For the metamorphosis and the morphometrics experiment, larvae were counted and checked for developmental abnormalities such as asymmetries and dwarf phenotypes at each water change. The latter phenotype is characterized by an extremely small body size, usually three to four times smaller than normally developing larvae. We prepared thyroxine (Sigma-Aldrich, T-1775, St. Louis, MO, USA) as described in Chino et al. (1994) and thiourea [Sigma-Aldrich, T7875, a thyroxine synthesis inhibitor that acts by blocking thyroid peroxidase (TPO) activity] in MFSW at appropriate concentrations.

Iodine incorporation and thin-layer chromatograms (TLC)

In the iodine experiment, we exposed larvae (October 9, day 7) for 18 h in 12-well plates to the following experimental treatment: [Thiourea 10^{-3} (10^{-3} M thiourea), Thiourea 10^{-5} (10^{-5} M thiourea), Thiourea 10^{-7} (10^{-7} M thiourea)]. We placed 30 randomly chosen larvae in each well, containing 4ml of solution. All solutions were made up in SW^{125} (MPFSW with I^{125} at 51,937 dpm; the carrier-free specific activity of I^{125} was 642.8GBq/mg). Samples containing I^{125} were counted on an ssMPD instrument (BioTraces Inc., Herndon, VA) in standard mode. In standard mode, digital signal processing is used to distinguish the I^{125} decay-specific characteristics from those of background events to give a background equivalent to 5dpm of I^{125} with about 45% efficiency.

After the exposure of larvae to SW^{125}, we transferred them from each well into a separate glass tube where they were washed five times with fresh, nonradioactive MFSW until the radioactivity in the supernatant was below 30dpm. Between each wash, larvae were centrifuged at 1980 *g* for 3min and kept on ice. Finally, we counted the radioactivity in the supernatant. If we were still able to detect a radioactivity signal in the supernatant, we performed additional washes until we could not detect any radioactivity in the supernatant. In order to test whether larvae incorporated I^{125} into T4, we prepared samples for TLC. We added 1 ml of ice cold MeOH to each sample and extracted at 4°C overnight. After mixing samples in a vortex for 2min, we centrifuged them at 1980 *g* for 10 min and collected the supernatant. We spiked each sample with 100 µl nonradioactive 10^{-4} M T4 (Sigma-Aldrich, T-1774) and then dried the samples with a SpeedVac® centrifuge. An additional negative control was prepared that contained 100 µl of the original SW^{125} plus 1 ml of ice-cold MeOH processed in the same way as the experimental samples. The dry pellet was dissolved in 30 µl 0.01N NaOH. All 30 µl (excluding the crystals) were loaded on a TLC plate (Whatman LK5D silica gel 150A with a fluorescence marker; Whatman #4851-840; Florham Park, NJ, USA) and run for 1.5 h in a 2-methylbutanol/*t*-butyl alcohol/25% NH3/ acetone, 7:14:14:56, v/v solvent. We visualized the cold T4 marker under UV light on a BioRad Fluor-S MultiImager system (Sunnyvale, CA, USA) and radioactive bands for the larval samples on aMolecular Dynamics Phosphorimager SI after 7 days of exposure. Overlaying the UV image with the image from the Phosphorimager allowed direct comparison of the radioactive bands with the T4 standard.

Morphometric analysis

We sampled five larvae from each of four replicate cultures per treatment and performed morphological analyses of larval characters: arm length (postoral arms, PO; postero-dorsal arms, PD), general body size (body rod, BR; body midline, BML), stomach size (stomach length, SL; stomach width, SW), and juvenile rudiment size (rudiment length, RL; rudiment width, RW). We measured these characters in all cases after larvae were fixed in 4% paraformaldehyde (a maximum of 72 h before measurement), dehydrated through an EtOH series (50–100%), and cleared in clove oil (Sigma-Aldrich, C8392). We then measured the cleared larvae using a technique described previously and applied for similar purposes by McEdward (1984). We mounted larvae on microscopic slides and viewed them using a compound Olympus microscope (Center Valley, PA, USA) with an attached camera lucida. We identified specific larval landmarks on a digitizing tablet in order to retrieve the *x* and *y*

information of the landmark (see Heyland et al. 2004). The z information was retrieved with a rotary encoder attached to the fine focus knob of the microscope (see McEdward 1984). The data (digitized *x*, *y*, *z* information from each individual landmark) were exported into an Excel™ spread sheet and the sizes of the morphological characters were calculated using general trigonometric analysis supported by Excel™ macrocommands. We staged development using a modification of the previously described method used for *D. excentricus* (Heyland and Hodin 2004). We considered larvae to be in stage 1 when skeletal spikes were present on the body rods, rudiments of postdorsal arms were present, and the hydrocoel had flattened. Stage 2 larvae were characterized by the formation of the dorsal arch. In stage 3 larvae, first adult skeletal elements were present such as spicules, skeletal plates, and fused pentaradial skeletal elements. At stage 4, juvenile spines were visible in the juvenile rudiment. Juvenile size was measured as the average of two maximal perpendicular test diameters (not including the spines).

Metamorphic competence

We tested for metamorphic competence over 4 days, from December 11th to 14th, 2001. At each time point, we randomly chose 90 larvae from each treatment and distributed them randomly into three replicates in 15-cm petri dishes with 80mM excess KCl in MFSW. After 6 h of incubation, we scored the number of postmetamorphs that had undergone metamorphosis during the assay (defined as the moment when tube feet stick out of the larva and attach firmly to the bottom of the culture dish). Based on our definition, metamorphic competence was only reached when more than 30% of larvae per replicate underwent the metamorphic transition.

Immunohistochemistry

Thyroid peroxidase is a critical enzyme for TH synthesis in vertebrates. For *C. rosaceus*, we used a monoclonal antibody that specifically binds to human TPO in order to analyze the distribution of similar molecules in developmental stages of the sea biscuit. Note that due to the relatively (40–50%) conserved animal heme domain of this enzyme, it is possible that cross-hybridization with different peroxidases can occur with this method. Larvae and juveniles from three developmental stages (first pluteus stage and two metamorphic stages) were fixed for 20min at room temperature (RT) in 4% paraformaldehyde in phosphate-buffered saline (PBS; 1 ×) and then postfixed in 100% ice-cold MeOH for 10min. Specimens were then washed three times in PBS and either stored at 4°C in PBS or immediately processed. We washed specimens (0.3% Triton X-100 in 1X PBS) three times in PBT and incubated them for 30min in blocking solution (5% normal goat serum in PBT). After the incubation, specimens were washed five times in PBT and then incubated with the primary antibody (1:1000, monoclonal mouse anti-human TPO; Research Diagnostics Inc., Concord, MA, USA) overnight at 4°C or 2h at RT (20°C). Specimens were then washed three times in PBT and incubated with the secondary antibody (1:200, anti-mouse Alexafluor™ 488, Molecular Probes™, Carlsbad, CA, USA) overnight at 4°C or at RT for 2 h. Finally, specimens were washed five times in PBT and incubated with DRAQ5™ (Biostatus Limited, Shepshed, UK) in PBS for 15 min, washed another three times, and then directly mounted in Vectashield™ to view with a confocal microscope (Leica, Vector, Burlingame, CA, USA).

Biochemical analysis

We quantified total energy (joules per individual) in eggs and juveniles using the acid dichromate oxidation with the micromodification by McEdward and Carson (1987) of the procedure described by Parsons et al. (1984). We placed individuals in 13-mm test tubes (Fisher Scientific™, Hampton, NH, USA) and removed excess seawater using a micropipette. All samples were rinsed in distilled water and then pipetted dry. We next added 100 μl of concentrated phosphoric acid, mixed by a vortex, and dried samples for 15 min at 105°C. After the samples cooled to RT, we added 200 μl 0.3% acid dichromate, mixed by a vortex thoroughly, and heated again for 15 min at 105°C. We then added 350 μl of distilled water, mixed by a vortex, and allowed them to cool to RT. Sample absorbance was measured at 440nm and energy content was determined based on a glucose standard (0–2 j). We had four replicates for each experimental treatment using 30 specimens (eggs or juveniles) per replicate.

Statistical analysis

We analyzed the growth trajectories of larvae from all treatments compared with the control using principal component analysis (PCA) and ANCOVA commands in SPSS™. We performed PCA using all eight morphological characters measured (PO, PD, BR, BML, SL, SW, RL, RW). We used the first two PCs that together accounted for more than 50% of the variance based on an eigenvalue larger than 10% of the overall sample variance. We then used ANCOVA with PC2 as the dependent variable and PC1 as the covariate. In this analysis, we tested whether the interaction between experimental treatment and the covariate is statistically significant. We also used MANOVA in combination with post hoc comparisons in order to test whether PCs are significantly different between treatments and the control.

RESULTS

Thiourea inhibits and thyroxine accelerates metamorphic competence in *C. rosaceus* larvae

We tested for metamorphic competence of *C. rosaceus* premetamorphic larvae on 3 days. On December 11 (7 days after hatching), only individuals from the Thyroxine and the Rescue treatment had reached metamorphic competence (more than 30% of individuals metamorphosed) (Fig. 1A). The ANOVA results using Bonferroni's correction for pairwise comparisons are as follows (note that values represent % difference between treatment and control, and a positive value represents an earlier metamorphosis in the treatment): Thyroxine–Control (63.17 ± 8.08; $P<0.001$); and Rescue–Control (76.51 ± 8.08; $P<0.001$). On December 13 and 14 (9–10 days after hatching), only larvae from the Control and Thiourea treatments 10^{-7}, 10^{-6}, and 10^{-5} M had reached the 30% threshold when we combined the data from those 2 days, and metamorphic competence was significantly lower in Thiourea 10^{-3} M and Thiourea 10^{-4} M compared with the Control (Fig. 1B). The ANOVA results using Bonferroni's correction for the pairwise comparisons are: Thiourea 10^{-3} M–Control (−50.96 ± 7.85; $P<0.001$) Thiourea 10^{-4} M–Control (−38.47 ± 7.85; $P<0.001$). We did not find any statistically significant difference in juvenile size and biochemical composition among any of the treatments using MANOVA with Bonferroni's

correction (P>0.10). Table 1 presents the mean values and one SE (standard error of the mean) of total energy content and juvenile sizes as a function of treatment and egg size.

In summary, these data demonstrate an acceleration of development in the thyroxine treatment compared with the control. Moreover, a significantly higher percentage of larvae reached metamorphic competence in the Thyroxine and Rescue treatments. Thiourea strongly inhibited metamorphic competence in a dose-responsive way. However, no effect of this acceleration was seen in terms of juvenile size or juvenile biochemical composition.

Thiourea and thyroxine induce phenotypically plastic response of larval and juvenile characters but neither one leads to a shift in the developmental timing of juvenile morphogenesis in *C. rosaceus*

We investigated how thiourea and thyroxine affect the larval morphology during development. In order to estimate relative investment into larval versus juvenile structures, we first performed a principal component analysis on all eight morphological characters (PO, PD, BR, BML, SL, SW, RL, and RW). We analyzed the correlation matrix and extracted eigenvalues larger than 1. This analysis resulted in two PCs that together explained more than 73% of the total variance of all characters. In Table 2, we report factor loadings for each component and morphological character. Based on the similar signs and magnitudes of the factor loadings, the structure of these two components indicates that PO, BML, SL, and SW form one group (larval characters) and RL and RW form another group (juvenile characters). The factor loadings of PD and BR are not consistent with those of any of these two groups.

Next, we used the two statistically independent compound variables (PC1 and PC2) to test whether the experimental treatments differentially affect the growth trajectories of larvae. To test this, we first performed an ANCOVA using PC1 as a covariate and PC2 as the dependent variable for each time point. Forty-eight and 72 h after hatching, the interaction between experimental treatment and the covariate was not statistically significant (48 h: F=0.62, P=0.69; 72 h: F=2.77, P=0.06). One-hundred and 140 h after hatching, the interaction was statistically significant (100 h: F=10.65, P<0.001; 140 h: F=32.08, P<0.001), indicating that the growth trajectories were differentially affected by the experimental treatment.

We then performed a MANOVA with post hoc comparisons in order to test whether the PC1 and PC2 from the experimental treatments were significantly different from the control. Because we used post hoc comparisons with Bonferroni's corrections, the values in parentheses indicated below represent the observed mean difference between treatment and control with adjusted standard errors. A negative value in mean difference means that the experimental value is larger than the control. A positive value means that the average experimental value is smaller than the control. Figure 2 represents the plots of PC1 and PC2 as a function of time and treatment. Forty-eight hours posthatching, the Rescue treatment was significantly different from the Control (0.43 ± 0.13; P<0.04) for PC1. Seventy-two hours posthatching, the Rescue treatment and the Thyroxine treatment were significantly different from the control (0.77 ± 0.13, P<0.001, and 0.70 ± 0.13, P=0.001) for PC1. One hundred hours posthatching, the Rescue treatment and the Thyroxine treatment were

significantly different from the Control (1.90 ± 0.26, $P<0.001$, and 1.73 ± 0.26, $P<0.001$) for PC1 and the Thyroxine treatment was significantly different from the Control (−0.65 ± 0.14, $P=0.004$) for PC2. One hundred and forty hours posthatching, Rescue, Thyroxine, Thiourea 10^{-3}, and Thiourea 10^{-5} treatment were all significantly different from the Control (1.57 ± 0.18, $P<0.001$; 1.70 ± 0.18, $P<0.001$; −1.07 ± 0.18, $P<0.001$; −0.71 ± 0.18, $P<0.001$) for PC1 and Rescue, Thyroxine, and Thiourea 10^{-3} were significantly different from the Control (−0.53 ± 0.15, $P=0.038$; −0.91 ± 0.15, $P<0.001$; 0.75 ± 0.15, $P=0.002$).

Finally, to analyze developmental rates we calculated the time that each individual larva needed to develop to developmental stages 1–4 (categorization defined above). We calculated the average time in hours for each treatment and the control and then plotted the value of each treatment against the value from the control (Fig. 3). This heterochrony plot does not show any significant difference in developmental timing between the experimental treatments and the control ($P<0.05$) using ANOVA.

Juvenile size and energy is not significantly affected by thiourea and thyroxine treatment

Total energy content was significantly decreased in juveniles compared with eggs (Table 1). We did not detect any statistically significant difference in juvenile size and energy between thyroxine, thiourea, rescue treatment, and control.

Mortality in cultures

No significant differences in survival between the treatments were found through 196 h posthatching when the survival rate was significantly lower in the 10^{-3} M Thiourea (3.12 ± 0.9%, $P=0.04$) treatment and the Rescue treatment (3.41 ± 0.9%, $P=0.02$) when compared with the control. Two hundred and forty-four hours after hatching, the survival rate was significantly lower in only the 10^{-3} M Thiourea treatment compared with the Control.

C. rosaceus larvae incorporate radioactive iodine and build thyroxine

We tested whether *C. rosaceus* larvae incorporate radioactive iodine (I^{125}) and use it to synthesize thyroxine 5 days after hatching. In addition, we tested whether the incorporation and/or synthesis of thyroxine can be inhibited by thiourea. We identified radioactive thyroxine on the TLC plate, which indicates that the incorporated radioactive iodine was used to synthesize T4 (Fig. 4). The band from the cold (nonradioactive) T4 migrated to the same position as the radioactive band seen under the phosphorimager after 7 days of exposure. In summary, we were able to show that thiourea does inhibit thyroid hormone synthesis in *C. rosaceus* larvae, suggesting that the inhibition of thyroxine synthesis occurs on the level of the iodine uptake.

Presence of thyroxine and TPO in perimetamorphic stages of *C. rosaceus*

TPO is the key enzyme for the synthesis of TH in vertebrates. For *C. rosaceus*, we used a monoclonal antibody directed against human TPO to analyze the distribution of putative TPO in developmental stages of the sea biscuit (green stain in Fig. 5). We used the nuclear stain DRAQ5™ (blue stain in Fig. 5) as a counter-stain to visualize nuclei of cells in larvae. We found that TPO immuno-positive staining occurs only in peri-metamorphic stages (i.e., pre and postmetamorphosis) at the base of adult spines (Fig. 5, B–D). TPO immuno-

reactivity was absent in 2-day-old larvae (Fig. 5A). Figure 5D shows the cellular extranuclear localization of TPO in *C. rosaceus*.

DISCUSSION

Our data suggest that facultative planktotrophic larvae of the sea biscuit *C. rosaceus* can synthesize thyroxine endogenously and that synthesis can be inhibited with the application of a known peroxidase inhibitor. The effects of exogenously applied hormone and synthesis inhibitor further imply that TH-like molecules play a critical role as developmental signals. Our data confirm earlier findings from the obligatorily feeding larva of the sand dollar *D. excentricus* that TH-like molecules are responsible for phenotypic plasticity in larval development. The phenotypic plastic response of *C. rosaceus* to TH is strongly reduced compared with *D. excentricus*, whereas TH synthesis inhibitor effects are more significant in *C. rosaceus* when compared with *D. excentricus*. This relationship suggests that the *C. rosaceus* has a higher capacity to synthesize TH endogenously than the obligatorily planktotroph *D. excentricus*, which obtains hormones from exogenous sources. At present, only data from these two species are available, so it is premature to draw a conclusion as to what extent this pattern can be generalized. However, if future data are consistent with these initial results, it would suggest that the evolution of nonfeeding development involves upregulation of endogenous TH synthesis in echinoids.

TH function in echinoids, a mechanism involved in development to metamorphosis

The juvenile develops as part of the larval body over the course of the larval period, which can last for several weeks in many echinoderm species. A number of physiological and ontogenetic processes have to be coordinated with the internal and external environment for the transformation from a larva to juvenile (i.e., metamorphosis) to take place (Bishop et al. 2006; Heyland and Moroz 2006). In this context, we consider TH signaling in echinoid larvae as a system co-opted to interpret environmental signals, such as food abundance (see discussion of phenotypic plasticity below), and coordinate the appropriate developmental response. Intriguingly, this system appears to act antagonistically to NO signaling, which appears to be an inhibitory signal during metamorphic competence (Bishop et al. 2006).

Among echinoderms, evidence for TH-related function exists for at least three of the five classes: sea urchins and sand dollars (Echinoidea), brittle stars (Ophiuroidea), and sea stars (Asteroidea), with conclusive evidence provided for echinoids (Chino et al. 1994; Johnson 1998; Saito et al. 1998; Suyemitsu 2000; Hodin et al. 2001; Heyland et al. 2004; Heyland and Hodin 2004) and one asteroid (Johnson and Cartwright 1996). For example, exogenous TH accelerates development to metamorphosis (Chino et al. 1994; Johnson 1998; Hodin et al. 2001; Heyland and Hodin 2004; Heyland et al. 2006), ultimately leading to an earlier metamorphic competence (Heyland et al. 2004) in sea urchins and sand dollars. Moreover, we previously provided evidence that thyroxine is required for metamorphosis in *Leodia sexiesperforata*, a sand dollar with relatively large eggs (Heyland et al. 2004). In species with smaller maternal investment than *L. sexiesperforata*, such as *D. excentricus*, TH treatment is not sufficient for development to metamorphosis (Heyland et al. 2004). Intriguingly, exogenous sources of hormone from phytoplankton can subsidize endogenous

synthesis (Chino et al. 1994; Heyland and Moroz 2005). However, this source is not available to nonfeeding larvae, raising the question of whether such larvae can synthesize sufficient hormone endogenously for metamorphic development. Our results show that facultative planktotrophic larvae of the sea biscuit *C. rosaceus* utilize endogenous TH synthesis in order to undergo the metamorphic transition. Moreover, data from the TLC analysis suggest that sea biscuit larvae incorporate iodine and synthesize thyroxine during metamorphic stages. This result provides evidence that thyroxine is a critical hormone involved in metamorphic regulation. At this point, we cannot conclude with certainty, however, whether thiourea inhibits the iodine uptake mechanisms or TH synthesis or both in echinoderms. Figure 4 shows a strong decrease in background iodine intensity that parallels the decrease in thyroxine signal. Future studies will have to address what processes are exactly involved in iodine uptake and whether this process can be inhibited by thiourea. The newly released sea urchin (*Strongylocentrotus purpuratus*) genome provides evidence for the presence of a sodium iodine symporter (NIS) Ortholog that would be a primary candidate for this function (GLEAN3_01273 at http://urchin.ni-dcr.nih.gov/blast/index.html). However, no evidence exists that thiourea inhibits NIS in any organism, suggesting that other mechanisms may be involved in iodine uptake in echinoderms. A recently published study by our laboratory further confirms this result for the sea urchin *Lytechinus variegatus* (Heyland et al. 2006) in that $KClO_4$ (potassium perchlorate), a known inhibitor of NIS, does not affect iodine uptake in this sea urchin species.

Exogenous TH effects on the development of facultative planktotrophic larvae of *C. rosaceus* are dramatically different from TH effects on the development of feeding larvae of *D. excentricus*. We previously reported a 42% acceleration of development in larvae of the sand dollar *D. excentricus* (Heyland and Hodin 2004) when treated with TH at 10^{-9} M. The same treatment accelerated development in *C. rosaceus* (a species that produces significantly larger eggs than *D. excentricus*) by only 10–28%. Moreover, we were not able to find any significant difference in juvenile size and total energy content in these larvae, which is in stark contrast to the TH effects on juvenile size in *D. excentricus* where we reported a 56% decrease in size (Heyland and Hodin 2004) upon thyroxine treatment. These findings suggest that developmental rate and juvenile size are much more plastic in feeding larvae than they are in facultative planktotrophic larvae. Moreover, we view these findings as evidence for a decreased dependence of facultative planktotrophic larvae from exogenous hormone sources. This hypothesis can be further tested using other closely related species from the Clypeasteroid clade. We previously published evidence for TH action in *L. sexiesperforata* (Heyland et al. 2004) and have preliminary evidence on *Melitta tenuis* (A. Heyland, unpublished). Together, these species provide a range of maternal investments and feeding modes that will allow us to further test this hypothesis. Finally, TH has been measured in *P. japonica* (Saito et al. 1998), another congeneric sand dollar with obligatorily nonfeeding larvae and high maternal investment. These findings show that *P. japonica* has the ability to synthesize hormones endogenously as well. Moreover, data by Saito et al. (1998) indicate that thiourea inhibits hormone synthesis.

TH signaling and phenotypic plasticity in facultative planktotrophic development

Larvae of the sand dollar *D. excentricus* use thyroxine as a signal for phenotypic plasticity (Heyland and Hodin 2004). When applied exogenously, the hormone simulates high food conditions in that it leads to a shift in investment from larval to juvenile structures. Larvae reared in low food environments show a shift in investment in the opposite direction (from juvenile to larval structures), resulting in relatively long larval arms (Strathmann et al. 1992; Hart and Strathmann 1994). For *C. rosaceus*, we show an increase in larval arm length after treatment with the TH synthesis inhibitor thiourea. The similarity in response of feeding larvae to low food conditions and *C. rosaceus* to inhibitor treatment (this study) suggests that facultatively feeding larvae have maintained vestiges of the phenotypically plastic response that is also induced by thyroxine. In feeding larvae, the cue is exogenous, whereas it appears to be endogenous for *C. rosaceus*. These findings further support the scenario that ancestral feeding larvae gained the ability to synthesize any hormones necessary for metamorphosis.

Although hormonal effects are clearly reflected in the growth trajectories of larval and juvenile structures, we did not find significant effects of any of our treatments on developmental rates other than time to metamorphosis. Our heterochrony analysis shows no shifts in developmental timing between the control and any experimental treatment. These findings are in contrast to the effects we detected for the sand dollar *D. excentricus* where we found significant heterochronic shifts in development in the thyroxine treatment. The capacity for endogenous hormone synthesis in *C. rosaceus* might make larval and juvenile tissues less sensitive to exogenous hormone. Alternatively, the difference could be explained by the faster development of *C. rosaceus* larvae and the subsequent lack of resolution to detect such changes with the method we used.

A question that needs further investigation is whether the phenotypically plastic response of larval and juvenile structures to endogenous hormone levels is a general characteristic of nonfeeding larvae, or whether it is only present in *C. rosaceus* with its specialized feeding mode (facultative planktotrophy). Because these sea biscuit larvae have the ability to feed, a certain amount of plasticity is retained because it could give larvae an adaptive advantage: Miner et al. (2002) observed previously that egg size and egg energy in *C. rosaceus* can vary significantly between females. This natural variation could lead to a situation where insufficient maternal reserves are compensated for during the larval period, making feeding obligatory in order to reach metamorphosis. In such cases, it may be advantageous for larvae to have a phenotypically plastic response to food abundance. Unfortunately, there is not sufficient information about facultative planktotrophy and its functional significance with regard to food uptake and digestion. Experiments comparing particle ingestion rates in inhibitor-induced, long-arm larvae, and control would provide data to answer some of these questions, as initially proposed by Hart and Strathmann (1994). Such experiments will allow us to further understand the role that TH plays in the evolution of derived (nonfeeding) life histories in echinoderms.

Finally, experimentation with feeding larvae is inherently different from the experimentation with facultative planktotrophic larvae or nonfeeding larvae. Feeding larvae can never be

reared to metamorphosis in the complete absence of food. Therefore, it is possible that feeding larvae can accumulate sufficient TH during the feeding period from algae (see also Chino et al. 1994; Heyland and Moroz 2005) that allows them to develop to metamorphosis when larvae are exposed to thyroid hormone synthesis inhibitors without food (see also Heyland and Hodin 2004).

Peroxidases: candidates for endogenous TH synthesis in invertebrates and their role in life history evolution

Whereas several components of the TH signaling system are present in all chordates, increasing evidence suggests that urochordates (sea squirts) and cephalochordates (lancelets) share critical elements of this signaling pathway. Zone 7 of the urochordate endostyle expresses genes related to vertebrate thyroid function including Orthologs of TPO (Ogasawara et al. 1999; Sasaki et al. 2003). Furthermore, some evidence suggests that the endostyle is involved in the biosynthesis of TH (reviewed in Eales 1997). Despite increasing indications that TH is synthesized and/or act as signaling molecules in various invertebrates (Eales 1997; Heyland et al. 2005), the mechanisms of TH biosynthesis in invertebrates other than basal chordates are essentially unknown.

Recently, we made some progress in cloning two new members of the animal peroxidase superfamily from the sea urchin *L. variegatus* and the sea hare *Aplysia californica*. Although these enzymes show high sequence similarity with TPO, they are not TPO Orthologs (Heyland et al. 2006). Moreover, extensive searches of the *S. purpuratus* genome have not revealed any clear TPO Ortholog. Nevertheless, other peroxidases from the superfamily have the capacity to synthesize TH. In fact, we have previously argued that different peroxidases could have been co-opted for TH synthesis in marine invertebrate phyla and that TPO is just one specialized enzyme co-opted for this function in vertebrates (Heyland and Moroz 2005; Heyland et al. 2006).

In this study, we used a human antibody to screen for the presence of TPO expression in larval stages of *C. rosaceus*. The catalytic domain of peroxidases shows approximately 40–50% conservation between vertebrates and invertebrates. The human antibody could therefore cross-react with various peroxidases. Intriguingly, this antibody labeled very few juvenile cells during peri-metamorphic stages (sensu Gosselin and Jangoux 1998). We now have several lines of evidence that these cells (juvenile and adult spines and other skeletal structures) are among those tissues showing high sensitivity to TH treatment (Heyland et al. 2004, 2006; Heyland and Hodin 2004).

Endogenous TH synthesis could be a preadaptation for the evolution of nonfeeding development among echinoids (Heyland et al. 2004, 2005). This hypothesis was built on the observation that different clades of echinoids seem to have evolved nonfeeding development at different frequencies. We predicted that one could find an overall enhanced capacity of endogenous hormone synthesis in clades that evolved nonfeeding development more frequently. Our new data provide support for this hypothesis in that both *C. rosaceus* (this study) and *D. excentricus* (Heyland and Hodin 2004) have the capacity to synthesize TH endogenously and are part of a larger clade (Clypeasteroids) including species that synthesize TH and/or show phenotypic effects to TH treatment (*L. sexiesperforata* and *P.*

japonica). This clade also evolved nonfeeding development multiple times. However, iodine incorporation and pharmacological data also show that a TPO-related peroxidase is likely involved in the process of TH synthesis in *C. rosaceus*. Recent data from our lab show similar results for the feeding larvae of the sea urchin *L. variegatus* and the mollusk *A. californica* (Heyland et al. 2006). These findings make endogenous TH synthesis via peroxidases a shared characteristic among deuterostomes and potentially among all metazoa. This has specific implications for the role of TH signaling as a mechanism underlying the evolution of nonfeeding development. Once selection has favored sufficiently large, yolk-rich eggs, upregulation of endogenous TH synthesis via peroxidases may result in accelerated development and differential shifts in investment from larval to juvenile structures. We predict that both feeding and nonfeeding larvae have the ability to synthesize TH endogenously; however, in lineages where selection has favored the evolution of increased maternal investment, an upregulation of endogenous TH synthesis via peroxidases may lead to the evolution of nonfeeding development.

Finally, the synthesis enzyme itself is not the only component of the putative TH signaling module in echinoid larvae. Receptor expression and the role of activation enzymes such as deiodinases or sulfotransferases that can change the iodination state of the hormones are critical factors as well, and it should be the goal of future studies to identify them and their expression patterns in both feeding and nonfeeding larvae of echinoderms and other animal phyla as well (see also Heyland et al. 2006).

CONCLUSIONS

We provide evidence for endogenous thyroxine (T4) synthesis in larvae of the sea biscuit *C. rosaceus*. Larvae incorporate iodine from the seawater to build T4, which plays a critical role in larval development and metamorphosis in several echinoderm species including *C. rosaceus*. Pharmacological and immunohistochemical evidence suggests that a peroxidase closely related to vertebrate TPO could be involved in the process of TH synthesis. Its expression in cell clusters at the base of juvenile spines during premetamorphic stages further emphasizes a role of TH signaling in echinoid metamorphosis. Comparing the ability to synthesize hormone endogenously between closely related species such as the sea biscuit *C. rosaceus* and the sand dollar *D. excentricus*, and the phenotypic plastic response of larval arms resulting from inhibition of endogenous hormone synthesis provides additional evidence for this conclusion. Finally, we propose that upregulation of endogenous TH synthesis in nonfeeding larvae may have been a critical factor for the evolution of nonfeeding development.

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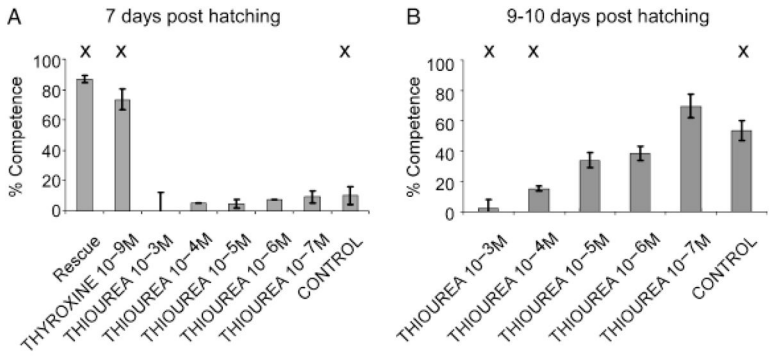

Fig. 1.

Thyroxine accelerates development to metamorphosis (A), whereas thiourea leads to an inhibition of metamorphosis in a dose-responsive manner (B). Larvae of *Clypeaster rosaceus* were induced to metamorphic competence using 80mM excess KCl on December 11 and December 13/14. (A) While the majority of larvae from the Thyroxine and the Rescue treatment underwent the metamorphic transition upon induction with KCl (i.e., were competent; see text for definition) on 7 days postfertilization, larvae from the Inhibitor treatments and the Control did not reach the threshold of 30% competence (see our definition in text). (B) Two to 3 days later, however, larvae from the Control and the 10^{-5}, 10^{-6}, and 10^{-7} M thiourea treatments reached metamorphic competence, whereas larvae from 10^{-3} to 10^{-4} M thiourea did not. Error bars correspond to ± one standard error of the mean. X's indicate statistically significant differences in percent metamorphosis.

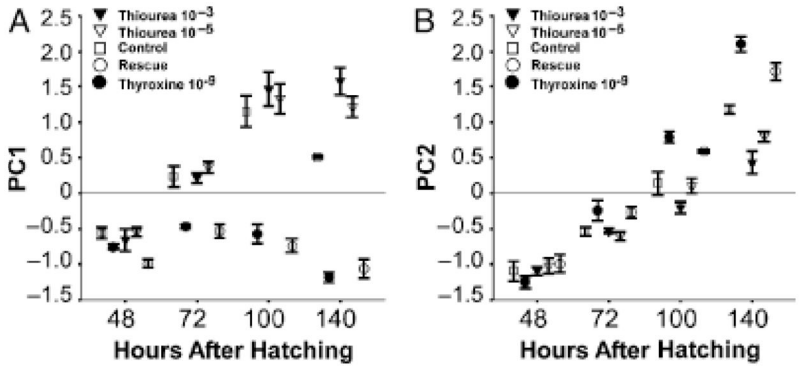

Fig. 2.

Thyroxine leads to increased growth of juvenile structures and a decreased growth of larval structures, whereas thiourea reverses this effect in larvae of the sea biscuit *Clypeaster rosaceus*. We plotted first and second principal component (PC) as a function of time and treatment. PC were derived from eight morphological characters measured on planktonic larvae of *C. rosaceus* (see Tables 2 and 3) for 48, 72, 100, and 140 h postfertilization. We view PC1 as representative for larval characters, whereas PC2 is representative for the juvenile rudiment (see Table 3). Larvae were exposed to the experimental treatments indicated in the figure legend: Thyroxine (10^{-9} M thyroxine); Thiourea 10^{-3} M (10^{-3} M thiourea); Thiourea 10^{-5} M (10^{-5} M thiourea); and Rescue (10^{-9} M thyroxine+10^{-3} M thiourea). Compared with the control, thyroid hormone synthesis inhibitor has a positive effect on the growth of larval structures (PC1), whereas thyroxine and the rescue treatment led to a negative effect on growth of larval structures (PC1), relative to the control. These effects are reversed for juvenile structures (PC2) and are statistically significant (for details, see text). These results suggest that thyroxine acts as an endogenous signal for the phenotypic plasticity of larval and juvenile structures in *C. rosaceus*.

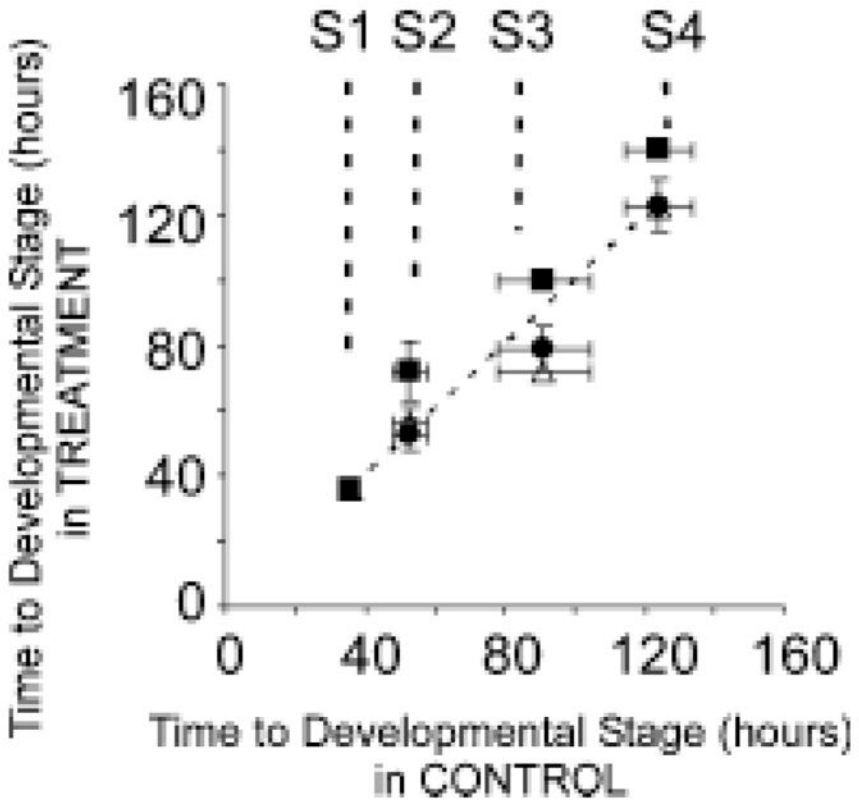

Fig. 3.

Thyroxine treatment does not lead to a consistent heterochronic shift in development for *Clypeaster rosaceus* facultative feeding larvae. The graph represents heterochrony plot for the following treatments: ●, Thyroxine (10^{-9} M thyroxine); ■, Thiourea 10^{-3} M (10^{-3} M thiourea) Δ, Rescue (10^{-9} M thyroxine+ 10^{-3} M thiourea). Each point in a heterochrony plot represents the mean value of time to a particular developmental stage (for developmental stages S1–S4, see text and Heyland and Hodin 2004). The *y*-axis information is the timing of larvae to a developmental stage from a given treatment. The *x*-axis represents the corresponding timing of control (i.e., unfed) larvae to reach the same developmental stage. Note that the error bars are SE of the mean based on four independent replicates. The dotted black line represents the standard and represents the developmental timing of control versus control. All points that lie below this black line represent accelerated development. All points above the black line represent decelerated development. No significant difference between treatments and the control were detected ($P<0.05$).

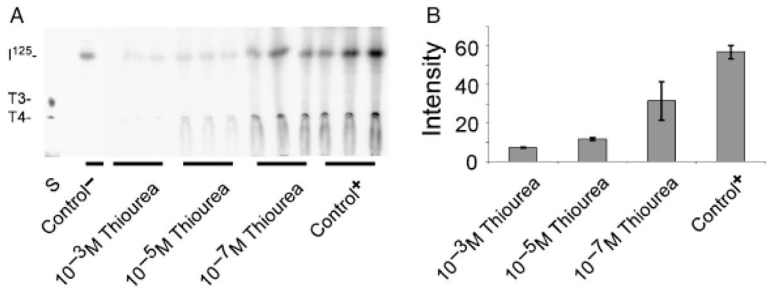

Fig. 4.

Larvae of *Clypeaster rosaceus* incorporate I^{-125} and synthesize thyroxine with it. (A) Thin layer chromatography (TLC) plate showing T4 (thyroxine) band but not T3 (3,3′,5-L-triiodothyronine). (B) Measurement of intensity of putative T4 bands for all treatments and the control. Both iodine incorporation (data not shown) and thyroxine synthesis are inhibited by thiourea (A, B). Larvae were exposed to I^{125} for 18 h in the presence (10^{-3} M Thiourea, 10^{-5} M Thiourea, 10^{-7} M Thiourea) and absence (Control+) of the thyroid hormone synthesis inhibitor thiourea. The negative control (Control−) consisted only of I^{125} with no larvae. S represents nonradioactive T4 (thyroxine) and T3 (3,3′, 5-triiodothyronine) standards that were run in the same and in different lanes and detected via UV light (see materials and methods). The intensity of bands was measured using imageJ™ software. Note that the upper band represents I^{125} as seen in the Control−.

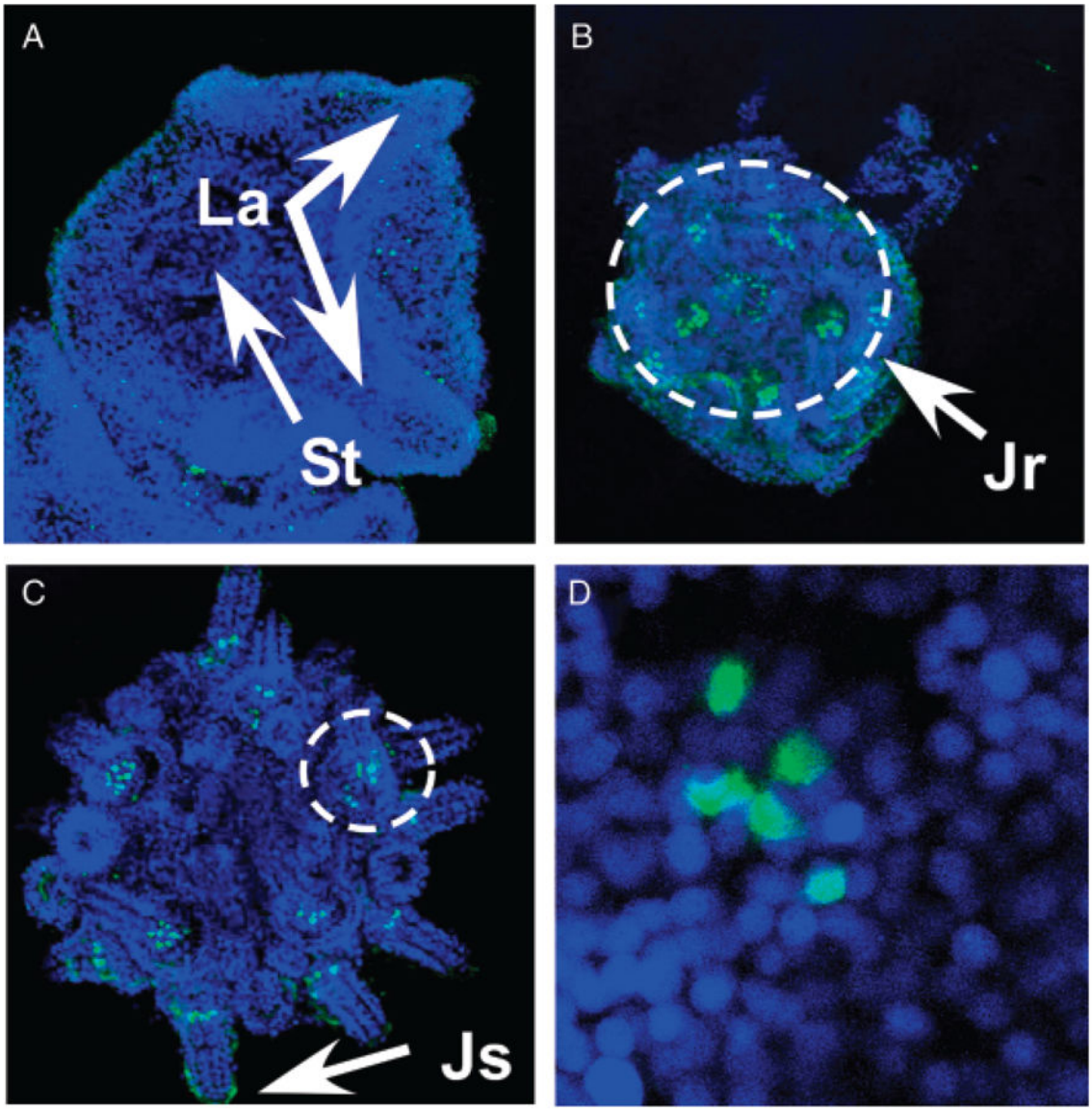

Fig. 5.

Immunohistochemical analysis of *Clypeaster rosaceus* developmental stages reveals the presence of blocking thyroid peroxidase (TPO)-like peroxidase in cell clusters at the base of the adult spines (green label: localization of TPO antibody, blue label: nuclear stain). Two-day-old *C. rosaceus* larva did not show any TPO (thyroid peroxidase) staining (A). Premetamorphic (9 days) larvae (B) and postmetamorphic juveniles (12 days) stained for TPO antibody (C). A close-up (D) shows that TPO antibody is localized to both intracellular and extracellular spaces. La, Larval arms; St, Stomach; Jr, Juvenile rudiment; Js, Adult spines.

Table 1

Total energy content (μg) and size (μm) of *Clypeaster rosaceus* eggs and newly metamorphosed juveniles

Treatment	Total energy (μg)	Size (μm)
Egg	1.67 ± 0.24 (n = 5)	293.81 ± 2.17 (n = 20)
Juveniles		
Control	0.60 ± 0.02 (n = 3)	279.47 ± 4.88 (n = 3)
Rescue	0.79 ± 0.07 (n = 3)	277.70 ± 7.74 (n = 6)
Thyroxine (10^{-9} M)	0.58 ± 0.08 (n = 3)	282.21 ± 5.37 (n = 6)
Thiourea (10^{-5} M)	0.63 ± 0.07 (n = 3)	282.67 ± 6.10 (n = 3)
Thiourea (10^{-3} M)	0.52 ± 0.06 (n = 3)	250.24 ± 4.20 (n = 3)

Note that no significant difference in total energy content was found between juveniles from different treatments but that all juveniles had less energy than eggs.

Table 2

Table of eigenvalues from principal component analysis from eight morphological characters measured on planktonic larvae of *Clypeaster rosaceus*: PO, postoral arms; PD, postero-dorsal arms; BR, body rod; BML, body midline; SL, stomach length; SW, stomach width; RL, rudiment length; RW, rudiment width

	Initial eigenvalues		
Component	**Total**	**% of Variance**	**Cumulative (%)**
1	3.463	43.283	43.283
2	2.449	30.610	73.893
3	0.621	7.767	81.660
4	0.441	5.514	87.174
5	0.330	4.124	91.298
6	0.284	3.553	94.851
7	0.231	2.891	97.742
8	0.181	2.258	100.000

We used components 1 and 2 for further analysis (Table 3).

Table 3

Factor loading of first and second principal component from principal component analysis (PCA) of eight morphological characters: PO, postoral arms; PD, postero-dorsal arms; BR, body rod; BML, body midline; SL, stomach length; SW, stomach width; RL, rudiment length; RW, rudiment width (see Table 2)

	Component	
Morphological character	**1**	**2**
PO	0.890	− 0.064
PD	0.785	0.425
BR	−0.004	− 0.874
BML	0.856	− 0.168
SL	0.835	− 0.241
SW	0.714	− 0.286
RL	0.336	0.788
RW	0.005	0.844

Based on sign and magnitude of factor loadings, we group PO, BML, SL, SWas larval characters and RL, RWas juvenile characters. In Fig. 2, transformed values for PC1 and P2 are plotted as a function of time and treatment.