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# Egg Energetics, Fertilization Kinetics, and Population Structure in Echinoids With Facultatively Feeding Larvae

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**Abstract.** Larvae of marine invertebrates either arise from small eggs and feed during their development or arise from large eggs that proceed to metamorphosis sustained only from maternal provisioning. Only a few species are known to posses facultatively feeding larvae. Of about 250 echinoid species with known mode of development, only two, *Brisaster latifrons* and *Clypeaster rosaceus*, are known to develop through facultatively planktotrophic larvae. To obtain more information on this form of development and its consequences, we determined egg size and egg energetic and protein content of these two species. We found that eggs of *B. latifrons* resemble those of species with nonfeeding larvae in these characteristics more than those of *C. rosaceus*. We also compared DNA sequences of the cytochrome oxidase (COI) gene from the Caribbean *C. rosaceus* to those of the sympatric planktotrophic developer *C. subdepressus* and also to those of the eastern Pacific species *C. europacificus* to estimate the degree of divergence between species with different developmental modes. Comparison of COI sequences of *C. rosaceus* from Panama and Florida revealed that there is no geographic differentiation in this species. Cross-fertilization experiments between *C. rosaceus* and *C. subdepressus* indicated that bidirectional gametic incompatibility has evolved between the two species.

## Introduction

The diversity of life is evident in the astonishingly varied forms of marine invertebrates and their larvae (Raff, 2008). Marine invertebrate larvae may differ in their embryology, morphology, and ecology. Individual species generally have larvae that fit into one of two modes: feeding larvae arise from small eggs that give rise to planktotrophic larvae, whereas nonfeeding larvae arise from larger eggs that proceed directly to metamorphosis. Both modes of development are observed in a wide range of taxa, indicating multiple evolutionary transitions between feeding and nonfeeding development.

Facultatively feeding larvae represent a third mode of development. They arise from eggs that contain enough energy to support them through metamorphosis in the absence of food, but they also retain the ability to feed. As a mode of larval development, facultative feeding is quite rare; confirmed cases are limited to eight species in three phyla (Allen and Pernet, 2007). Of more than 250 echinoid (sea urchin, heart urchin, and sand dollar) species with known mode of development, two-thirds have obligately feeding larvae and the other third have nonfeeding larvae. Only two species, *Brisaster latifrons* and *Clypeaster rosaceus*, develop from facultatively feeding larvae (Emlet, 1986, 1990; Hart, 1996). These two species belong to different echinoid orders (Spatangoida and Clypeasteroida, respectively), and so represent two evolutionarily distant examples of facultatively planktotrophic larvae. The echinoid examples of this rare developmental mode provide an opportunity to examine how developmental features change with mode of development, and to ask if this developmental mode has been reached by similar changes in these distinct lineages.

Because all echinoids with feeding larvae develop *via* the distinctive pluteus larva, and because of extensive similar-

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ities between the sea urchin pluteus and the feeding larvae of other echinoderms, a feeding pluteus is inferred to be the ancestral form of development in echinoids (Strathmann, 1978). Nonfeeding echinoid larvae have evolved from feeding larvae at least 14 times (Emlet, 1990). Although the re-evolution of feeding larvae from nonfeeding larvae may be possible in some groups (Marshall *et al.*, 1994; Rouse, 2000), the switch from feeding to nonfeeding larval development appears to be irreversible in echinoids. Nonfeeding echinoid larvae lose the complex ciliary band required for feeding, fail to form a complete gut, and acquire novel features related to direct development (Strathmann, 1978; Raff, 1996); re-evolving the ancestral features is highly unlikely.

Echinoids with facultatively feeding larvae provide an opportunity to study life-history and developmental intermediates between feeding and nonfeeding larvae. Emlet (1986) showed that *C. rosaceus* has facultatively feeding larvae and compared their development with that of the obligately feeding larvae of *C. subdepressus*. Reitzel and Miner (2007) showed that though *C. rosaceus* larvae can feed, they do not assimilate much food as larvae. Heyland *et al.* (2006) found that time to metamorphosis in *C. rosaceus* is influenced by thyroxine levels. Allen *et al.* (2006) determined that time to metamorphosis is largely insensitive to experimental manipulation of egg size or food ration. Smith *et al.* (2007) discovered that *C. rosaceus* forms a large left coelom (a precursor to the adult rudiment) earlier in development than *C. subdepressus* does, an embryological modification that likely contributes to the rapid time to metamorphosis in *C. rosaceus*. Hart (1996) described the development of *B. latifrons.*

In the present paper we extend the previous work of others in several ways. First, we examine egg characteristics of *B. latifrons*, *C. rosaceus*, and *C. subdepressus*, a sympatric congener of *C. rosaceus* that develops *via* an obligately feeding larva. We use mitochondrial DNA sequences to estimate how long ago *C. rosaceus* and *C. subdepressus* diverged. We compare the fertilization dynamics of these two species to each other and to other echinoids. Last, we use mitochondrial DNA sequences to determine whether the short larval duration of *C. rosaceus* has resulted in population subdivision across the Caribbean. We then discuss insights gained from the study of *B. latifrons* and *C. rosaceus* on the patterns of change in larval features involved in the evolution of developmental mode.

## Materials and Methods

### *Samples*

*Clypeaster rosaceus* (Linnaeus) and *C. subdepressus* (Gray) were collected in October and November 2004 and in June 2005 at 2–5-m depth at Bocas del Toro, Panama, and transferred to the laboratory, where they were maintained in sea tables for as long as one week. Animals were spawned by intracoelomic injection of 0.5 mol $l^{-1}$ KCl, supplemented in *C. rosaceus* by vigorous shaking. B. Miner (Western Washington University) kindly provided tissue samples from *C. rosaceus* individuals from Long Key Channel, Florida. *Brisaster latifrons* (Agassiz) was dredged at 200-m depth off Meadow Point in Puget Sound, Washington, in March 2004, transferred to sea tables at the Friday Harbor Laboratories, and induced to spawn by intracoelomic injection of 0.5 mol $l^{-1}$ KCl.

### *Egg size and egg energy content*

After spawning, eggs were washed several times in filtered seawater (FSW). An aliquot of eggs was transferred to a slide and covered with a coverslip supported by modeling clay at the corners to prevent compression of the eggs. Mean egg sizes were determined for eight *C. rosaceus* and four *C. subdepressus* females from a sample of 20 eggs per female. *C. rosaceus* and *B. latifrons* eggs were frequently not completely spherical, so egg diameters were measured for both the long and short axes. Egg volume was then calculated as that of an oblate spheroid. For comparison to earlier studies, the egg diameter of a sphere with volume equal to that of the spheroid was also calculated. *C. subdepressus* eggs were generally spherical, so egg diameter was measured for only one axis.

To calculate energy content per egg, we used the dichromate oxidation method as described by Miner *et al.* (2002) with the following modifications: eggs were briefly rinsed in distilled $H_2O$, and 3–5 replicates of a known number of eggs (20/replicate for *C. rosaceus*, 25/replicate for *B. latifrons*, and 100/replicate for *C. subdepressus*) per female were then frozen in a minimal volume of $dH_2O$. Energy per egg was then measured as described, with glucose as a standard.

Egg protein concentrations were determined using the Bradford reagent (Sigma B-6916) according to the manufacturer's directions, with the following modifications: 3–5 replicates of a known number of eggs per female (6 or 10 for *C. rosaceus*, 10 for *B. latifrons,* and 25 for *C. subdepressus*) were frozen in 100 $\mu$l of filtered seawater. Protein concentrations per egg were calculated using a standard absorption curve generated with bovine serum albumin (Sigma A-2153).

### *Fertilization experiments*

Thirty experimental crosses within and between *C. rosaceus* and *C. subdepressus* were performed in October 2004. Individual animals were used in one or two crosses on a single day, and were crossed with both heterospecific and conspecific animals at a range of sperm concentrations. Eggs were collected by inverting females over beakers of 0.45 $\mu$m FSW and subsequently washed several times in FSW. They were then resuspended at a concentration of

approximately 250 eggs/ml. Three milliliters of this egg suspension was placed in each of 12 wells of a 24-well cell culture plate.

Sperm were collected "dry" from the gonopores of males. A series of five 5-fold sperm dilutions in FSW was prepared, beginning with a 1:250 dilution of dry sperm. A 50-$\mu$l sample of each sperm dilution was added to the appropriate conspecific and heterospecific egg suspensions, and the culture plate was briefly swirled to mix the sperm and eggs. After 10 min, the volume in each well was raised to 12 ml with FSW. The sperm-egg mixes were then allowed to sit at room temperature for at least 2 h, by which time cleavage had begun. One hundred eggs per well were then examined to determine if they had cleaved. Obviously immature oocytes (evidenced by a large germinal vesicle) were ignored.

The remnant of the first sperm dilution was preserved by the addition of paraformaldehyde. Fixed sperm samples were briefly mixed using a vortex mixer, and a 10-$\mu$l aliquot was transferred onto a hemacytometer. The sperm concentration was measured after the samples had settled for 15 min.

### *Fertilization kinetics calculations*

To quantify levels of gametic compatibility within and between *C. rosaceus* and *C. subdepressus,* we calculated the linear regression of logit-transformed fertilization percentages against the log sperm concentration (McCartney and Lessios, 2002). From these regressions we then calculated $F_{50}$ (the number of sperm per microliter required to fertilize 50% of the eggs) and $F_{90}$ (the number of sperm per microliter required to fertilize 90% of the eggs) values for each cross. Additionally, to arrive at a single $F_{50}$ and $F_{90}$ value for each of the four possible crosses (female $\times$ male: *C. rosaceus* $\times$ *C. rosaceus*, *C. rosaceus* $\times$ *C. subdepressus*, *C. subdepressus* $\times$ *C. rosaceus*, and *C. subdepressus* $\times$ *C. subdepressus*), we calculated a single linear regression between sperm concentration and percent fertilization for all values tested for a particular class of crosses.

### *Mitochondrial DNA sequencing, phylogenetic analysis, and population structure*

A 640-bp fragment of the mitochondrial cytochrome oxidase I (COI) gene was amplified and sequenced using the primers COIa (5′-AGTATAAGCGTCTGGGTAGTC-3′) and COIf (5′-CCTGCAGGAGGAGGAGAYCC-3′) as described in Lessios *et al*. (1999) from 12 individuals of *C. rosaceus* (8 from Panama and 4 from Florida) and 9 of *C. subdepressus* (all from Panama). The same gene fragment was sequenced from 8 *C. europacificus* individuals collected on the Pacific coast of Panama and from one *Mellita longifissa* (Clypeasteroida:Mellitidae) for use as an outgroup. The sequences have been deposited in Genbank (accession numbers EU669832–EU669861).

After combining identical sequences and excluding the outgroup, we used Modeltest (ver. 3.7; Posada and Crandall, 1998) to identify the best model of nucleotide substitution for the data (GTR + $\Gamma$ as selected by the Akaike Information Criterion). We then used these parameters to construct a phylogenetic tree using the neighbor-joining distance method in PAUP* (ver. 4.0b10; Swofford, 2003). We bootstrapped the data (1000 replicates) using these parameters and neighbor-joining. We calculated *F* statistics in Arlequin (ver 2.0; Schneider *et al.*, 2000) and compared haplotypes to determine the degree of divergence between Florida and Panama populations of *C. rosaceus*.

## Results

### *Egg size and energy content*

The mean egg diameter of *Clypeaster rosaceus* was 283.4 $\mu$m (Table 1). Our estimate of mean egg energy in *C. rosaceus* was 0.076 J/egg (Table 1). *C. rosaceus* eggs contained an average of 0.79 $\mu$g of protein. Egg diameter in *C. subdepressus* was 155.5 $\mu$m. Egg energy in this species was 0.0051 J/egg and protein content was 0.12 $\mu$g/egg (Table 1). Egg diameter of *B. latifrons* was 357.4 $\mu$m. *B. latifrons* egg energy was 0.251 J/egg, and protein content 2.63 $\mu$g/egg (Table 1).

Eggs of echinoderms with feeding larvae are generally less energetically dense (in J/$\mu$l of egg volume) than those of echinoderms with nonfeeding larvae, regardless of the assay method used (Pernet and Jaeckle, 2004). After excluding *Perknaster fuscus* and *Notasterias armata* from the dataset in McEdward and Morgan (2001) (as in Pernet and Jaeckle, 2004), the mean egg energetic density for the two feeding types of echinoderms was 5.9 J/$\mu$l for 21 species with feeding larvae and 12.1 J/$\mu$l for 23 species with nonfeeding larvae. Our estimates of egg energetic density are 2.6 J/$\mu$l for *C. subdepressus*, 6.4 J/$\mu$l for *C. rosaceus,* and 10.5 J/$\mu$l for *B. latifrons* (Fig. 1).

### *Fertilization kinetics*

For conspecific crosses, the numbers of conspecific sperm per microliter required to fertilize 50% of *C. rosaceus* and *C. subdepressus* eggs ($F_{50}$ values) were 101 and 50, respectively (Table 2). *C. rosaceus* and *C. subdepressus* are gametically incompatible in both directions, as indicated by the ratio of heterospecific to conspecific $F_{50}$ values. *C. rosaceus* eggs are incompatible with *C. subdepressus* sperm (Fig. 2); it takes 15 times more *C. subdepressus* sperm to fertilize 50% of *C. rosaceus* eggs (Table 2). *C. subdepressus* eggs are extremely incompatible with *C. rosaceus* sperm (Fig. 2); the $F_{50}$ ratio for this cross is $3.6 \times 10^4$ (Table 2).

**Table 1**

*Egg size, egg energy content, and egg protein content for* Clypeaster rosaceus, C. subdepressus, *and* Brisaster latifrons

| Species | Date | Female | Mean egg diameter (um) | Mean energy (J/egg) | Mean protein (ug/egg) |
|---|---|---|---|---|---|
| *Clypeaster rosaceus* | 10/7/2004 | 1 | 287.1 | 0.073 | 0.60 |
| | | 2 | 289.5 | 0.073 | 0.72 |
| | | 3 | 299.2 | 0.073 | 0.69 |
| | | 4 | 284.3 | 0.075 | 1.06 |
| | | 5 | 279.4 | 0.061 | 0.81 |
| | 11/4/2004 | 6 | 277.5 | 0.088 | 0.94 |
| | | 7 | 269.4 | 0.077 | 1.00 |
| | | 8 | 280.6 | 0.089 | 0.47 |
| | | Mean +/− S.D. | 283.4 +/− 8.9 | 0.076 +/− 0.009 | 0.79 +/− 0.21 |
| *C. subdepressus* | 11/4/2004 | 1 | 160.5 | 0.0058 | 0.11 |
| | | 2 | 156.5 | 0.0050 | 0.15 |
| | | 3 | 151 | 0.0046 | 0.13 |
| | | 4 | 154 | 0.0049 | 0.09 |
| | | Mean +/− S.D. | 155.5 +/− 4.0 | 0.0051 +/− 0.0005 | 0.12 +/− 0.03 |
| *Brisaster latifrons* | 3/27/2004 | 1 | 338.1 | 0.217 | 2.32 |
| | | 2 | 349.9 | 0.214 | 2.20 |
| | | 3 | 388.5 | 0.318 | 3.13 |
| | | 4 | 353.1 | 0.253 | 2.88 |
| | | Mean +/− S.D. | 357.4 +/− 21.7 | 0.251 +/− 0.048 | 2.63 +/− 0.45 |

In *C. rosaceus* × *C. rosaceus* crosses at high sperm concentrations we occasionally observed eggs with fertilization envelopes that were not cleaving after 2 h, suggesting that polyspermy may have been a problem with *C. rosaceus* at high sperm concentrations, and may have decreased our estimates of fertilization success at the highest sperm concentrations.

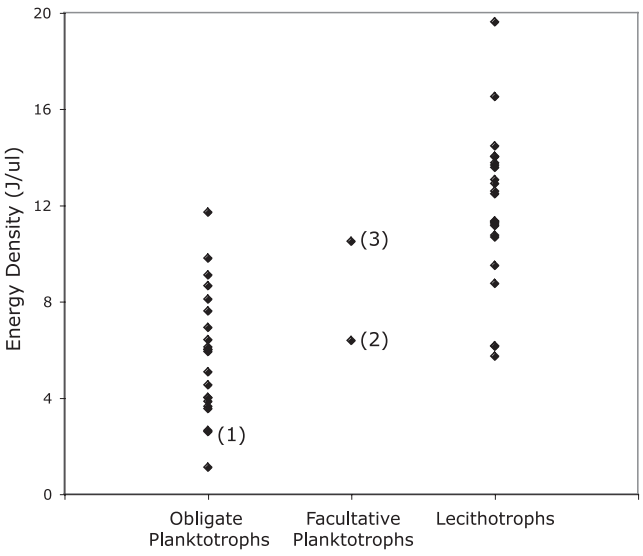


**Figure 1.** Egg energetic density (in J/$\mu$l egg volume) for 26 species of echinoderms, separated by their mode of development. The values for *Clypeaster subdepressus* (1), *C. rosaceus* (2), and *Brisaster latifrons* (3) were determined in this study. All other egg energy and volume values are from McEdward and Morgan (2001).

*Phylogenetic relationships, genetic divergence, and population structure*

*C. rosaceus* and *C. subdepressus* are not closely related (Fig. 3). Mean COI divergence between *C. rosaceus* and *C. subdepressus* (based on the GTR + $\Gamma$ model) was 21.9%, which indicates a distant relationship between the two species by the standard of genetic distances among species in other echinoid genera (Lessios *et al.*, 2001, 2003; Zigler and Lessios, 2004). *C. subdepressus* is more closely related to the Eastern Pacific *C. europacificus* (8.7% divergence). Florida and Panama populations of *C. rosaceus* do not appear to be genetically distinct, as two of four haplotypes present in Florida are shared with Panama, and there is little overall diversity among the eight observed *C. rosaceus* haplotypes (mean difference 0.29% between haplotypes). These observations are consistent with the absence of significant population structure in *C. rosaceus* between the two sides of the Caribbean ($F_{ST} = 0.014$, $P = 0.39$).

## Discussion

*Gamete characteristics*

Our measurements of egg size for *Clypeaster rosaceus*, *C. subdepressus*, and *Brisaster latifrons* and for protein content in *B. latifrons* are consistent with previously reported values (Table 1). Our estimate of egg energy for *C. rosaceus* falls between four previous estimates: we studied Panamanian individuals and estimated egg energy at 0.076 J/egg, Emlet (1986) estimated that Panamanian *C. rosaceus* contain 0.047 J/egg (cited in Miner *et al.*, 2002), whereas

**Table 2**

*Summary of fertilization results for* Clypeaster rosaceus *and* C. subdepressus

| Female | Male | $n$ | $R^2$ | $F_{(reg)}$ | $F_{50}$ | $F_{90}$ |
|---|---|---|---|---|---|---|
| *C. subdepressus* | *C. subdepressus* | 31 | 0.796 | 113.18*** | 50 | 335 |
| *C. subdepressus* | *C. rosaceus* | 9 | 0.456 | 5.87* | $1.8 \times 10^6$ | $3.0 \times 10^8$ |
| *C. rosaceus* | *C. subdepressus* | 28 | 0.424 | 19.14*** | 1523 | 70509 |
| *C. rosaceus* | *C. rosaceus* | 32 | 0.497 | 29.62*** | 101 | 4670 |

$n$ = number of sperm concentrations tested, $F_{(reg)}$ = significance values of the regression between log sperm concentration and logit-transformed percent fertilization: *$P < 0.05$, ***$P < 0.001$. $F_{50}$ and $F_{90}$ indicate the number of sperm/ml required to fertilize 50% and 90% of eggs, respectively.

Miner *et al.* (2002) and Heyland *et al.* (2006) estimated that Floridian *C. rosaceus* contain 0.106 J/egg and 0.065 J/egg, respectively. The value from Heyland *et al.* (2006) was converted from reported values of organic C using the conversion 1 $\mu$g C = $3.9 \times 10^{-2}$ J (after McEdward and Carson, 1987). The reported egg diameters are similar: 274 $\mu$m (Miner *et al.*, 2002), 280 $\mu$m (Emlet, 1986), 283 $\mu$m (this study), and 294 $\mu$m (Heyland *et al.*, 2006). Larger eggs do not contain more energy per egg, considering that the values reported by Miner *et al.* (2002) are the smallest for egg diameter but the highest for egg energetic content. There is no clear geographic effect in egg energy when comparing opposite sides of the Caribbean, nor would we expect one given the apparent gene flow between these two sites. Seasonal or lunar effects are also unlikely to explain the reported differences, as all four studies worked with *C. rosaceus* during the same time of the year (September to early November), and Lessios (1991) showed that *C. rosaceus* in Panama does not spawn on a lunar cycle. Methodological differences alone do not account for the different results, as both Heyland *et al.* (2006) and this study used the dichromate oxidation methods of Miner *et al.* (2002). The dichromate oxidation method has been criticized for failing to completely oxidize proteins, which leads to an underestimation of egg energy, particularly in species with small eggs (Pernet and Jaeckle, 2004). This effect is relatively less important when studying species with larger, lipid-rich eggs, where proteins make up a smaller proportion of total egg mass (Jaeckle, 1995), but future studies of egg energy in *Clypeaster* should consider biochemical component analysis, as suggested by Pernet and Jaeckle (2004).

A trend toward an increase in egg energetic density with increasing egg diameter is evident when comparing *C. subdepressus*, *C. rosaceus*, and *B. latifrons* (Fig. 1). Due to these differences in energetic density, a *C. rosaceus* egg, with about 6 times greater volume than a *C. subdepressus* egg, contains about 15 times more energy. Similarly, a *B. latifrons* egg has twice the volume of a *C. rosaceus* egg, but contains more than 3 times as much energy. These differences are likely due to the larger eggs containing a higher proportion of energetically dense lipids, and correlates with the observation that although *C. subdepressus* and *C. rosaceus* eggs are negatively buoyant in seawater, *B. latifrons* eggs float.

### *Fertilization kinetics of* Clypeaster rosaceus *and* Clypeaster subdepressus

Despite their larger size, eggs of *C. rosaceus* are not easier to fertilize than those of *C. subdepressus*, and the

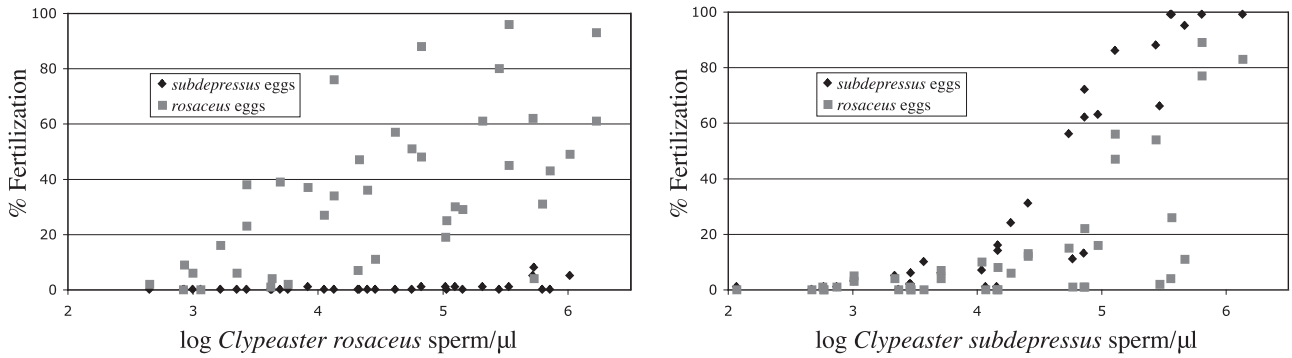


**Figure 2.** Results of fertilization experiments involving *Clypeaster rosaceus* and *C. subdepressus*. Percent fertilization of *C. rosaceus* and *C. subdepressus* eggs by *C. rosaceus* sperm (left panel). Percent fertilization of *C. rosaceus* and *C. subdepressus* eggs by *C. subdepressus* sperm (right panel).

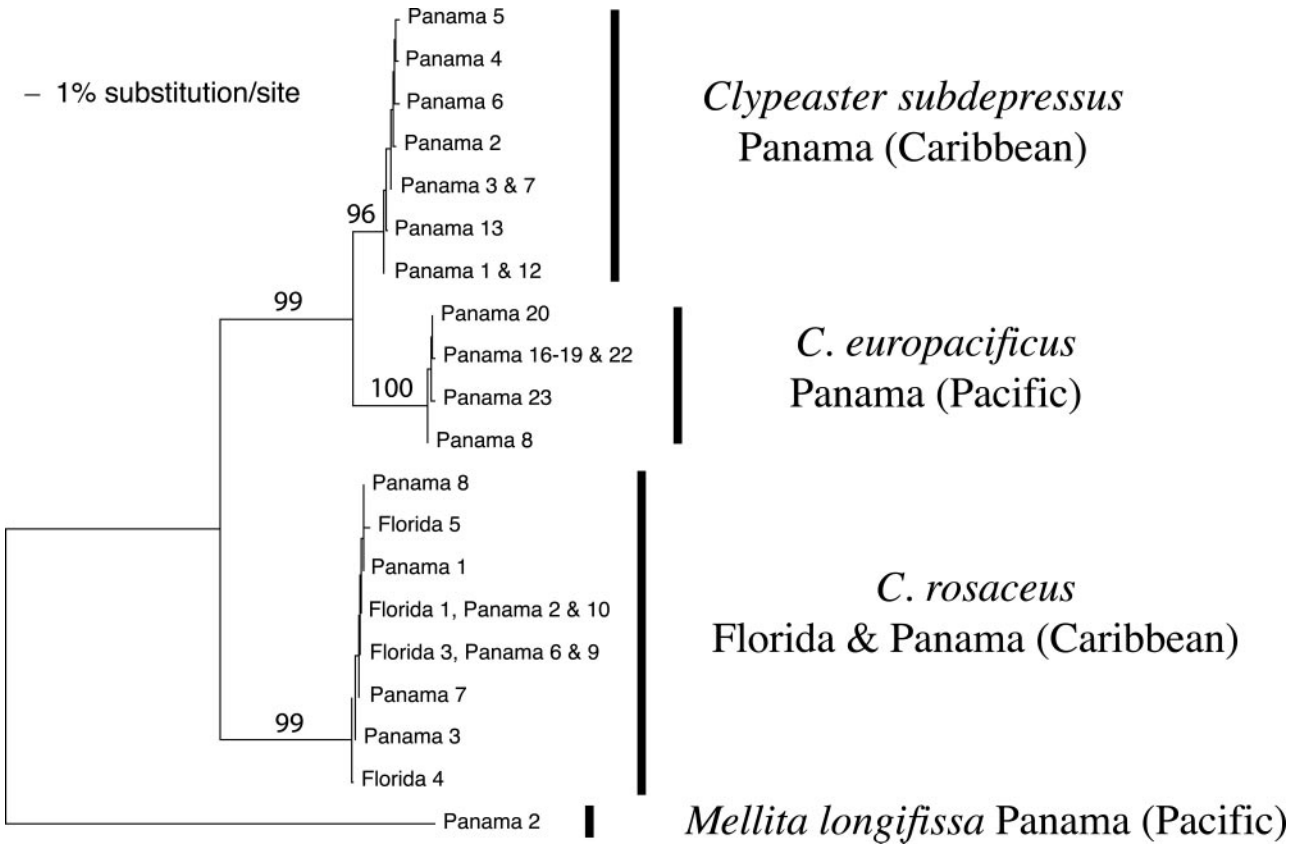


**Figure 3.** Neighbor-joining tree based on GTR + Γ distances between cytochrome oxidase sequences from three species of *Clypeaster*. Bootstrap values less than 70% are not shown. The tree is rooted with a sequence from *Mellita longifissa*.

conspecific $F_{50}$ values of both species are well within the range typical for echinoderms with obligately feeding larvae (Harper and Hart, 2005). Adult *C. rosaceus* and *C. subdepressus* live in similar habitats: *C. subdepressus* is commonly found in sand, whereas *C. rosaceus* is typically found in turtle grass beds adjacent to sand fields. As a result, *C. rosaceus* and *C. subdepressus* live side by side in the shallow waters of the Caribbean. Lessios (1984, 1991) showed that both species are ripe much of the year in Panama, with peak levels of readiness to spawn from July to October. This combination of adult habitat preferences and spawning periods raised the possibility that these species, despite their differences in larval development, would have had the potential to cross-fertilize in nature in the absence of gametic incompatibility.

Our results indicate that *C. rosaceus* and *C. subdepressus* are gametically incompatible, with *C. subdepressus* eggs particularly resistant to fertilization by *C. rosaceus* sperm. Judging from the large genetic divergence between these species, they have had ample time to evolve bidirectional gametic incompatibility (Zigler *et al.*, 2005; Lessios, 2007). *C. rosaceus* eggs are more vulnerable to fertilization by *C. subdepressus* sperm, but with an $F_{50}$ ratio of 15 between heterospecific and conspecific fertilizations, it is unlikely that many *C. rosaceus* eggs are fertilized by *C. subdepressus* sperm in nature. The only other comparison of gamete compatibility between congeneric echinoids with different modes of development was conducted in *Heliocidaris*. In this genus, the larger eggs of *H. erythrogramma*, a species with nonfeeding larvae, are better protected from fertilization by *H. tuberculata*, a species with small eggs and obligately feeding larvae, than are eggs in the reciprocal cross (Zigler *et al.*, 2003). In *Clypeaster*, the smaller eggs of *C. subdepressus* are better protected from fertilization by *C. rosaceus*. On the basis of these two comparisons, there does not seem to be a consistent pattern of larger eggs being better protected from cross-fertilization by sympatric congeners.

### *Divergence time between* Clypeaster rosaceus *and* Clypeaster subdepressus

*C. rosaceus* and *C. subdepressus* are not sister species, as *C. europacificus* and *C. subdepressus* are more closely related to each other (Fig. 3). As *C. subdepressus* is found only in the Caribbean and *C. europacificus* is found

only in the Eastern Pacific, it is reasonable to assume that they diverged at or before the final closure of the Isthmus of Panama, 3 million years ago (mya) (Coates and Obando, 1996). Consistent with this idea, the genetic divergence between *C. europacificus* and *C. subdepressus* is similar to that observed for other presumed trans-Isthmian (geminate) species pairs of sea urchins (Lessios *et al.*, 2001). With COI divergence between *C. rosaceus* and *C. subdepressus* 2.5 times greater than that between *C. subdepressus* and *C. europacificus*, we can roughly estimate that *C. rosaceus* and *C. subdepressus* diverged at least 7–8 mya, and potentially longer, if *C. subdepressus* and *C. europacificus* diverged at some point before 3 mya, or if saturation at the third codon position of the COI gene has led us to underestimate the actual genetic divergence between *C. subdepressus* and *C. rosaceus*.

Given that *C. rosaceus* develops *via* a pluteus and all of its congeners with a known mode of development develop from smaller eggs into obligately feeding plutei (Mortensen, 1937; Emlet, 1986; Vernon *et al.*, 1993; Amemiya and Arakawa, 1996), it is likely that this species evolved from ancestors with smaller eggs and an obligately feeding pluteus. The mode of development of *C. europacificus* is not known, but if we assume that the common ancestor of the three species of *Clypeaster* we studied developed *via* an obligately feeding larva, the divergence time estimate of *C. rosaceus* and *C. subdepressus* places a maximum limit on how long it has been since *C. rosaceus* evolved a facultative feeding larva.

### *Population genetic structure in* Clypeaster rosaceus

One might expect that the short minimum time to metamorphosis in *C. rosaceus* would lead to genetic divergence across the range of the species, as has been observed in other echinoderms with short larval lives (reviewed in Hart, 2002). This, however, is not the case, as we found no evidence of divergence between Panama and Florida. Instead, geographic variation of *C. rosaceus* resembles that of obligately planktotrophic species, which lack population structure across the Caribbean (Lessios *et al.*, 1999, 2001, 2003; Zigler and Lessios, 2004). Such a pattern indicates that even though larvae of *C. rosaceus* are capable of rapid metamorphosis, there has been sufficient trans-Caribbean gene flow to prevent divergence from evolving.

### *Insights from the study of species with facultatively feeding larvae*

*C. rosaceus* and *B. latifrons* are extraordinary representatives of a rare mode of development. What has the study of these species revealed about echinoid development in general and about echinoid species with facultatively feeding larvae in particular? Also, how do we interpret the developmental and life-history features of these species relative to echinoid species with feeding or nonfeeding larvae?

At one extreme of planktotrophic echinoid larvae (in terms of egg size), *C. rosaceus* and *B. latifrons* embryos indicate how flexible the pluteus form can be. The most commonly studied echinoid species have egg diameters of $\approx$100 $\mu$m. *C. rosaceus* and *B. latifrons* eggs, with volumes 20 and 45 times greater, respectively, than an egg with a diameter of 100 $\mu$m, still develop *via* the typical pluteus form (Emlet, 1986; Hart, 1996). Although similar in form to those of species with smaller eggs, *C. rosaceus* plutei differ in several ways that are intermediate between feeding and nonfeeding larvae. Plutei of obligately planktotrophic species typically take weeks to reach metamorphosis, but *C. rosaceus* plutei can metamorphose in as few as 5 days (Emlet, 1986). This rapid time to metamorphosis is likely related both to a smaller allocation of embryonic resources toward feeding structures (in terms of total pluteus length and postoral arm length [Emlet, 1986]), and to a larger allocation of resources to early formation of the adult rudiment (*via* the early formation of a large left coelom [Smith *et al.*, 2007]) when compared to *C. subdepressus*. In contrast, time to metamorphosis in *B. latifrons* is not accelerated relative to co-occurring echinoids with obligately feeding larvae (Hart, 1996).

Comparing *C. rosaceus* and *B. latifrons* makes it clear that not all eggs that develop into facultatively feeding larvae are built in the same fashion. *C. rosaceus* eggs are not very different from the eggs of obligate planktotrophs. By our measurements, *C. rosaceus* egg energetic density, though twice that of *C. subdepressus,* falls in the middle of the range of values reported for echinoids with feeding larvae. Like the eggs of other echinoids with feeding larvae, *C. rosaceus* eggs are negatively buoyant. In contrast, *B. latifrons* eggs are more similar to those of echinoids with nonfeeding larvae, in terms of egg size, energetic density, and buoyancy.

In summary, the eggs, embryos, and life histories of *C. rosaceus* and *B. latifrons* exhibit distinct mixtures of features from echinoids with feeding and nonfeeding larvae. *C. rosaceus* resembles echinoid species with obligately feeding larvae in its egg energetic density, egg buoyancy, fertilization kinetics, and population structure. At the same time, *C. rosaceus* resembles echinoid species with nonfeeding larvae in its time to metamorphosis, reduced allocation to larval feeding structures, and early formation of a left coelom. We know less about *B. latifrons*, but what we do know indicates that the situation in this species is reversed relative to that of *C. rosaceus*: *B. latifrons* eggs are similar to those of species with nonfeeding larvae in energetic density and buoyancy, but its time to metamorphosis is similar to that of echinoids

with obligately feeding larvae. There are, however, some similarities between the larvae of the two species, as neither is particularly effective at gathering energy in the plankton: *C. rosaceus* does not assimilate much food as a larva (Reitzel and Miner, 2007), and *B. latifrons* has reduced capture rates relative to other planktotrophic echinoids (Hart, 1996). Rather than being similarly intermediate for developmental and life-history features that differ between echinoid species with feeding and nonfeeding development, *C. rosaceus* and *B. latifrons* exhibit distinct combinations of features of both modes of development, indicating different evolutionary paths to facultative feeding.

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## Literature Cited

**Allen, J. D., and B. Pernet. 2007.** Intermediate modes of larval development: bridging the gap between planktotrophy and lecithotrophy. *Evol. Dev.* **9:** 643–653.

**Allen, J. D., C. Zakas, and R. D. Podolsky. 2006.** Effects of egg size reduction and larval feeding on juvenile quality for a species with facultative-feeding development. *J. Exp. Mar. Biol. Ecol.* **331:** 186–197.

**Amemiya, S., and E. Arakawa. 1996.** Variation of cleavage pattern permitting normal development in a sand dollar, *Peronella japonica*: comparison to other sand dollars. *Dev. Genes Evol.* **206:** 125–135.

**Coates, A. G., and J. A. Obando. 1996.** The geologic evolution of the Central American Isthmus. Pp. 21–56 in *Evolution and Environment in Tropical America,* J. B. C. Jackson, A. G. Coates, and A. Budd, eds. University of Chicago Press, Chicago.

**Emlet, R. B. 1986.** Facultative planktotrophy in the tropical echinoid *Clypeaster rosaceus* (Linnaeus) and a comparison with obligate planktotrophy in *Clypeaster subdepressus* (Gray) (Clypeasteroida:Echinoidea). *J. Exp. Mar. Biol. Evol.* **95:** 183–202.

**Emlet, R. B. 1990.** World patterns of developmental mode in echinoid echinoderms. Pages 329–335 in *Advances in Invertebrate Reproduction 5*, M. Hoshi and O. Yamashita, eds. Elsevier, Amsterdam.

**Harper, F. M., and M. W. Hart. 2005.** Gamete compatibility and sperm competition affect paternity and hybridization between sympatric *Asterias* sea stars. *Biol. Bull.* **209:** 113–126.

**Hart, M. W. 1996.** Evolutionary loss of larval feeding: development, form and function in a facultatively feeding larva, *Brisaster latifrons*. *Evolution* **50**: 174–187.

**Hart, M. W. 2002.** Life history evolution and comparative developmental biology of echinoderms. *Evol. Dev.* **4**: 62–71.

**Heyland, A., A. M. Reitzel, D. A. Price, and L. L. Moroz. 2006.** Endogenous thyroid hormone synthesis in facultative planktotrophic larvae of the sand dollar *Clypeaster rosaceus*: implications for the evolutionary loss of feeding. *Evol. Dev.* **8**: 568–579.

**Jaeckle, W. B. 1995.** Variation in the size, energy content, and biochemical composition of invertebrate eggs: correlates to the mode of larval development. Pp. 1–48 in *Ecology of Marine Invertebrate Larvae*, L. R. McEdward, ed. CRC Press, Boca Raton, FL.

**Lessios, H. A. 1984.** Annual reproductive periodicity in eight echinoid species on the Caribbean coast of Panama. Pp. 303–311 in *Proceedings of the 5th International Echinoderm Conference,* B. F. Keegan and B. D. S. O'Connor, eds.. A. A. Balkema, Boston.

**Lessios, H. A. 1991.** Presence and absence of monthly reproductive rhythms among eight Caribbean echinoids off the coast of Panama. *J. Exp. Mar. Biol. Ecol.* **153:** 27–47.

**Lessios, H. A. 2007.** Reproductive ecology and reproductive isolation in sea urchins. *Bull. Mar. Sci.* **81:** 191–208.

**Lessios, H. A., B. D. Kessing, D. R. Robertson, and G. Paulay. 1999.** Phylogeography of the pantropical sea urchin *Eucidaris* in relation to land barriers and ocean currents. *Evolution* **53:** 806–817.

**Lessios, H. A., B. D. Kessing, and J. S. Pearse. 2001.** Population structure and speciation in tropical seas: global phylogeography of the sea urchin *Diadema*. *Evolution* **55:** 955–975.

**Lessios, H. A., J. Kane, and D. R. Robertson. 2003.** Phylogeography of the pantropical sea urchin *Tripneustes*: contrasting patterns of population structure between oceans. *Evolution* **57:** 2026–2036.

**Marshall, C. R., E. C. Raff, and R. A. Raff. 1994.** Dollo's Law and the death and resurrection of genes. *Proc. Natl. Acad. Sci. USA* **91:** 12283–12287.

**McCartney, M.A., and H. A. Lessios. 2002.** Quantitative analysis of gametic incompatibility between closely related species of neotropical sea urchins. *Biol. Bull.* **202:** 166–181.

**McEdward, L. R., and S. F. Carson. 1987.** Variation in egg organic content and its relationship with egg size in the starfish *Solaster stimpsoni*. *Mar. Ecol. Prog. Ser.* **37:** 159–169.

**McEdward, L. R., and K. H. Morgan. 2001.** Interspecific relationships between egg size and the level of parental investment per offspring in echinoderms. *Biol. Bull.* **200:** 33–50.

**Miner, B. G., J. D. Cowart, and L. R. McEdward. 2002.** Egg energetics for the facultative planktotroph *Clypeaster rosaceus* (Echinodermata:Echinoidea), revisited. *Biol. Bull.* **202:** 97–99.

**Mortensen, T. 1937.** Contributions to the study of the development and larval forms of echinoderms, III. *K. Dan. Vidensk. Selsk. Skr. Naturv. Math. Afd. Ser. 9*, **7**(1)**:** 1–65.

**Pernet, B., and W. B. Jaeckle. 2004.** Size and organic content of eggs of marine annelids, and the underestimation of egg energy content by dichromate oxidation. *Biol. Bull.* **207:** 67–71.

**Posada, D., and K. A. Crandall. 1998.** Modeltest: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818.

**Raff, R. A. 1996.** *The Shape of Life. Genes, Development, and the Evolution of Animal Form.* University of Chicago Press, Chicago.

**Raff, R. A. 2008.** Origins of other metazoan body plans: the evolution of larval forms. *Philos. Trans. R. Soc. Lond. B* **363:** 1473–1479.

**Reitzel, A. M., and B. G. Miner. 2007.** Reduced planktotrophy in larvae of *Clypeaster rosaceus* (Echinodermata: Echinoidea). *Mar. Biol.* **151:** 1525–1534.

**Rouse, G. W. 2000.** The epitome of handwaving? Larval feeding and hypotheses of metazoan phylogeny. *Evol. Dev.* **2:** 222–233.

**Schneider, S., D. Roessli, and L. Excoffier. 2000.** Arlequin ver. 2.000:

a software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

**Smith, M. S., K. S. Zigler, and R. A. Raff. 2007.** Evolution of direct-developing larvae: selection vs. loss. *Bioessays* **29:** 566–571.

**Strathmann, R. R. 1978.** The evolution and loss of feeding larval stages of marine invertebrates. *Evolution* **32:** 894–906.

**Swofford, D. L. 2003.** *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods).* Version 4. Sinauer Associates, Sunderland, MA.

**Vernon, J. D., J. B. McClintock, T. S. Hopkins, S. A. Watts, and K. R. Marion. 1993.** Reproduction of *Clypeaster ravenelii* (Echinodermata: Echinoidea) in the northern Gulf of Mexico. *Invertebr. Reprod. Dev*. **24:** 71–78.

**Zigler, K. S., and H. A. Lessios. 2004.** Speciation on the coasts of the new world: phylogeography and the evolution of bindin in the sea urchin genus *Lytechinus*. *Evolution* **58:** 1225–1241.

**Zigler, K. S., E. C. Raff, E. Popodi, R. A. Raff, and H. A. Lessios. 2003.** Adaptive evolution of bindin in the genus *Heliocidaris* is correlated with the shift to direct development. *Evolution* **57:** 2293–2302.

**Zigler, K. S., M. A. McCartney, D. R. Levitan, and H. A. Lessios. 2005.** Sea urchin bindin divergence predicts gamete compatibility. *Evolution* **59:** 2399–2404.