Thyroid hormones determine developmental mode in sand dollars (Echinodermata: Echinoidea)

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SUMMARY Evolutionary transitions in larval nutritional mode have occurred on numerous occasions independently in many marine invertebrate phyla. Although the evolutionary transition from feeding to nonfeeding development has received considerable attention through both experimental and theoretical studies, mechanisms underlying the change in life history remain poorly understood. Facultative feeding larvae (larvae that can feed but will complete metamorphosis without food) presumably represent an intermediate developmental mode between obligate feeding and nonfeeding. Here we show that an obligatorily feeding larva can be transformed into a facultative feeding larva when exposed to the thyroid hormone thyroxine. We report that larvae of the subtropical sand dollar *Leodia sexiesperforata* (Echinodermata: Echinoidea) completed metamorphosis without exogenous food when treated with thyroxine, whereas the starved controls (no thyroxine added) did not. *Leodia sexiesperforata* juveniles from the thyroxine treatment were viable after metamorphosis but were significantly smaller and contained less energy than sibling juveniles reared with exogenous food. In a second starvation experiment, using an *L. sexiesperforata* female whose eggs were substantially larger than in the first experiment (202 ± 5 vs. $187 \pm 5\,\mu m$), a small percentage of starved *L. sexiesperforata* larvae completed metamorphosis in the absence of food. Still, thyroxine-treated larvae in this experiment completed metamorphosis faster and in much higher numbers than in the starved controls. Furthermore, starved larvae of the sand dollar *Mellita tenuis*, which developed from much smaller eggs ($100 \pm 2\,\mu m$), did not complete metamorphosis either with or without excess thyroxine. Based on these data, and from recent experiments with other echinoids, we hypothesize that thyroxine plays a major role in echinoderm metamorphosis and the evolution of life history transitions in this group. We discuss our results in the context of current life history models for marine invertebrates, emphasizing the role of egg size, juvenile size, and endogenous hormone production for the evolution of nonfeeding larval development.

INTRODUCTION

Two extreme developmental modes are found among marine invertebrate taxa. At one end of the spectrum is larval planktotrophy, where females release many small energy-poor eggs that require external nutrition to grow and develop to the benthic settlement stage. At the other extreme is lecithotrophy, where females produce few large energy-rich eggs that require no additional nutrition from the environment to reach the settlement stage (Thorson 1950; Strathmann 1985; McEdward 1997; McEdward and Miner 2003).

Planktotrophy and lecithotrophy are often viewed as distinct optima in the adaptive landscape of maternal investment strategies (Mortensen 1921; Vance 1973a,b; Christiansen and Fenchel 1979; Roughgarden 1989; Havenhand 1995; Sewell and Young 1997; McEdward and Miner 2003). However, several studies suggested that reproductive success can also be optimized at intermediate levels of maternal investment (McEdward 1997; Levitan 2000). For example, McEdward (1997) presented a fecundity-time model that used the concept of facultative feeding to account for a continuum of reproductive strategies between planktotrophy and lecithotrophy. McEdward proposed to subdivide the planktonic stage into a nonfeeding, a facultative feeding, and an obligate feeding period (Fig. 1). Two key predictions of the McEdward model found support from work on subtropical sand dollars and sea biscuits (Echinodermata: Echinoidea: Clypeasteroida) (Eckert 1995; Herrera et al. 1996): (a) that the relative length of the facultative feeding period is positively correlated with egg size and (b) that the relative length of the obligate feeding period is negatively correlated with egg size. For example, larvae from the sand dollar *Mellita tenuis* (family Mellitidae) develop from relatively small eggs (approximately 111 μm, but see Materials and Methods for details) and have a short

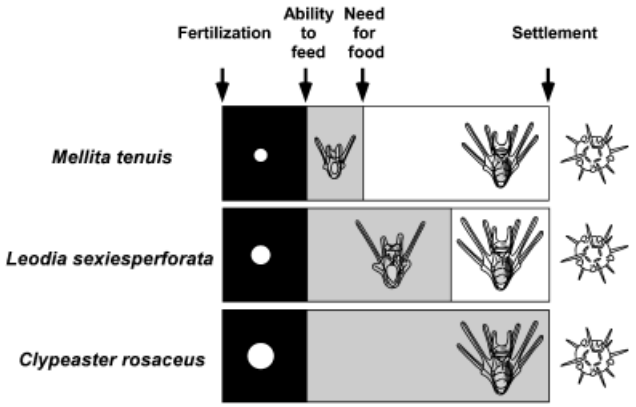

Fig. 1. The concept of facultative feeding (McEdward 1997) exemplified by three closely related echinoid species developing from different egg sizes. The planktonic period can be subdivided into three parts based on the ability and the need of larvae to feed: a nonfeeding period (fertilization through ability to feed), a facultative feeding period (ability to feed through need to feed), and an obligate feeding period (need for food through settlement). Compared with *Leodia sexiesperforata* and *Clypeaster rosaceus*, *Mellita tenuis* develops from relatively small eggs (96–133 μm, average of 111 ± 12 μm; $n = 2$) and its facultative period is very short. *Leodia sexiesperforata* develops from much larger eggs (157–204 μm, average of 178 ± 14 μm; $n = 6$) and develops to early rudiment stages in the absence of exogenous food, although a few larvae from the largest eggs can complete metamorphosis in the absence of food (see text). *Clypeaster rosaceus* develops from 260–280 μm eggs, yielding a true facultative feeding larva, which can develop to metamorphosis in the absence of food (i.e., their facultative feeding period extends all the way to settlement). Unlike nonfeeding larvae, however, *C. rosaceus* larvae have the ability to feed. Note that the lengths of the developmental periods in these three species as shown are relative values and are not intended to indicate absolute time. Note also that juvenile diameters in these three species are similar (270–280 μm) (note that these values include our own measurements together with published data; see text).

facultative feeding period and a long obligate feeding period. Larvae of the sand dollar *Leodia sexiesperforata* (family Mellitidae) develop from larger eggs (approximately 178 μm, but see Materials and Methods for details) and reach relatively late developmental stages without feeding (i.e., they have a long facultative period). Still, both *L. sexiesperforata* and *M. tenuis* larvae are considered obligate planktotrophs. By contrast, larvae from the sea biscuit *Clypeaster rosaceus* (family Clypeasteridae; egg size 260–280 μm) are functional lecithotrophs, because their larvae complete metamorphosis when starved. However, these larvae (unlike obligate lecithotrophs) retain the ability to feed and therefore are called facultative planktotrophs (Emlet 1986; Miner et al. 2002) (Fig. 1). From an evolutionary point of view, facultative feeding has been hypothesized to be an intermediate life history strategy between planktotrophy and lecithotrophy (Emlet 1986; Hart 1996). Still, the fact that there are only two confirmed facultative-feeding echinoids, *Clypeaster rosaceus* and the heart urchin *Brisaster latifrons*, has made it difficult to rigorously test this hypothesis in a phylogenetic context (cf. Hart et al. 1997).

Most authors agree that lecithotrophic development has evolved multiple times independently from planktotrophy within the echinoids and other marine invertebrate taxa (Strathmann 1985; Wray 1995; Hart et al. 1997; McEdward 1997; but see Lacalli 1993; McHugh and Rouse 1998). Although many scenarios have been proposed to account for this evolutionary transition (Turner and Lawrence 1979; Strathmann et al. 1992; Jaeckle 1995; Wray 1996; George 1999; Levitan 2000; Villinski et al. 2002), no laboratory or field manipulations (including egg size manipulations) have succeeded in transforming an obligate planktotroph into a functional lecithotroph. Here we test a novel hypothesis, that treatment of an obligatorily planktotrophic larva with the vertebrate thyroid hormone (TH) thyroxine (T4) is sufficient to induce this transformation in the absence of any larval feeding.

THs such as T4 and 3,3′,5-L-triiodothyronine (T3) are abundant in the planktonic algae that feeding larvae consume (Chino et al. 1994; Heyland 2004), and empirical studies show that these hormones have profound effects on echinoderm life history traits, including developmental rate (Chino et al. 1994; Suyemitsu et al. 1997; Saito et al. 1998; Hodin et al. 2001; Heyland and Hodin 2004) and size at settlement (Heyland and Hodin 2004). Recent data also suggest that some echinoid larvae can synthesize THs endogenously, and that lecithotrophs seem to have a greater relative capacity for TH synthesis than do planktotrophs (Suyemitsu et al. 1997; Saito et al. 1998; Heyland 2004; Heyland and Hodin 2004). Such results indicate that obtaining TH from the environment is connected to the ability of feeding larvae to undergo the transition from larva to juvenile. But is the addition of TH sufficient for a large-egg planktotroph to complete metamorphosis and settle in the total absence of food?

Here we show that larvae of *L. sexiesperforata* can complete metamorphosis in the absence of any food if they are exposed to T4, whereas the larvae of *M. tenuis* cannot. Moreover, in a repeat of the *Leodia* experiment with a female that produced larger eggs (202 ± 5 μm), we were able to confirm the above-mentioned results. However, a few of these larvae reared in the absence of food and without hormone were also able to reach metamorphic competence. TH treatment is sufficient to turn obligatorily planktotrophic larvae derived from relatively large eggs into functional lecithotrophs. We discuss these data in the context of life history theory, juvenile size, and the evolution of alternative life cycles in echinoids specifically and marine invertebrates in general.

MATERIALS AND METHODS

We use the term thyroid hormone based on the evidence provided by Chino et al. (1994), Saito et al. (1998), and Heyland (2004). All

these studies confirm the presence of both T4 and T3 in different echinoid species and four species of unicellular algae, using high-pressure liquid chromatography purification and/or radioimmunoassay.

Animal collection, culturing of larvae, and experimental treatments

We collected adult *M. tenuis* (Clark 1940) by SCUBA west of Cedar Key, Florida in May 2001 and adult *L. sexiesperforata* (Leske 1778) by snorkeling off Long Key, Florida in May 2001 (Leodia experiment 1) and May 2004 (Leodia experiment 2). We maintained animals in aquaria with recirculating seawater at the University of Florida (Gainesville) until we set up fertilizations (*M. tenuis* on 17 May 2001; *L. sexiesperforata* on 5 June 2001 and 12 May 2004; one male and one female from each species in each experiment), using previously described procedures (Strathmann 1987). Additionally, we measured the egg sizes of five females in May 2001 and one female in May 2004 to obtain an idea of the variability in egg size in this species. Egg sizes in *L. sexiesperforata* ranged from 157 to 204 μm, with an average egg size of 178 ± 14 μm. From two females of *M. tenuis*, egg sizes ranged from 96 to 133 μm, with an average egg size of 111 ± 12 μm. Egg sizes for females used in these experiments are indicated in Table 1.

In Leodia experiment 1 we distributed embryos into three 2 l glass jars filled with 0.2 μm Millipore (Bedford, MA, USA) filtered seawater (MPFSW) after fertilization (fertilization success > 98%). Hatching occurred by 12 h after fertilization for both species, after which we transferred larvae to 1 l MPFSW at 1 larva/10 ml. We then set up four replicate cultures per experimental treatment for each species as follows: FOOD treatment (6000 cells/ml of the unicellular green alga *Dunaliella tertiolecta*, no T4), STARVED treatment (no food, no T4), and STARVED+T4 treatment (no food, 10^{-9} M T4) (Sigma, St. Louis, MO, USA). After settlement occurred in the FOOD treatment, we maintained cultures from the other treatments for a maximum of 2 weeks or until more than 50% of larvae had undergone settlement or died (7 June for *M. tenuis* and 19 June for *L. sexiesperforata*). We changed water (using MPFSW) in the cultures every 48 h, transferring larvae individually with glass Pasteur pipettes. In the STARVED+T4 treatment we added fresh T4 (see below) at each water change, and in the FOOD treatment we added fresh algae. Culturing temperatures for larvae and adults were 26 ± 1°C in both experiments. We monitored abnormalities in larvae but detected no differences among treatments. The protocol for Leodia experiment 2 was essentially the same, except that we used 0.45 μm filtered seawater and the fertilization success was 100%. The total duration of this experiment was from 12 May to 6 June 2004.

We made up T4 stock solutions using the following protocol (modified from Chino et al. 1994 and reported in Heyland and Hodin 2004). We diluted 0.0155 g L-thyroxine (T-1775, Sigma-Aldrich) in 10 ml 0.01 N NaOH and slowly warmed up to 50°C under continuous stirring. Once all traces of T4 were dissolved (approximately 10 min), we added 190 ml of dH_2O and stirred again for 5 min. Aliquots of this 10^{-4} M solution were immediately frozen at −20°C for future use. Before each water change we thawed one aliquot of T4 stock solution, diluted it 1:100 in MPFSW, and added 1 ml of 10^{-6} M working stock to 1 l of MPFSW (for a final concentration of 10^{-9} M), mixing well with mechanical agitation.

Biochemistry

We performed biochemical analyses (protein, lipid, and carbohydrate) on newly spawned fertilized eggs and newly metamorphosed juveniles from Leodia experiment 1 only. We prepared replicate samples in microgrinders (100–1000 μl capacity; Fisher Scientific, Atlanta, GA, USA) removing any seawater with a micropipette. Note that we ran four replicates each of 25 eggs per sample for *L. sexiesperforata* and 40 eggs per sample for *M. tenuis*. For *L. sexiesperforata* juveniles from the FOOD and STARVED+T4 treatments, we used 20 specimens per replicate for protein and lipid analysis and 30 specimens per replicate for carbohydrate analysis. Each measurement was replicated four times for each biochemical species (Table 1).

We quantified protein with the Coomassie brilliant blue G-250 binding assay (Bradford 1976) with bovine serum albumin as the standard (0.0–7.5 μg carbon). We homogenized samples in 700 μl distilled water and transferred 600-μl aliquots from each replicate into 13 × 100–mm glass test tubes. We added 50 μl of protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to each tube, mixed the contents thoroughly, and took spectrophotometric measurements (595 nm, 1-cm path length). (Bio-Rad's protein assay is based on the color change of Coomassie brilliant blue G-250 dye in response to various concentrations of protein.) We calculated protein content as the weight of bovine serum albumin (μg) yielding equivalent color change. To calculate total protein content for the original sample, we corrected for the difference in volume between the 600-μl aliquot and the full volume of distilled water used in the extraction.

We measured lipid content using the acid-dichromate oxidation technique (Parsons et al. 1984) with tripalmitin as the standard (0.0–50.0 μg carbon). We homogenized samples in 200 μl

Table 1. Egg size and energy is significantly higher in *Leodia sexiesperforata* than in *Mellita tenuis*

Species	Egg Size (μm) ($n = 20$)	Egg Volume (mm^3) ($n = 20$)	Egg Energy (μg)		
			Lipids ($n = 5$)	Proteins ($n = 5$)	Carbohydrates ($n = 5$)
Mellita tenuis	100 ± 2	$4.20 \pm 0.06 \times 10^{-3}$	0.21 ± 0	$4.9 \pm 0.6 \times 10^{-2}$	$7.8 \pm 2.5 \times 10^{-2}$
Leodia sexiesperforata	187 ± 5	$2.57 \pm 0.05 \times 10^{-2}$	0.63 ± 0.02	0.14 ± 0.01	0.10 ± 0.01

Values represent means ± 1 standard deviation of the mean. Egg size diameters were measured from 20 individual eggs; egg energy contents for *M. tenuis* were measured from 40 eggs per independent replicate (5 replicates), and from 25 eggs for *L. sexiesperforata* per independent replicate (5 replicates). L, lipids; P, proteins; C, carbohydrates.

chloroform and methanol at a 2:1 (v/v) ratio. Next, we added 50 µl of distilled water to each grinder and extracted the lipids by additional agitation and grinding with the pestle (Bligh and Dyer 1959). Phases were allowed to separate, and we transferred 100-µl aliquots of the organic phase from each sample into 13 × 100–mm acid-washed (0.3% acid dichromate) test tubes and dried them using a dry bath incubator at 65°C for 2 h. Lipids were oxidized with potassium dichromate (0.30%) in concentrated sulfuric acid (400 µl, 15 min, 105°C). We diluted samples in 900 µl distilled water and took spectrophotometric measurements (440 nm, 1-cm path length). We calculated lipid content as the weight of tripalmitin (µg) yielding equivalent reduction in dichromate oxidation. We calculated total lipid content for the original sample as described above for total protein content.

We assayed for carbohydrate content using the phenol-sulfuric acid method (Dubois et al. 1956) with dextran as the standard (0.0–20.0 µg carbon). We homogenized samples in 150 µl of distilled water and transferred 100-µl aliquots of each into 1.5-ml plastic centrifuge tubes. We then added 100 µl of liquefied phenol (Fisher Scientific) to each tube, followed immediately by 500 µl of concentrated sulfuric acid (H_2SO_4). Tubes were capped, mixed, and left at room temperature for 10 min. All samples were heated in a dry bath incubator at 30°C for 20 min and mixed thoroughly and then measured spectrophotometrically (490 nm, 1-cm path length). The phenol-sulfuric acid method is a colorimetric test that quantifies production of a yellow-orange product from reducing sugars and polysaccharides. We calculated carbohydrate content as the weight of dextran (µg) yielding equivalent color change. We calculated total carbohydrate content for the original sample as described above for total protein content.

Morphological analysis

These analyses were all performed for Leodia experiment 1 only. We sampled five *L. sexiesperforata* larvae from each of four replicate cultures per treatment (Table 1) at 25, 73, 126, 143, and 263 h after hatching (note that we sampled 10 larvae from one replicate from the STARVED treatment at 263 h; all larvae from the other three replicates had died). We performed morphological analyses on larval characters (PO, postoral arms; PD, postdorsal arms; BM, body midline), stomach size (SS; see below), and juvenile character rudiment size (RS; see below) (Fig. 2). We measured these characters in all cases in larvae that we had previously fixed in 4% paraformaldehyde (a maximum of 72 h before measurement), dehydrated through an EtOH series (50–100%), and cleared in clove oil (C8392, Sigma-Aldrich). We then measured the cleared larvae using a technique previously described and applied for similar purposes by McEdward (1984, 1985). We mounted larvae on a microscopic slide and viewed them using a compound microscope (Olympus, Pittsburgh, PA, USA) with an attached camera lucida. We identified specific larval landmarks (Fig. 2) on a digitizing tablet to retrieve the x- and y-information of the landmark. The z-information was retrieved with a rotary encoder attached to the fine focus knob of the microscope (McEdward 1984, 1985). The data (digitized x,y,z information from each individual landmark; Fig. 2) were exported into an Excel (Microsoft, Redmond, WA, USA) spreadsheet and we calculated the sizes of the morphological characters (see above) using general trigonometric analysis supported by Excel macro commands. Although PO, PD, and BM are all used as linear measurement, SS and RS were calculated as the square root of the cross-sectional area of an ellipsoid (using longest and shortest diameter of the stomach and longest and shortest diameter of the rudiment, respectively, as the axes of the ellipsoid; Fig. 2). We also staged development using one stage of the juvenile rudiment that is easily recognizable: the occurrence of first juvenile skeletal elements. We measured two morphological characters from freshly metamorphosed larvae: the spine length and the two largest test diameters that were orthogonal to one another using the system described above (microscope and digitizer). We then used these measured diameters to calculate juvenile size as the surface of an ellipsoid in mm^2.

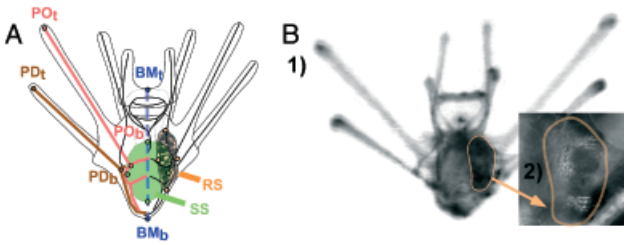

Fig. 2. Morphological structures in *Leodia sexiesperforata* larvae. (A) Cartoon of the *L. sexiesperforata* larva shown in B1. Five morphological characters are emphasized in color: brown, postdorsal arms (PD); red, postoral arms (PO); blue, body midline (BM); green, stomach size (SS); orange, rudiment size (RS). Lowercase b stands for base and lowercase t, for tip. We calculated SS and RS as the surface of an ellipsoid in which SL, SW, RL, and RW (not indicated) are the diameters of the ellipsoid. SL, longest diameter of stomach; SW, shortest diameter of stomach; RL, longest diameter of rudiment; RW, shortest diameter of rudiment. (B2) Dark-field close-up image of the echinus rudiment (developing adult structures) of the larva shown in B1. The bright spots indicate adult skeletal elements (skeletal plates and spines in this case).

Settlement and metamorphosis

As previously described (Heyland and Hodin 2004), we distinguished between settlement and metamorphosis. Although metamorphosis is a longer term process encompassing the morphogenetic transition from the bilateral larva to the pentameral juvenile, we define settlement operationally as the time when the larvae attach to the bottom of a glass jar and can resist a suction challenge with a Pasteur pipette. In all experiments we induced settlement by exposing larvae to 40 mM excess KCl. If larvae settled within 6 h, we considered them to have settled. *Leodia sexiesperforata* larvae usually settled after 2 to 4 h.

Statistics

We plotted each morphological character against RS using scatter plots and compared the distribution of points for the two treatments (STARVED+T4 and STARVED) using correlation analysis with subsequent Z-transformation for comparison of correlation coefficients. For comparison of juvenile size, spine length in juveniles, numbers of spines, and biochemical composition of juveniles, we used two-tailed independent sample *t*-test and MANOVA commands in SPSS (Chicago, IL, USA) based on

estimated marginal means. Note that all egg measurements are indicated in the text as means ± standard deviation unless stated differently. In cases where more than one female was measured, we noted the range of egg sizes additionally to the average.

RESULTS

We exposed larvae of *L. sexiesperforata* and *M. tenuis* to the following three treatments: FOOD, STARVED, and STARVED+T4. We repeated both experiments once. Larvae from the first *L. sexiesperforata* experiment (May 2001, *Leodia* experiment 1) developed from eggs with a diameter of 187 ± 5 µm (Table 1). In the second *L. sexiesperforata* experiment (May 2004, Leodia experiment 2), larvae developed from 202 ± 5 µm eggs. *Mellita tenuis* larvae from the first experiment (May 2001) developed from 100 ± 2 µm eggs (Table 1), whereas larvae from the second experiment (June 2001) developed from 121 ± 5 µm eggs. For Leodia experiment 1, we present a complete analysis of egg size, energetic composition of eggs, larval development, settlement, and energetic composition of postmetamorphic juveniles for the STARVED+T4 and the FOOD treatments (Tables 1 and 2; Fig. 3). For *M. tenuis* we only present the finding relevant in comparison with *L. sexiesperforata*, namely, that no larvae from either *M. tenuis* experiment completed metamorphosis in the absence of food, with or without excess T4 (STARVED treatment and STARVED+T4 treatment).

In Leodia experiment 1, 57 ± 6% (standard error) of the larvae from the STARVED+T4 treatment and more than 50% from the FOOD treatment settled upon induction of settlement with 40 mM excess KCl at 170 h after hatching (Table 2). No larvae from the STARVED treatment ever settled upon induction with KCl (Fig. 3B). These STARVED larvae died within 2 weeks (336 h) in culture. In *M. tenuis*, >50% of the larvae from the FOOD treatment settled upon induction with 40 mM excess KCl 288 h after hatching. No larvae from the STARVED+T4 or STARVED treatments settled during the 21 days of this experiment (17 May to 7 June) in response to induction with KCl; by the end of the experiment, all larvae had died. In Leodia experiment 2 (May 2004), >50% of larvae from the STARVED+T4 treatment settled after 4 days, whereas >50% of larvae from the FOOD treatment settled after 6 days. However, in contrast to Leodia experiment 1 (see above), 12% of the larvae from the STARVED treatment in Leodia experiment 2 settled after 12

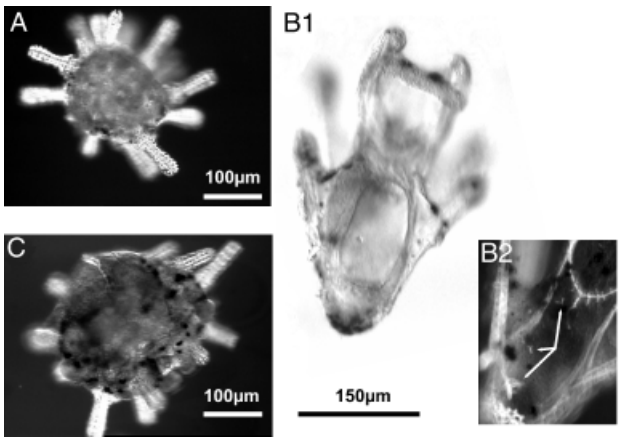

Fig. 3. Starved larvae of *Leodia sexiesperforata* can complete metamorphosis in the absence of food when treated with thyroxine (A, STARVED+T4 treatment), whereas larvae reared in the absence of food and thyroxine cannot (B1, STARVED treatment; B2, dark-field close-up image from the larva in B1). Larvae fed with a normal food ration of 6000 cells/ml *D. tertiolecta* in the absence of excess hormone (FOOD treatment) metamorphose at a much larger size (C) than larvae from the STARVED+T4 treatment (A). The STARVED larva in B1 developed early juvenile skeletal structures (arrows indicate spicules and plates). Note that the larval structures in STARVED are partially resorbed (compare larva in B1 with the healthy-looking larva depicted in Fig. 2). Note also that all these larvae originated from an initial egg size of 187 ± 5 µm (see Table 1 and text for further detail).

Table 2. Thyroid hormone treatment of *Leodia sexiesperforata* larvae results in earlier completion of metamorphosis at a reduced size and energy content

			Energy (µg)		
Treatment	Age_M (h)	$Size_M$ (mm^2) ($n = 10$)	Lipids	Proteins	Carbohydrates
STARVED+T4	142	$3.15 \pm 0.07 \times 10^{-4}$	0.51 ± 0.14 ($n = 4$)	0.21 ± 0.01 ($n = 4$)	$6.9 \pm 0.7 \times 10^{-2}$ ($n = 4$)
FOOD	170	$4.83 \pm 0.05 \times 10^{-4}$	1.84 ± 0.59 ($n = 5$)	0.41 ± 0.06 ($n = 5$)	0.35 ± 0.04 ($n = 5$)

Juveniles of *L. sexiesperforata* from the STARVED+T4 (10^{-9} thyroxine, no food) treatment completed metamorphosis earlier (Age_M) at a smaller size ($Size_M$) and had significantly less energy than juveniles from the FOOD treatment (no excess thyroxine, 6000 cells/ml *Dunaliella tertiolecta*). Larvae from the STARVED treatment (no thyroxine, no food) did not reach the juvenile stage during the course of the experiment (see Materials and Methods). Values are means ± 1 standard error. Numbers of independent replicates are indicated in parentheses. We used 20 juveniles per protein and lipid replicate and 30 juveniles for carbohydrates. For juvenile size ($Size_M$), parentheses indicate replicate individual larvae for energy, independent replicate measurements (see Materials and Methods). We calculated juvenile size at metamorphosis as surface area based on two perpendicular diameters (see Materials and Methods). L, lipids; P, proteins; C, carbohydrates.

days, and this percent of settled larvae was maintained until the end of the experiment on 6 June 2004.

In summary, we found that larvae of *L. sexiesperforata* have the capacity to complete metamorphosis in the absence of food when excess TH is present, whereas starved *M. tenuis* larvae cannot complete metamorphosis, either with or without excess hormone. Our data also suggest that a small percentage of *L. sexiesperforata* larvae can complete metamorphosis in the absence of food, but only when these larvae are derived from sufficiently large eggs (in Leodia experiment 2: 202 ± 5 μm). Even so, most of these latter larvae will only complete metamorphosis if additional TH is provided.

Postmetamorphic *L. sexiesperforata* juveniles from the STARVED+T4 treatment (Leodia experiment 1) were significantly smaller than those from the FOOD treatment ($t_{1,18} = -19.71$; $P < 0.001$). Juveniles from the STARVED+T4 treatment had significantly lower lipid ($d = 1.33 \pm 0.53$; $P = 0.024$), carbohydrate ($d = 0.28 \pm 0.04$; $P < 0.001$), and protein ($d = 0.21 \pm 0.06$; $P = 0.004$) contents when compared with the FOOD treatment (d represents difference in energy content ± standard error of the mean, derived from an analysis of variance with marginal estimated means; see Materials and Methods) (Table 2; Fig. 3).

Although *L. sexiesperforata* juveniles derived from the STARVED+T4 treatment (Leodia experiment 1) were smaller than those from the FOOD treatment, they otherwise appeared normal and functional (e.g., they were capable of moving their tube feet and spines and could adhere to the walls of their culture bowls; we also observed this in Leodia experiment 2). To further assess juvenile development, we measured and counted the spines in Leodia experiment 1. We did not find any significant difference between average spine length from the STARVED+T4 treatment (61.45 ± 17.12 μm; $n = 9$) compared with the FOOD treatment (76.05 ± 25.80 μm; $n = 9$) ($t_{1,16} = 0.47$; $P = 0.64$), nor did we detect a significant difference ($t_{1,18} = 0.20$; $P = 0.85$) in spine number (14.3 ± 0.7 STARVED+T4 vs. 14.5 ± 0.7 FOOD treatment).

To assess developmental strategies among treatments, we investigated *L. sexiesperforata* larvae (Leodia experiment 1) for their relative investment into larval structures (larval arm length and body midline length) or the stomach versus the juvenile rudiment (for a comparable analysis, see Strathmann et al. 1992 and Heyland and Hodin 2004). We consider the stomach to be both a larval and a juvenile structure because the larval stomach is partially retained in the juvenile (Chia and Burke 1978). Figure 4 shows that developmental trajectories for PO, PD, BM, and SS relative to RS are different between the STARVED and STARVED+T4 treatments. Pearson's correlation coefficients and two-tailed test value for the STARVED treatment were as follows: RS-PO ($r_{1,13} = 0.42$; $P = 0.16$), RS-PD ($r_{1,13} = 0.85$; $P < 0.01$), RS-BM ($r_{1,13} = 0.47$; $P = 0.10$), RS-SS ($r_{1,13} = -0.67$; $P = 0.01$). Pearson's correlation coefficients and two-tailed test value for T4 treatment were as follows: RS-PO ($r_{1,12} = -0.91$; $P < 0.01$), RS-PD ($r_{1,12} = -0.65$; $P = 0.02$), RS-BM ($r_{1,12} = -0.94$; $P < 0.01$), RS-SS ($r_{1,12} = -0.98$; $P < 0.01$). We then compared these Pearson's correlation coefficients from the STARVED+T4 and STARVED treatments using Fisher's Z-transformation. Z values are as follows: RS-PO ($Z = 4.23$; $P < 0.01$), RS-PD ($Z = 4.44$; $P < 0.01$), RS-BM ($Z = 4.83$; $P < 0.01$), RS-SS ($Z = 3.08$; $P < 0.01$).

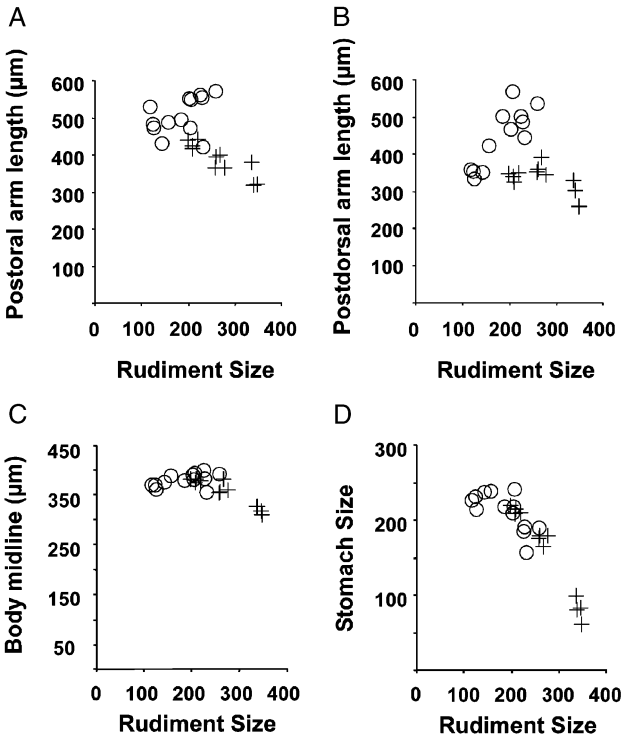

Fig. 4. Relative allocation of energy is "switched" from larval to juvenile structures when larvae are treated with exogenous hormone. (A–D) Correlation of morphological characters (*y*-axis) against rudiment size (*x*-axis). See text for the results of a correlation analysis. +, STARVED; O, STARVED+T4.

The aforementioned results show that development through metamorphosis was significantly accelerated with TH treatment in *L. sexiesperforata*. This acceleration was accomplished both by a shift in investment from larval to juvenile structures (see above) and as precocious development of juvenile structures. At 25 h after hatching, 100% of the larvae from the STARVED+T4 treatment (Leodia experiment 1) had begun to build juvenile skeletal structures, whereas none of the STARVED larvae had done so. Furthermore, only 25% of STARVED larvae had juvenile skeletal structures 126 h after hatching, 20% at 143 h after hatching, and 33% at 263 h after hatching.

DISCUSSION

We show that an obligatorily feeding larva has the ability to become metamorphically competent and settle in the absence of food when T4 is provided. Measurements of several morphological characters suggest a relative shift in energy allocation from larval to juvenile structures in larvae exposed to T4 compared with control larvae. This change in developmental strategy suggests that a change in energy investment from larval to juvenile structures permits hormone-treated larvae to attain metamorphic competence in the absence of food. Our results indicate that THs along with egg size are critical determinants of developmental mode and suggest plausible scenarios for the evolution of lecithotrophy within planktotrophic lineages.

Egg size and TH as determinants of developmental mode in echinoids

By definition, planktotrophic larvae require exogenous nutrition before they can undergo the metamorphic transition and settle to the benthos. Lecithotrophic larvae, which either do not need to feed (facultative feeders) or cannot feed (obligate lecithotrophs), develop from significantly larger eggs than their obligatorily planktotrophic relatives. Such lecithotrophic taxa have been hypothesized to have evolved many times independently from planktotrophic ancestors within the echinoids (sea urchins, sand dollars, and their kin) and other invertebrate groups (Strathmann 1985; Wray 1995; Hart et al. 1997; McEdward 1997; but see Lacalli 1993; McHugh and Rouse 1998). These observations have led to the hypothesis that there is a taxon-specific critical egg size that determines developmental mode in echinoids and in other marine invertebrates as well (Mortensen 1921; Vance 1973a,b; Emlet et al. 1987; Havenhand 1995; Sewell and Young 1997; McEdward and Miner 2003).

Still, if egg size is the only determinant of developmental mode in echinoids (noting the widespread controversy concerning how well egg size represents maternal investment; for discussion see McEdward and Morgan 2001), then we would predict a critical egg size, above which we would find strictly lecithotrophic development. We know from comparative and experimental data that no such critical threshold exists for echinoids. The larvae of the heart urchin *Brisaster latifrons* have the ability to feed but can also complete metamorphosis in the absence of exogenous food (Hart 1996). But *B. latifrons* larvae develop from much larger eggs (345 μm diameter) than the nonfeeding (obligatorily lecithotrophic) sand dollar species *Peronella japonica* (276 μm diameter, which is at the lower end of the egg size range for lecithotrophs in echinoids). Moreover, blastomere separation experiments done 50 years ago with *P. japonica* showed that nonfeeding larvae can complete metamorphosis from half (220 μm) or quarter sized (176 μm) embryos (Okazaki and Dan 1954). Although some differences in juvenile morphology were observed in some of these partial embryos, they are clearly far below the hypothesized critical egg size for lecithotrophic development in echinoids. Thus, egg size does not correlate strictly with developmental mode in echinoids.

Our data presented here provide further evidence that egg size is not the only factor determining developmental mode in echinoids, though it is clearly important. The addition of T4 can allow an obligatorily planktotrophic larva to complete metamorphosis in the absence of food. The ability to do so, however, certainly depends on egg size. Furthermore, the evidence to date suggests that different echinoid species differ in the ability of their larvae to synthesize THs endogenously, with lecithotrophic larvae having a greater capacity for endogenous hormone synthesis than their planktotrophic relatives (Suyemitsu et al. 1997; Saito et al. 1998; Hodin et al. 2001; Heyland and Hodin 2004).

Together, these results provide a plausible explanation for why there is no clear threshold of egg size in echinoids that results in lecithotrophic development. Determination of development mode is most likely a combination of factors such as egg size and the ability to synthesize signaling molecules such as TH endogenously. To further test this hypothesis, one should apply a similar experimental design as we used in this study to a diversity of echinoderm species developing from different egg sizes. Moreover, we propose generating half or quarter sized embryos from species such as *L. sexiesperforata* and the facultative feeding sea biscuit *Clypeaster rosaceus* and testing them for their ability to complete metamorphosis in the absence of food. If any of these size-reduced embryos are unable to develop to settlement under these conditions (and we can be reasonably certain that reduced sized *L. sexiesperforata* embryos would be unable), we suggest exposing these larvae to T4 and then testing for their ability to complete metamorphosis.

Our data with two batches of *L. sexiesperforata* eggs provide a tantalizing link between internal hormone synthesis and egg size as codeterminants of developmental mode. *Leodia sexiesperforata* from our smaller-egg batch were unable to undergo metamorphosis at all in the absence of food, unless they were provided with additional hormone. By contrast, a small percentage of starved *L. sexiesperforata* larvae from our larger-egg batch did complete metamorphosis, even in the absence of additional hormone. These data suggest the intriguing possibility of a partial correlation of endogenous hormone synthesis with egg size, both within and among species. We discuss in further detail below the notion that this correlation may vary among echinoid taxa and might underlie taxon-specific differences in the likelihood of evolving lecithotrophy. Furthermore, these data indicate that *L. sexiesperforata* could be considered a poecilogenous species, like the spionid polychaete *Streblospio*, where both obligate

planktotrophs and lecithotrophs can be found, even within the same population (Levin 1986). Experiments with additional *L. sexiesperforata* crosses are required to more fully evaluate this possibility.

Juvenile size in echinoids

Emlet et al. (1987) reviewed time to settlement and size at settlement from over 200 echinoid and asteroid species (for a comparable analysis see Levitan 2000). These meta-analyses revealed that (a) juvenile size among echinoid species with feeding larvae is relatively constant (approximately $380 \pm 70\,\mu m$) over a 3-fold range in egg diameters (70–210 μm), (b) time to settlement is highly variable in planktotrophic echinoid larvae, (c) planktotrophic and lecithotrophic asteroids have variability in size at settlement but less variability in time to settlement, and (d) lecithotrophic asteroids show a strong positive correlation between egg size and juvenile size, whereas planktotrophic echinoids and asteroids do not. From these data the question emerges about the link between egg and juvenile size and possible constraints on juvenile size.

Emlet et al. (1987) and Emlet and Hoegh-Guldberg (1997) proposed that egg size might play a fundamentally different role in planktotrophic and lecithotrophic development. Although planktotrophic larvae can potentially settle earlier when egg size is increased, lecithotrophic larvae, which already develop at the maximal developmental rate (McEdward 1997), could use increased energy from the egg to build a larger juvenile. Our data from *L. sexiesperforata* show that TH-treated planktotrophic larvae settle at a much smaller size than has been previously observed in the laboratory or in the field.

Empirical evidence has supported the hypothesis that better quality juveniles frequently have higher fitness by outperforming juveniles of poorer quality. These better quality juveniles, either assessed by higher energy content or by juvenile size, can result in increased juvenile growth rates (Miller and Emlet 1999; Roberts and Lapworth 2001; Phillips 2002), increased survival (Emlet 1986; Emlet and Hoegh-Guldberg 1997), increased intra- and interspecific competitive ability (Connell 1985), or a size refuge from predation (Stoner 1990; Gosselin 1997). Such costs associated with small juvenile size could explain why it would be disadvantageous for larvae to metamorphose at the smallest size possible, despite the initial survival advantage associated with a shorter developmental time in the plankton (Rumrill 1990; Lamare and Barker 1999).

We propose the testable hypothesis that juvenile size in planktotrophic echinoids is constrained and that this constraint can be experimentally relaxed by exposing larvae to THs. Experimentally induced small juveniles could be exposed to different competition and predation regimes to measure their mortality in the natural environment when compared with control full-sib juveniles. Such an experiment is presumably not possible by simply manipulating food level or food type, because such treatments provide only subtle variation in juvenile size (Sinervo and McEdward 1988; Hart 1996; Hodin et al. 2001).

Our data suggest that TH from an exogenous source (phytoplankton in the field, addition of TH to the water in our experiments) signals to a feeding echinoid larva when it has reached the appropriate stage to undergo settlement (Heyland and Hodin 2004). This particular environmental signal could be more reliable than alternative signals such as the number of ingested particles because different food types (different algae species) can vary significantly in caloric content (Hinegardner 1969; Strathmann 1971, 1987; Jonasdottir 1994; McEdward and Herrera 1999). However, caloric content alone is unlikely to be the signal for the attainment of competence to settle: our TH-treated *L. sexiesperforata* larvae completed metamorphosis despite being deprived of an external energy source, and half and quarter embryos from *P. japonica* yielded settled juveniles. It is possible that there is a strong correlation between caloric and TH contents in planktonic algae, something that has not been investigated to date.

TH and the evolution of lecithotrophy

Hormones play critical roles in the development of insects (ecdysteroids and juvenile hormone) and amphibians (THs) where they regulate complex morphological transitions such as larval and pupal molts and metamorphosis to the adult stage (reviewed in Nijhout 1994; Tata 1998). Furthermore, these same hormones have been hypothesized to underlie the evolution of alternative life history strategies within these two animal groups (insects: Nijhout 1999; Hodin and Riddiford 2000; Truman and Riddiford 2002; amphibians: Frieden 1981; Kühn and Jacobs 1989; Yaoita and Brown 1990; Galton 1992; Hanken et al. 1997; Jennings and Hanken 1998; Rose 1999; Callery and Elinson 2000; reviewed in Heyland et al., in press). Although there is considerable support for the hypothesis that, among echinoids, planktotrophy is the ancestral life history strategy and lecithotrophy is derived (Strathmann 1978, 1985; Hart 1996; but see Lacalli 1993), little information exists on the mechanisms that could have led to such a radical change in mode of life.

In echinoderms, several authors, including ourselves, investigated the hormonal regulation of metamorphosis (Chino et al. 1994; Johnson and Cartwright 1996; Suyemitsu et al. 1997; Johnson 1998; Saito et al. 1998; Hodin et al. 2001; Heyland and Hodin 2004) and the possible involvement of these hormones in life history evolution as well (Hodin et al. 2001; Heyland and Hodin 2004). T4 and T3 accelerate larval development through metamorphosis and lead to precocious metamorphosis and settlement in sand dollars (Suyemitsu

et al. 1997; Saito et al. 1998; Hodin et al. 2001; Heyland and Hodin 2004) and sea urchins (Chino et al. 1994; Johnson 1998). Although lecithotrophic sand dollar larvae seem to synthesize hormones endogenously (Suyemitsu et al. 1997; Saito et al. 1998), planktotrophic sea urchin larvae depend largely on hormones from phytoplankton, which contains significant amounts of T4 and T3 (Chino et al. 1994; Heyland 2004). Such findings indicate that THs may have played a critical role for the evolution of lecithotrophy in echinoids and potentially for other marine invertebrates as well (Hodin et al. 2001; Heyland and Hodin 2004).

Based on this information and the facultative feeding model presented by McEdward (1997), we envision a scenario for how a derived lecithotrophic mode of development might have arisen. The relative length of the facultative feeding period of *C. rosaceus* is extended compared with closely related obligate planktotrophs such as *M. tenuis* and *L. sexiesperforata* (Fig. 1). Here we consider two proximate mechanisms by which the facultative feeding period could be extended: by increasing egg size and by accelerating development so that later developmental stages can be reached with the available maternal energy. This latter option depends largely on the energetic and physiological costs associated with accelerated development and how these costs compare with the costs associated with staying in the plankton for an extended period of time. Still, based on our scenario, the first mechanism requires increased maternal investment, whereas the second mechanism could be achieved by upregulation of endogenous TH synthesis at a given egg size. Our morphological analyses in this and a previous study (Heyland and Hodin 2004) provide evidence that TH treatment induces an acceleration of development of juvenile structures relative to larval structures. The fact that this acceleration of development allows *L. sexiesperforata* larvae to eliminate the obligate feeding period suggests that upregulation of endogenous TH synthesis (second mechanism) may be the proximate mechanism underlying the evolution of lecithotrophy in planktotrophic species with sufficiently large eggs.

To begin to tease apart the relative importance of egg size and endogenous TH synthesis on the relative lengths of the facultative and obligate feeding periods as well as for the evolution of lecithotrophy, we propose the following. First, a broader range of echinoids (as well as other echinoderms) should be investigated for the correlation between endogenous hormone synthesis, egg size, and the relative lengths of these feeding periods. Second, more large-egg planktotrophs (from diverse taxa) should be exposed to TH treatments such as those reported here. Finally, we propose to feed larvae with algae pretreated with inhibitors of TH synthesis, so that they will either contain very little or no hormone. We would predict, for example, that if larvae of *L. sexiesperforata* are fed with such algae, then they would be inhibited in their ability to reach the juvenile stage.

It has been known for a considerable time that feeding larvae with different algal species can result in different times to settlement in a variety of echinoids (Hinegardner 1969; Strathmann 1971; McEdward and Herrera 1999) and, indeed, that some algae are unable to support growth through metamorphosis at all. Testing our hypothesis that these algae might differ in caloric content (as has been typically assumed) as well as in TH content will have wide-ranging implications, not only for the relationship between hormones and life history evolution, but also for our understanding of what exactly is required to make a juvenile out of a larva. Such studies would also offer what we believe is a more holistic picture of developmental hormones, namely, morphogenetic compounds in an appropriate ecological context.

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