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The evolution of larval developmental mode: insights from hybrids between species with obligately and facultatively planktotrophic larvae

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SUMMARY Life history characteristics play a pervasive role in the ecology and evolution of species. Transitions between feeding and non-feeding larval development have occurred many times in both terrestrial and marine phyla, however we lack a comprehensive understanding of how such shifts occur. The sea biscuits *Clypeaster rosaceus* and *Clypeaster subdepressus* employ different life history strategies (facultatively feeding larvae and obligately feeding larvae, respectively) but can hybridize. In this study, we examined the development of hybrid larvae between these two species in order to investigate the inheritance of larval developmental mode. Our results show that both reciprocal hybrid crosses developed via the feeding mode of their maternal species. However, as feeding larvae can obtain both energy and hormones from algal food, we tested how hormones alone affected development by setting up a treatment where we added exogenous thyroid hormone, but no food. In this treatment the offspring of all four crosses (two homospecific and two heterospecific crosses) were able to metamorphose without algal food. Therefore we hypothesize that although hybrid developmental mode was inherited from the maternal species, this result was not solely due to energetic constraints of egg size.

INTRODUCTION

Development through a free-living larval stage is a widespread component in life histories of both marine and terrestrial metazoans. Most marine invertebrates have larvae that fall into one of two categories: feeding, planktotrophic, or non-feeding, lecithotrophic larvae. Planktotrophic larvae come from small eggs and can spend weeks to months feeding in the plankton before settling. Lecithotrophic larvae develop from large eggs and metamorphose in a matter of days. In echinoderms—presumably as in all metazoans—feeding larvae represent the ancestral life-history state, whereas non-feeding larvae have independently evolved many times (Strathmann 1978; Wray and Bely 1994; Hart 1995; Wray 1996; Raff and Byrne 2006). Larval developmental mode in marine invertebrates plays a critical and pervasive role in micro- and macro-evolutionary processes as it affects species ranges, population connectivity, as well as rates of molecular evolution, speciation, and extinction (Vermeij 1982; Strathmann 1985; Wray and Raff 1991; Jeffery and Emlet 2003). Despite the number of times this transition has occurred, we have little mechanistic understanding of the evolution of alterative developmental modes.

In addition to planktotrophy and lecithotrophy, some echinoderm species develop through an intermediate developmental mode, facultative planktotrophy (Emlet 1986; Hart 1996). Facultatively planktotrophic larvae can feed, but do not need to, as they can metamorphose solely based on energy provided in their egg. Larvae that are facultatively planktotrophic develop from an intermediate egg size and exhibit intermediate developmental timing to metamorphosis; however, they retain a pluteus morphology and therefore phenotypically resemble planktotrophic larvae (Emlet 1986; Hart 1996; Wray 1996). Facultatively planktotrophic larvae provide a unique opportunity to examine the order and timing of changes that occur in the evolution of lecithotrophy (Allen and Pernet 2007; Snoke-Smith et al. 2007; Zigler et al. 2008; Collin 2012; Zigler and Raff 2013).

An increase in egg size is consistently associated with the evolution of non-feeding development. Previous models explaining the evolution of alternative developmental modes in marine invertebrate life histories have focused on changes in egg size as the primary corollary of development (Vance 1973; Christiansen and Fenchel 1979; Wray 1996; McEdward 1997). However, experimental egg size reductions show that changes in egg size do not hinder the ability of larvae to develop (Allen et al. 2006). Additionally, in echinoderms, the loss of a feeding larval form is accompanied by the gain of many characters, including early formation of a large left coelom (Snoke-Smith et al. 2007), changes in embryonic axis formation (Raff et al.

1999), and the ability to produce endogenously hormones that stimulate metamorphosis (Heyland et al. 2004). These developmental changes must involve genetic regulation, suggesting that the evolution of non-feeding larvae is more complicated than simply increasing maternal investment (Allen and Pernet 2007; Snoke-Smith et al. 2007; Zigler et al. 2008; Zigler and Raff 2013).

Changes in hormonal regulation appear to play an important role in the evolution of life history characteristics throughout metazoans (Manzon et al. 2001; Pfennig 1992; Chino et al. 1994; Heyland and Moroz 2005). For example, thyroid hormones cue metamorphosis in echinoderm larvae (Chino et al. 1994; Johnson and Cartwright 1996; Heyland and Hodin 2004). Non-feeding echinoderm larvae (including facultative planktotrophs) can synthesize their own thyroid hormones, whereas feeding larvae must obtain hormones from their food (Heyland et al. 2006). Thus, the evolution of endogenous thyroid hormone synthesis should be incorporated into models of the evolution of non-feeding larval forms.

Taken together, existing evidence suggests that changes in maternal investment (Vance 1973; Wray 1996; Levitan 2000), hormonal regulation (Chino et al. 1994; Heyland and Hodin 2004), and genetic regulation of development (Nielsen et al. 2000; Zigler and Raff 2013) are all involved in the evolution of non-feeding larval development. Integrating these studies has proved challenging, however, as few study systems have been able to investigate egg size and zygotic genetic effects together. Here we present a study system in which the relative importance of these factors in controlling developmental mode can be examined. The sea biscuit *Clypeaster rosaceus* is one of only two echinoderm species known to possess facultatively planktotrophic larvae. *Clypeaster rosaceus* co-occurs with its congener *Clypeaster subdepressus,* which possesses obligately planktotrophic larvae. This species pair diverged around 8 million years ago, has a 15-fold difference in egg energy content, and can be hybridized in the laboratory (Zigler et al. 2008). No study has followed the hybrids past fertilization, however, and the developmental mode of the hybrid offspring remains unknown.

Using larvae of these two species and their hybrids, we addressed two main questions. First, we tested how developmental mode is inherited. Offspring from both reciprocal hybrid crosses have half of their genome from each species; however they come from different maternal species, and therefore their egg sizes differ substantially. If the offspring from the two hybrid crosses develop similarly to their maternal species, this would suggest that a maternal effect, such as egg size, maternally deposited mRNA, or genetic imprinting controls developmental mode. Alternatively, if the offspring from two hybrid crosses develop similarly to each other, this would suggest that zygotic genetics control developmental mode. To address this question, we reared larvae from all four crosses (two homospecific, two heterospecific) in fed and starved larval cultures to determine their developmental mode. Second, we asked whether a difference in endogenous production of hormones affected the observed developmental differences between the crosses. To do this, we set up two additional treatments: thyroid hormone addition and thyroid hormone inhibition. If offspring from the crosses that develop as obligate planktotrophs are able to metamorphose with only the addition of thyroid hormone, then the a lack of hormone production, rather than an energetic constraint, would be important in limiting their ability to metamorphose without feeding.

METHODS

Collection

We conducted these experiments at the Smithsonian Tropical Research Institute (STRI) in Bocas Del Toro, Panama in August and September 2013. We collected adult specimens of *Clypeaster rosaceus* and *Clypeaster subdepressus* at STRI point (9.3497°N, 82.2632°W). Following collection, we immediately transported adults back to flow-through seawater tanks at ambient water temperatures (27–29°C; http://biogeodb.stri.si.edu/physical_monitoring) and maintained them there until spawning.

Fertilization

We ran three replicate experiments throughout August and September using different parents in each. For each experiment, we used gametes from one male and one female of each species to set up crosses. We injected adults with 2–3 ml of 0.5 M KCl solution to obtain gametes. After injection, we placed females over beakers containing 0.45 μm filtered seawater (FSW) for egg collection; we pipetted sperm dry directly off the aboral side of males into 1.5 ml collection tubes. After eggs had been spawned, we washed the eggs in FSW twice before pipetting them into beakers containing 100 ml of fresh FSW. We pipetted eggs of each female into two separate beakers for concentrations of approximately 50 eggs/ml. In order to set up all four possible crosses, we added conspecific sperm to one beaker and heterospecific sperm to the other. In this article we designate each cross with initials from the maternal species listed first (*C. rosaceus* eggs × *C. rosaceus* sperm = CR × CR; *C. subdepressus* eggs × *C. subdepressus* sperm = CS × CS; *C. rosaceus* eggs × *C. subdepressus* sperm = CR × CS; and *C. subdepressus* eggs × *C. rosaceus* sperm = CS × CR). We kept sperm dry until immediately before fertilizations when we added one drop of concentrated sperm to 10 ml of FSW. For crosses between conspecifics, we added 0.1 ml dilute sperm solution to each of the beakers containing eggs. To compensate for low fertilization success in heterospecific crosses, we added excess sperm, as described in Zigler et al. 2008, to achieve fertilization. After

exposure to the sperm for 10 min, we washed the eggs twice with FSW to minimize the chance of polyspermy. Two hours post fertilization, we examined 50 embryos from each cross to determine the proportion that cleaved. For larval rearing, we only used offspring from crosses in which both homospecific combinations achieved at least 85% cleaving zygotes.

Larval husbandry

Directly after hatching (12 h post fertilization), we pipetted 200 swimming larvae of each cross into 1000 ml plastic beakers. We maintained cultures at ambient air temperature (25–29°C; http://biogeodb.stri.si.edu/physical_monitoring) on a Strathmann (1987) stirring rack. Forty cultures were set up per experiment—10 cultures of each cross. One of four treatments (described below) was randomly assigned to each culture resulting in three replicate fed, three replicate starved, three replicate unfed thyroid hormone treatment, and one unfed hormone inhibited culture (only one could be set up due to constraints on the number of cultures we could rear at once) for each cross. Thus, the treatments were as follows: Fed: We added 2500 cells/ml of each of the two algal species *Isochrysis galbana* and *Rhodomonas* spp. to each of the fed cultures. Starved: Nothing was added to starved cultures beyond fresh FSW. Thyroid hormone: We added the thyroid hormone, thyroxine (Sigma Aldrich T-1775), at a concentration of 10^{-9} M (Heyland et al. 2006). Nothing else was added beyond fresh FSW. Hormone inhibited: We added the thyroid hormone synthesis inhibitor, thiourea, to cultures to block endogenous production of thyroid hormone. We mixed thiourea (Sigma–Aldrich T-8656) in FSW and added it to beakers for a concentration of 10^{-5} M (Chino et al. 1994).

Every other day, we changed the water in each larval culture by siphoning out 80% of the water through a 100 μm mesh filter and adding fresh FSW. After adding FSW to each culture, treatments were administered by adding food, hormone, or hormone inhibitor accordingly. We used KCl as a settlement cue; we added 50 mM excess KCl to induce metamorphosis in cultures where larvae had developed an advanced juvenile rudiment (Strathmann 1987; Pearce and Scheibling 1994). Larvae were continuously exposed to KCl after initial addition. We inspected cultures daily for juveniles until the whole culture had settled or died.

Measurements

To measure egg size, we photographed ten unfertilized eggs from both females used in each experiment under a compound microscope at 100× magnification directly after they were released. Every other day we also photographed five larvae from each culture on a compound microscope at 100× magnification until all the larvae had settled, died, or until 12 days post fertilization (dpf) when all larvae had stopped growing larger. Juveniles were photographed on the day they settled. We used imageJ (version 1.48) to measure egg diameter, larval body length, larval post-oral arm length, total length of larvae, and test diameter of juveniles from each picture (Fig. 1).

Larval cultures were maintained until all larvae settled as juveniles or died; the number of juveniles that metamorphosed out of the 200 larvae originally placed in each culture determined survivorship. Larvae in all cultures either settled or died by 30 dpf.

Statistical analyses

We compared egg size between females using a linear model in R (version 3.1.0), with female as a fixed effect. We tested for effect of cross and treatments on larval phenotype, juvenile size, and time to metamorphosis using a linear model in R with maternal species, paternal species, treatment, and their interactions as fixed effects; we modeled replicate experiment as a random effect. Due to variable rates of development between crosses, we only analyzed larval measurements from the 8-arm stage of each cross (Table 1).

We compared the number of juveniles that settled in each culture using a quasibinomial regression in R, followed by a Chi-Square goodness of fit test to determine the significance of each term in the model. We modeled the effects of maternal

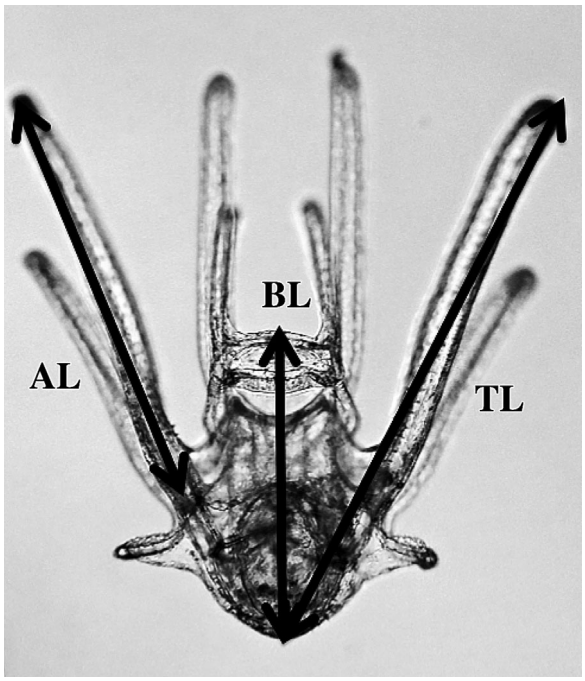

Fig. 1. Larval measurements. Body length (BL) was measured from the midpoint of the larva to the top of the oral hood. Arm length (AL) was measured from the tip of each post oral arm to where the arm met the body. Total length (TL) was measured from the tip of the longest arm to the body midline.

Table 1. Developmental timing of all crosses. Embryonic stages are measured in hours post fertilization (hpf) while larval stages are measured in days post fertilization (dpf). Developmental timing in the fed cultures of each cross is shown here. Time to metamorphosis shows the range over which larvae were seen to metamorphose in the fed treatment. For each cross, initials of the maternal species are listed first and the paternal species second

Stage	CR × CR	CR × CS	CS × CR	CS × CS
Two-cell	2 hpf	2 hpf	1.5 hpf	1.5 hpf
Hatched blastula	12 hpf	12 hpf	8 hpf	8 hpf
Prism	1 dpf	1 dpf	1 dpf	1 dpf
Four arm	2 dpf	2 dpf	3 dpf	3 dpf
Six arm	3 dpf	3 dpf	5 dpf	6 dpf
Eight arm	4 dpf	4 dpf	8 dpf	10 dpf
Metamorphosis	5–9 dpf	6–10 dpf	15–20 dpf	16–28 dpf

species, paternal species, treatment, and their interactions as fixed effects with replicate experiment as a random effect.

In each of these analyses, when significant effects were found, we ran Tukey's HSD post hoc test to test for pairwise differences.

RESULTS

Development

In both *Clypeaster subdepressus* and *C. rosaceus* egg diameter significantly differed between females of the same species ($F_{2,\ 27} = 9.356$, $P < 0.001$; and $F_{2,\ 27} = 17.36$, $P < 0.001$, respectively) (Table 2). However, intraspecific differences in egg size did not result in developmental mode differences between experiments. In all three replicate experiments, larvae from each of the four crosses developed into normal echinoplutei (Fig. 2) and were able to metamorphose. As with previous work (Emlet 1986), *C. rosaceus* larvae metamorphosed in both the presence and absence of food, whereas larvae from *C. subdepressus* only metamorphosed in cultures containing food. Larvae from both hybrid crosses employed the same developmental mode as their maternal species: hybrids from *C. rosaceus* eggs (CR × CS) metamorphosed in both the fed and starved treatments, whereas hybrids from *C. subdepressus* eggs (CS × CR) only metamorphosed in the fed treatment (Fig. 3).

Table 2. Mean ± SE diameter of eggs used in each experiment. Measurements are shown in micrometers. Means with different superscripts (a, b, c, d, or e) were significantly different from one another in Tukey's HSD post-hoc test ($P < 0.05$)

	C. subdepressus	*C. rosaceus*
Experiment 1	152 ± 0.81[a]	290.0 ± 2.23[c]
Experiment 2	145.8 ± 1.19[b]	282.3 ± 1.01[d]
Experiment 3	145.9 ± 1.35[b]	298.2 ± 2.19[e]

Hormone addition and hormone inhibition both had strong effects on larval development (Fig. 3). The addition of exogenous thyroid hormone resulted in metamorphosis despite a lack of food in all four crosses, including the two from *C. subdepressus* eggs, which did not metamorphose in the starved treatment. Alternatively, adding the thyroid hormone synthesis inhibitor, thiourea, to unfed cultures resulted in fewer larvae metamorphosing from the CR × CR cross and no larvae metamorphosing from the CR × CS cross. Thiourea had no detectable effect on larvae from *C. subdepressus* eggs: larvae in the unfed thyroid hormone inhibited cultures developed the same as larvae in starved cultures, all of which died by the 17th dpf.

Maternal species, paternal species, treatment, and the interaction of maternal and paternal species all had significant effects on time to metamorphosis ($F_{1,\ 60} = 879.8$, $P < 0.001$; $F_{1,\ 60} = 88.3$, $P < 0.001$; $F_{3,\ 60} = 215.9$, $P < 0.001$; and $F_{1,\ 60} = 27.3$, $P < 0.001$, respectively). The results of replicate experiment did not significantly differ ($F_{2,\ 60} = 1.55$, $P = 0.221$).

Maternal species had a larger effect on developmental timing than paternal species (Table 1), with larvae from *C. subdepressus* mothers metamorphosing on average 5.8 days later than larvae from *C. rosaceus* mothers (95%CI = 5.75–5.91 days), and larvae from *C. subdepressus* fathers metamorphosing on average 1.9 days later than larvae of *C. rosaceus* fathers (95% CI = 1.52–2.36 days) (Fig. 3).

In every cross, thyroid hormone had a strong effect on developmental timing, resulting in earlier metamorphosis than any other treatment ($P < 0.001$ for all pairwise comparisons) (Fig. 3). Conversely, blocking endogenous production of thyroid hormone by addition of thiourea significantly delayed metamorphosis in larvae from the CR × CR cross relative to all other treatments ($P < 0.05$ for all three pairwise comparisons).

As it usually happens in echinoid larval cultures, not all larvae reached metamorphosis. The percent that metamorphosed was <50% in every cross (Fig. 3); this reduced survivorship may have been due to a variety of factors including imperfect stirring, loss of larvae during water changes, and stress on the larvae that were used for measurements. However, the larvae and juveniles that we observed and measured appeared healthy and normal. We modeled the number of juveniles that settled using a quasibinomial regression followed by a chi-square goodness of fit test. Maternal species, paternal species, treatment, and the interaction of maternal by paternal species significantly affected the fit of the model ($\chi^2 = 34.35$, $P < 0.001$; $\chi^2 = 30.26$, $P < 0.001$; $\chi^2 = 18.21$, $P < 0.001$; and $\chi^2 = 37.73$, $P < 0.001$, respectively), whereas replicate experiment did not ($\chi^2 = 5.32$, $P = 0.069$).

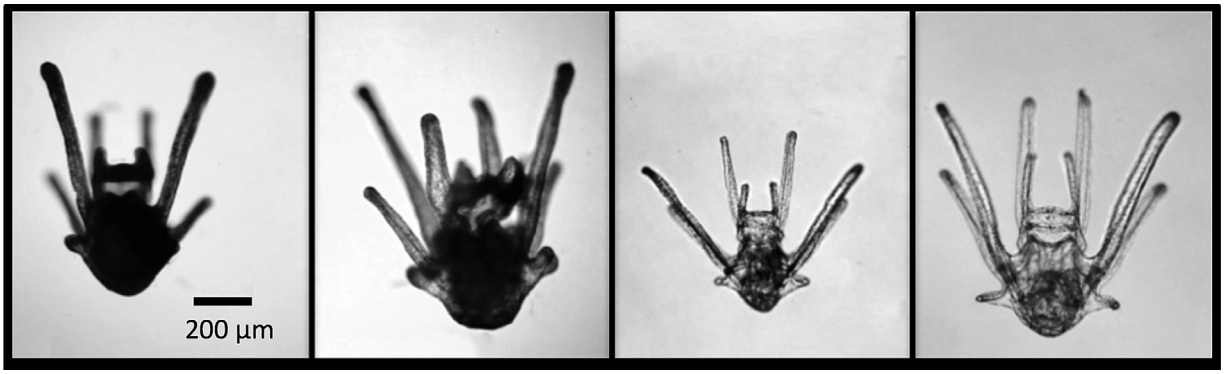

Fig. 2. Eight-armed larvae from each cross. From left to right: (1) CR × CR, (2) CR × CS, (3) CS × CR, and (4) CS × CS; for each cross, initials of the maternal species are listed first and the paternal species second. All pictures are scaled to the scale bar on the left. The hybrid larvae were over- or undersized compared to the parental species larvae.

Both hybrid crosses yielded significantly fewer juveniles in the fed treatment than either parental species ($P < 0.01$ for all pairwise comparisons) (Fig. 3).

Fed, starved and thyroid hormone treatments produced similar numbers of juveniles for the CR × CR cross (fed vs. starved: $P = 0.98$; fed vs. thyroid hormone: $P = 0.99$; thyroid hormone vs. starved: $P = 0.96$). However addition of the thyroid hormone synthesis inhibitor, thiourea, resulted in significantly fewer juveniles than any other treatment ($P < 0.05$ for all comparisons). Fed, starved and thyroid hormone treated CR × CS larvae all resulted in similar numbers of juveniles ($P > 0.85$ for all pairwise comparisons).

In both the CS × CS and CS × CR crosses, only larvae from the fed and thyroid hormone treatments settled as juveniles. In CS × CS cultures, significantly more larvae metamorphosed in the fed treatment relative to the thyroid hormone treatment ($P < 0.001$). However in CS × CR cultures the opposite was true: more CS × CR larvae metamorphosed in the thyroid hormone treatment than in the fed treatment ($P < 0.001$) (Fig. 3).

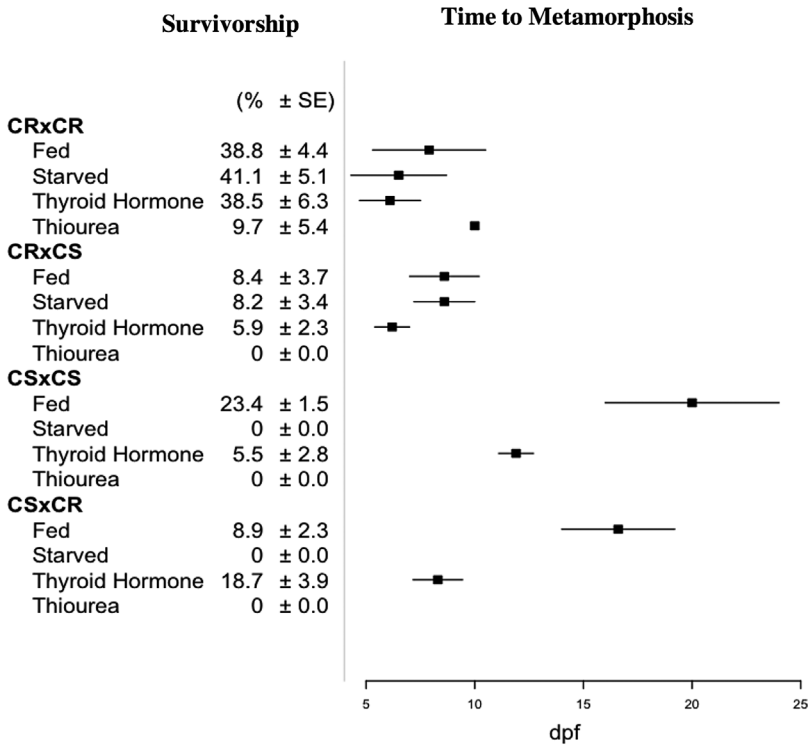

Fig. 3. Survivorship and time to metamorphosis for all crosses. The second column shows survivorship as the mean percent of larvae (out of 200) ± 95%CI that metamorphosed into juveniles. $N = 9$ replicate cultures for all treatments except thiourea where $n = 3$. The last column is a dot plot with mean time to metamorphosis in days post fertilization (dpf) ± 95%CI. For each cross, initials of the maternal species are listed first and the paternal species second.

Morphometrics

Post-oral arm length, body length and total length were all measured from five larvae in every culture at the 8-arm stage; larval measurements were compared using a linear model in R followed by a Tukey's HSD post-hoc test. Maternal species, paternal species, treatment, and replicate experiment all significantly effected all three measurements (Table 3).

Larvae of all four crosses had significantly different body lengths and arm lengths ($P < 0.001$ for each pairwise Tukey test) with CR × CS being the largest and CS × CR being the smallest (Fig. 4). The total lengths of larvae from the two homospecific crosses (CR × CR and CS × CS) did not significantly differ from one another ($P = 0.967$); however the hybrid CR × CS cross produced significantly larger larvae than any other cross ($P < 0.001$ for all pairwise comparisons) while the CS × CR cross produced larvae that were significantly smaller ($P < 0.001$ for both pairwise comparisons).

The addition of thyroid hormone to starved larval cultures resulted in larvae with significantly smaller arm, body and total lengths than any other treatment ($P < 0.001$ for all pairwise comparisons) (Figs. 4 and 5). The starved and thiourea treatments did not significantly differ from one another (total length: $P = 0.98$; arm length: $P = 0.803$; and body length: $P = 0.717$).

We measured juvenile test diameter on the day each juvenile settled and compared the measurements using a linear model in R with a Tukey's HSD post-hoc test. Maternal species, paternal species, treatment, the interaction of maternal and paternal species, and replicate experiment all had significant effects on size at metamorphosis (Table 3).

Juveniles from the CR × CR and CR × CS crosses were not significantly different in size from one another ($P = 0.70$); however, juveniles from every other cross significantly differed ($P < 0.001$ for all pairwise comparisons) (Fig. 6). Juveniles from the thyroid hormone treatment were significantly smaller than juveniles from any other treatment ($P < 0.01$ for all pairwise Tukey tests). Juvenile sizes in the fed and starved treatments did not differ significantly ($P = 0.408$). CR × CR was the only cross in which larvae from the hormone inhibited treatment metamorphosed; CR × CR juveniles from the hormone inhibited treatment were not significantly different in size than those from the fed or starved cultures ($P = 0.71$ and $P = 0.86$, respectively).

DISCUSSION

The transition between feeding and non-feeding larval development has occurred numerous times throughout metazoan phyla with sweeping ecological and evolutionary implications. *Clypeaster rosaceus* and *C. subdepressus* represent an exceptional opportunity for understanding the evolution of larval development in echinoderms as they are the only known pair of

Table 3. ANOVA values from linear models on juvenile and larval morphometric data. Maternal species, paternal species, treatment, and the interaction of maternal and paternal species were all modeled as fixed effects while replicate experiment was modeled as a random effect

Measurement	Effect	df	Sum of squares	*F*-value	*P*-value
Body length	Maternal species	1, 95	455,520	519.9	<0.001
	Paternal species	1, 95	47,627	54.4	<0.001
	Treatment	3, 95	201,961	76.8	<0.001
	Replicate experiment	2, 95	8893	5.07	0.008
	Maternal x paternal species	1, 95	1221	1.39	0.240
Arm length	Maternal species	1, 95	214,629	44.6	<0.001
	Paternal species	1, 95	577,296	119.9	<0.001
	Treatment	3, 95	892,729	61.8	<0.001
	Replicate experiment	2, 95	76,066	7.91	<0.001
	Maternal x paternal species	1, 95	5770	1.19	0.276
Total length	Maternal species	1, 95	911,618	171.7	<0.001
	Paternal species	1, 95	894,175	168.4	<0.001
	Treatment	3, 95	1,420,148	89.1	<0.001
	Replicate experiment	2, 95	96,643	9.10	<0.001
	Maternal x paternal species	1, 95	7957	1.49	0.224
Juvenile disk diameter	Maternal species	1, 70	172,864	343.5	<0.001
	Paternal species	1, 70	3321	6.60	0.012
	Treatment	3, 70	56,485	37.4	<0.001
	Replicate experiment	2, 70	5257	5.22	0.008
	Maternal x paternal species	1, 70	12,115	24.1	<0.001

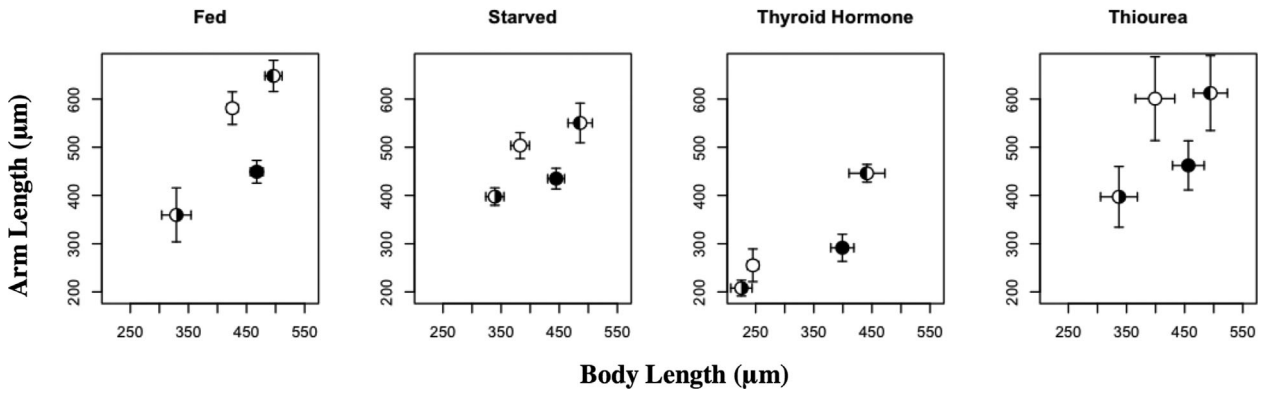

Fig. 4. Arm length versus body length of 8-armed larvae. Mean ± 95%CI shown for all four crosses: CR × CR (●), CR × CS (◐), CS × CR (◑), and CS × CS (○), initials of the maternal species are listed first and the paternal species second. Panels are broken up by treatment. $N = 9$ replicate cultures for all treatments except thiourea where $n = 3$. The two hybrid crosses fall outside of the range of arm and body lengths seen in the two parental crosses. Thyroid hormone resulted in smaller arm and body lengths than any other treatment.

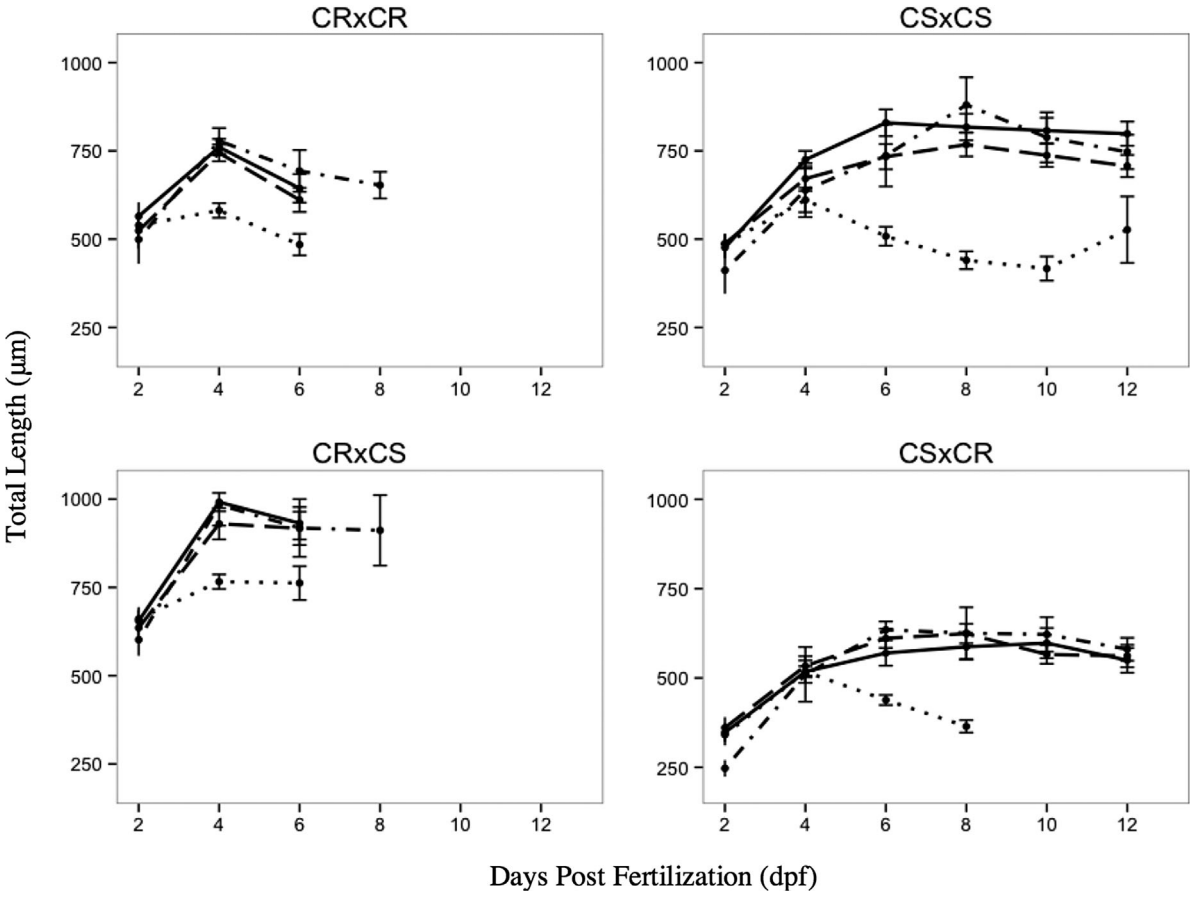

Fig. 5. Total length of larvae on each day post fertilization. Measurements represent the mean ± 95%CI. $N = 9$ cultures for each data point from the fed (——), starved (— —), and thyroid hormone (▪ ▪ ▪) treatments. For points from the hormone inhibited, thiourea, treatment (– · – · ·) $n = 3$. Measurements stopped being taken on day 12 as larvae had stopped growing. For each cross, initials of the maternal species are listed first and the paternal species second.

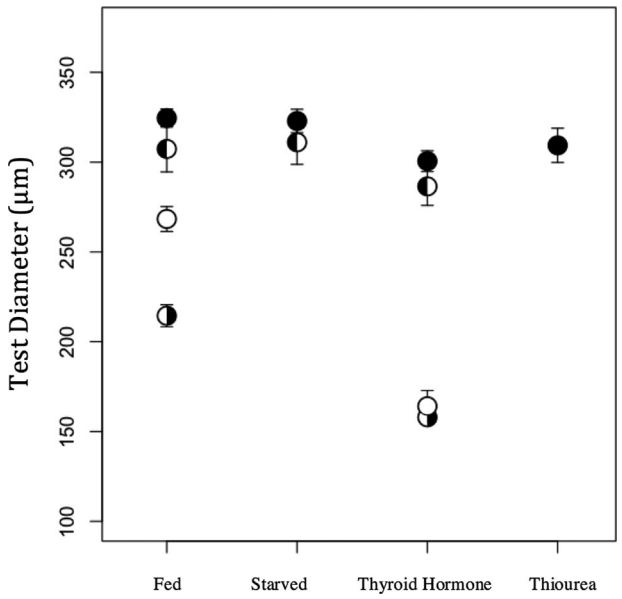

Fig. 6. Juvenile test diameter at metamorphosis. Points represent mean ± 95%CI. Some individuals from all four crosses (CR × CR [●], CR × CS [◐], CS × CR [◑], and CS × CS [○], initials of the maternal species are listed first and the paternal species second) developed into juveniles in the fed and thyroid hormone treatments. No offspring from CS × CS or CS × CR reached metamorphosis when starved. Only offspring of the CR × CR cross metamorphosed in the hormone inhibited, thiourea, treatment. $N = 9$ replicae cultures for fed, starved and thyroid hormone treatments, while $n = 3$ for the thiourea tretment.

echinoderms with differing developmental modes that can produce two viable hybrid crosses. Additionally, species with facultatively planktotrophic larvae provide a window for understanding the initial transition between obligately planktotrophic and lecithotrophic development.

Development

The evolution of non-feeding larval development in marine invertebrates is consistently associated with an increase in egg size (Vance 1973; Christiansen and Fenchel 1979; Wray 1996). Indeed, both hybrid crosses in this study developed like their maternal species, suggesting that either egg size or some other maternally inherited element (such as genetic imprinting or maternally deposited mRNA into the embryos) controls developmental mode. However, the experimental results of this study indicate that egg size alone does not fully explain developmental mode. The offspring of all four crosses metamorphosed without food when thyroid hormone was added, demonstrating that even the relatively small eggs characteristic of *C. subdepressus* provide sufficient energy to the embryo so that it can develop to metamorphosis without algal food.

There certainly is some minimum egg size needed to complete larval development—when starved larvae from the sand dollar *Mellita tenuis* (egg diameter ~100 μm) are given excess thyroid hormone, they still do not complete development through metamorphosis (Heyland et al. 2004). However, several studies demonstrate that the minimum egg size observed in non-feeding larval development is much larger than the minimum egg size sufficient to complete development (Emlet and Hoegh-Guldberg 1997; Heyland et al. 2004; Allen et al. 2006). Most notably, *Clypeaster rosaceus* has small eggs compared to echinoderms with non-feeding larval development, yet four-cell embryos can be divided into quarters and the resultant larvae can still settle as juveniles without food (Allen et al. 2006). Blastomere separations generally have little effect on development; the main impact of reducing egg size seems to be a reduction in size at settlement (Hart 1996; Allen et al. 2006).

Lecithotrophic eggs contain a much higher proportion of lipids such as wax esters than planktotrophic eggs (Byrne et al. 1999; Villinski et al. 2002); these lipids deposited in the eggs are not used during larval development, but instead are important for juvenile fitness (Emlet and Hoegh-Guldberg 1997; Byrne et al. 1999; Villinski et al. 2002). Thus, egg size in non-feeding larvae may be primarily influenced by selection on size at settlement, rather than for providing energy necessary for larval development (Emlet 1986; Hart 1996; Allen et al. 2006; Villinski et al. 2002). In the results presented here, juveniles that settled from larvae treated with thyroid hormone were significantly smaller than juveniles resulting from any other treatment. Therefore, although egg size in *C. subdepressus* may be large enough to complete larval development when hormones are added, the resultant juveniles are extremely small and therefore likely to be less fit than their fed counterparts (Emlet and Hoegh-Guldberg 1997; Allen et al. 2006).

If large eggs are not necessary for larvae to develop without food, some other maternally inherited element must be driving developmental patterns. Factors that are maternally deposited into eggs, such as mRNA and proteins, are crucial for proper embryonic development (Evans et al. 1983; Tadros and Lipshitz 2009; Li et al. 2010) and may be involved in the evolution of alternative developmental modes (Henry and Raff 1990; Byrne et al. 1999; Nielsen et al. 2000). In hybrid larvae from crosses between planktotrophic and lecithotrophic sea urchin species of *Heliocidaris*, at least one reciprocal cross is lethal due to differences in maternally specified embryonic axes (Raff et al. 1999, 2003). Embryos of *C. rosaceus* have different proportions of their germ layer allocated to ectoderm and endo-mesoderm relative to germ layer allocation in embryos of obligately planktotrophic larvae, which may support the early formation of a large left coelom (Zigler and Raff 2013). Changes in early developmental patterning such as these may be crucial for the evolution of novel developmental pathways.

Individuals with differing developmental modes can also be crossed in some poecilogonous species; poecilogony is a rare

developmental mode where multiple developmental modes are present in a single species (Levin 1984). In the poecilogonous marine polycheate *Streblospio benedicti* the length of the larval gut (a proxy for developmental mode) is almost entirely maternally specified (Zakas and Rockman 2014). Conversely, zygotic genetic effects explain phenotypic characteristics such as size of larval chaetae (Zakas and Rockman 2014). These results are consistent with the patterns of inheritance we found in *Clypeaster*.

Morphometrics

The significant effects of experiment on larval and juvenile phenotypes (Table 3) potentially reflect differences in egg size between experiments (Table 2). Nevertheless, the same phenotypic patterns (discussed below) were consistent across experiments.

Both hybrid crosses displayed intermediate developmental rates relative to the two parental species (Fig. 3 and Table 1). Phenotypically, however, the hybrids expressed arm, body, and total lengths well outside the range of phenotypes observed in either parental species (Figs. 2 and 4). Extreme hybrid phenotypes are often explained by one of two processes: (1) genetic imprinting, or (2) compensatory evolution.

Genetic imprinting is an epigenetic process causing differential gene expression based on the sex of the parent that transmitted the allele (Moore and Haig 1991). Imprinting results in unequal expression of maternally and paternally derived alleles in offspring, and is crucial for proper development in many organisms (O'Neill et al. 2000; Kono et al. 2004). Imprinting has been most widely studied in placental mammals (Moore and Haig 1991; Giannoukakis et al. 1993; Vrana et al. 1998; Kono et al. 2004), but it has also been documented in a variety of other organisms including frogs, fish, insects, and plants (Chakraborty 1989; Chandra and Nanjundiah 1990; Haig and Westoby 1991; McGowan and Martin 1997; Alleman and Doctor 2000; Shen et al. 2012). When species have different patterns of imprinting, hybrids often mis-express imprinted genes. For example, in the mice *Peromyscus polionotus* and *P. maniculatus,* imprinting of growth factors results in crosses where one hybrid is ~40% smaller than either parent, and the offspring of the second cross are oversized to a lethal extent, despite the two parents being similarly sized (Vrana et al. 1998). This pattern resembles the *Clypeaster* hybrid phenotypes presented here: larvae from the two parental species were of similar total length, whereas larvae from the CR × CS cross were significantly larger than both parents, and those from the CS × CR cross were significantly smaller. If genes related to larval development are imprinted in these species, this could at least partially explain the phenotypes of the hybrid crosses.

Alternatively, compensatory evolution between these two species could account for the hybrid phenotypes. Compensatory evolution occurs when two species retain similar levels of gene expression by different regulatory mechanisms (True and Haag 2001; Landry et al. 2007). When the two different regulatory backgrounds are combined in a single offspring, changes in *cis*- x trans-regulatory elements result in over or under expression of genes. Compensatory evolution is a widespread phenomenon, often contributing to hybrid incompatibility and breakdown (Maisnier-Patin and Andersson 2004; Haag 2007; Takahasi et al. 2011). Compensatory evolution can result in extreme levels of gene transcription in hybrids, outside the range seen in either parent.

These two processes have distinct genetic signatures. Imprinting is a *cis*-regulatory mechanism that results in a genes being expressed in a hemizygous state, whereas compensatory evolution results in both copies of a gene being mis-expressed. Future analysis of gene expression in the hybrid larvae can reveal which (if either) of these processes may be contributing to the expressed hybrid phenotypes.

Thyroid hormone

In both echinoid and asteroid echinoderm larvae, the addition of thyroid hormone accelerates larval development, reduces the length of larval feeding arms, accelerates development of a juvenile rudiment, and reduces juvenile size at metamorphosis (Chino et al. 1994; Johnson and Cartwright 1996; Heyland and Hodin 2004). Previous work has also shown that planktotrophic larvae of *Leodia sexiesperforata*, a sand dollar with an egg size intermediate between those of *Clypeaster rosaceus* and *C. subdepressus* (Lessios 1990), can metamorphose with only the addition of thyroid hormone (Heyland et al. 2004). Consistent with these studies on echinoderm development, we found that the addition of thyroid hormone to cultures altered developmental timing, larval phenotypes, juvenile size, and developmental mode in all four of our crosses.

Thyroid hormone had a much more striking effect on developmental timing, larval measurements and juvenile size in crosses originating from *C. subdepressus* eggs than in crosses originating from *C. rosaceus* eggs (Figs. 3 and 6). In contrast, blocking endogenous production of thyroid hormone by addition of thiourea had relatively little detectable effect on larvae from *C. subdepressus* eggs, whereas it delayed and inhibited metamorphosis in the offspring of the two crosses from *C. rosaceus* eggs. This suggests that exogenous thyroid hormone plays an important role in development of planktotrophic larvae whereas endogenous thyroid hormone production is much more important for facultatively planktotrophic larvae.

Hormone systems play a key role in regulating life cycle transitions of plants and animals (Gudernatsch 1912; Manzon et al. 2001; Heyland et al. 2005). Among metazoans, including annelids, molluscs, arthropods, echinoderms, and chordates, thyroxine and other thyroid hormones control many life history events, including regulating metamorphosis (Pfennig 1992; Chino et al. 1994; Eales 1997). The results presented here

further support the importance of thyroid hormone in larval development and metamorphosis of echinoderms. Additional research on the thyroid hormone pathway, its evolution and regulation would greatly enhance our understanding of the mechanisms leading to the evolution of non-feeding larval development.

CONCLUSIONS AND FUTURE DIRECTIONS

The results presented here show that maternal factors determine larval developmental mode in these crosses. This result is consistent with mounting evidence that maternal effects greatly contribute to early developmental patterning, influencing developmental mode (Henry and Raff 1990; Byrne et al. 1999; Zigler and Raff 2013; Zakas and Rockman 2014). However, we do not credit egg size solely as the reason for this maternal effect, as addition of the thyroid hormone, thyroxine, is sufficient to result in metamorphosis without food, even in our obligately planktotrophic crosses. Instead, maternally inherited factors such as mRNAs, proteins, or genetic imprinting may underlie the patterns observed here.

Future comparative transcriptomic work on eggs, embryos and larvae from *Clypeaster rosaceus*, *C. subdepressus*, and their hybrids has the potential to clarify the genetic mechanisms underlying larval developmental mode. By characterizing patterns of gene expression in the hybrid larvae, we can determine whether imprinting or other gene regulatory differences exist between these two species and how these differences affect development. Additionally, we are interested in whether genes involved in the thyroid hormone pathway are differentially regulated between these two species. The results presented here move us closer to understanding both the inheritance and evolution of developmental mode, and we believe that this system has the potential to contribute many more transformative insights.

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