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Environmental Induction of Polyembryony in Echinoid Echinoderms

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Abstract. Polyembryony, or the production of multiple offspring from a single zygote, is a widespread phenomenon in the animal kingdom. Various types of polyembryony have been described in arthropods, bryozoans, chordates, cnidarians, echinoderms, and platyhelminthes. We describe the induction of polyembryony in embryos of the sand dollar *Echinarachnius parma* and the pencil urchin *Eucidaris tribuloides* in response to elevated temperature and reduced salinity. Data on the environmental variation in temperature and salinity that normally occurs during the spawning season, combined with the range of laboratory conditions over which polyembryony was induced, suggest that polyembryony may occur frequently in these species under natural conditions. We tested an additional two species of echinoids for similar responses, but found little evidence for polyembryony in the green urchin *Strongylocentrotus droebachiensis* or the variegated urchin *Lytechinus variegatus*, suggesting that polyembryony is not a universal response of echinoids to fluctuations in temperature and salinity. The unexpected developmental changes that we observed in response to present-day fluctuations in temperature and salinity suggest that ongoing and future environmental shifts may drive substantial changes in marine invertebrate developmental patterns, and that these changes will be different across taxa.

Introduction

Polyembryony is the splitting of a single product of sexual reproduction (zygote, embryo, or larva) into multiple offspring with identical genotypes. It can result from a variety of mechanisms, including both embryonic and post-embryonic cloning (Craig *et al*., 1997). In many situations, polyembryony can be adaptive, such as when maternal egg production is constrained (Loughry *et al*., 1998), and when more information about the embryonic environment is available to offspring than to their mothers (Craig *et al*., 1997). Polyembryony is taxonomically widespread, occurring in at least six metazoan phyla: Arthropoda, Bryozoa, Chordata, Cnidaria, Echinodermata, and Platyhelminthes (reviewed by Craig *et al*., 1997; Zhurov *et al*., 2007).

In some species, polyembryony is an obligate part of development. For example, in the nine-banded armadillo, a single embryo always divides to produce a litter of four identical quadruplets (Loughry *et al*., 1998). Some parasitoid wasps also display obligate polyembryony, producing embryos that can divide into as many as 2000 individuals (Zhurov *et al*., 2007). However, in wasps, unlike armadillos, the number of individuals that each embryo produces is dependent on biotic interactions, such as intraspecific competition within the host (Segoli *et al*., 2009). Outside of wasps and armadillos, polyembryony is often facultative, occurring as a consequence of both abiotic and biotic stimuli. For example, the embryos of the coral *Acropora millepora* may fragment when they are exposed to turbulent water conditions at the two-, four-, or eight-cell stage, and yield normal (albeit smaller) larvae and juveniles (Heyward and Negri, 2012). In addition to cloning as a consequence of abiotic stress, animals can also clone in response to biological cues in potentially adaptive ways. For example, larvae of the sand dollar *Dendraster excentricus* (Eschscholtz, 1831) clone in response to cues from mucus collected from potentially predatory fish (Vaughn and Strathmann, 2008). In this case, the reduced size of the resulting clone is hypothesized to reduce attacks from the visually oriented predators that induce this response (Vaughn and Strathmann, 2008), consistent with data showing that smaller

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larvae are less susceptible to predatory fish (Allen, 2008; Vaughn, 2010). Cloning that affects offspring size and/or stage could carry tradeoffs; for instance, diminished size may reduce vulnerability to visual predators, but increase risks from other, non-visual predators (Allen, 2008), and/or significantly extend the period of planktonic development.

Among marine invertebrates, examples of polyembryony are most widespread within echinoderms. In addition to the sand dollar example described above, larval cloning (*i.e.,* a specific type of polyembryony) has been reported in multiple species of sea stars (Bosch *et al*., 1989; Jaeckle, 1994; Vickery and McClintock, 2000; Knott *et al.,* 2003), brittle stars (Balser, 1998), and sea cucumbers and sea urchins (Eaves and Palmer, 2003). Importantly, cloning has been documented in field-collected specimens, suggesting this phenomenon is not just an artifact of laboratory culture conditions (*e.g.,* Bosch *et al*., 1989; Jaeckle, 1994; Balser, 1998; Knott *et al*., 2003). In those cases where cues have been tested, polyembryony can be induced during the larval stage by either biotic stimuli, such as changes in food level or predator cues (Vickery and McClintock, 2000; Vaughn and Strathmann, 2008; McDonald and Vaughn, 2010), or abiotic changes, such as moderate temperature increases (Vickery and McClintock, 2000).

Despite these reports of larval cloning, there is only one description of polyembryony occurring in echinoderms at the embryonic stage without special efforts to induce the phenomenon. Mortensen (1938) briefly described polyembryony at the blastula stage in embryos of the cidaroid urchin *Prionocidaris baculosa* (Lamarck, 1816), which were fertilized and observed in the laboratory. While there is a long and storied history of experimental induction of polyembryony in the embryos of echinoid echinoderms (Driesch, 1892; Harvey, 1940; Okazaki and Dan, 1954; Vacquier and Mazia, 1968a, b; Horstadius, 1973; Sinervo and McEdward, 1988; Hart, 1995; Allen *et al*., 2006; McAlister, 2007; Moran and Allen, 2007; Alcorn and Allen, 2009; Allen, 2012), Mortensen's (1938) work opens the possibility that polyembryony may occur spontaneously during early development and under natural conditions.

Polyembryony during early development is likely to be the result of a disruption of blastomere adhesion. As an echinoid embryo develops, adjacent blastomeres are linked by adhesive bonds; in many cases, the developing embryo is also surrounded by an extracellular matrix, termed the hyaline layer. Previous work has shown that chemical degradation of disulfide (S-S) cell adhesion bonds can induce polyembryony in echinoid echinoderms (Mazia, 1958; Vacquier and Mazia, 1968a, b); however, the frequency of polyembryony following such disruption depends on the strength of interaction between blastomeres and the hyaline layer. When hyaline-blastomere connections are strong, as in regular urchins, degradation of cell adhesion bonds has little effect on embryos (Vacquier and Mazia, 1968b). When hyaline-blastomere interactions are weak, as in irregular urchins such as sand dollars, degradation of cell adhesion bonds leads to rampant polyembryony (Mazia, 1958; Vacquier and Mazia, 1968a). These findings led Vacquier and Mazier (1968a, b) to hypothesize that the strength of interaction between blastomeres and the hyaline layer is correlated with the ability to chemically induce polyembryony during early development in echinoids. Some echinoids (*e.g.,* primitive cidaroid urchins) appear to lack a hyaline layer altogether (Schroeder, 1981; Bennett *et al*., 2012), and thus hyaline-blastomere interactions may be nonexistent in those species. Phylogenetic effects may predispose some groups of echinoids to polyembryony, and preclude the phenomenon in others.

Environmental stressors may also disrupt cell adhesion and induce polyembryony in echinoids. Temperature and salinity vary widely during the spawning season of many nearshore marine invertebrates, and reduced salinity is likely to reduce the availability of Ca^{+2} and disrupt intercellular connections. In this study, we tested the effects of different levels of temperature and salinity on the frequency of polyembryony during early development in four species of echinoid echinoderms. Two of these species, the sand dollar *Echinarachnius parma* (Lamarck, 1816) and the pencil urchin *Eucidaris tribuloides* (Lamarck, 1816), have weak or nonexistent hyaline-blastomere interactions. In the other two species, the green urchin *Strongylocentrotus droebachiensis* (O. F. Müller, 1776) and the variegated urchin *Lytechinus variegatus* (Lamarck, 1816), hyaline-blastomere interactions are strong. We discuss our results in the context of environmental conditions that are prevalent during spawning and early development in these species, in order to evaluate the possibility that polyembryony is a common or widespread occurrence in nature. Finally, we evaluate support for the hypothesis that weak hyaline-blastomere interactions promote polyembryony during the early developmental stages of echinoids.

Materials and Methods

Experiments were conducted at the Bowdoin College Coastal Studies Center (CSC), Orrs Island, Maine, and at the College of William and Mary, Williamsburg, Virginia. Adult *Echinarachnius parma* were hand-collected from the intertidal zone at Cedar Beach, Bailey Island, ME (43° 44′ N, 69° 59′ W) and by subtidal dredge from St. Helena Island, Stonington, ME (44° 07′N, 68° 38′ W). Adult *Strongylocentrotus droebachiensis* were collected by dredge from the waters surrounding Stonington, ME. Adult *Lytechinus variegatus* and *Eucidaris tribuloides* were collected subtidally from the waters surrounding Big Pine Key, Florida. Subtidal animals were collected by Gulf of Maine, Inc. (*E. parma* and *S. droebachiensis*) or by Carolina Biological Supply (*L. variegatus* and *E. tribuloides*) and

shipped overnight for use in the experiments. In Maine, experimental animals (*E. parma*) were housed in flow-through seawater under ambient conditions of temperature (15–20 °C) and salinity (28–32 ppt) until spawning. In Virginia, experimental animals were housed in recirculating aquaria at either 12 °C and 32 ppt (*E. parma*, and *S. droebachiensis*) or at 22 °C and 32 ppt (*L. variegatus* and *E. tribuloides*) until spawning.

Adults of all four echinoid species were induced to spawn by intracoelomic injection of 1 ml of 0.5 mol l^{-1} KCl. Gametes were collected by pipette or by inverting spawning adults over beakers of 0.45-μm filtered seawater (FSW). Seawater was kept at a range of temperature (8–32 °C) and salinity (20–35 ppt) combinations that varied depending on the species being tested (Table 1). In Maine, FSW was collected from the flow-through seawater system at the CSC; in Virginia, FSW was made from a commercial artificial seawater mix (Instant Ocean Sea Salt; Spectrum Brands, Inc., Blacksburg, VA) and deionized water.

To assess how the frequency of polyembyrony varies with abiotic environmental conditions, eggs from all species were fertilized over a range of temperature and salinity combinations. The specific conditions that were tested varied among species over the following ranges: *E. parma,* 19–24 °C and 20–30 ppt; *E. tribuloides,* 22–30 °C and 26–32 ppt; *L. variegatus,* 24–32 °C and 24–35 ppt; and *S. droebachiensis* 8–11 °C and 22–32 ppt (Table 1). Each trial listed in Table 1 is the result of an independent cross between one male and one female animal. Fertilizations occurred in glass bowls filled with 100 ml of FSW at the temperature and salinity combinations described above and in Table 1. For each unique temperature and salinity combination in each trial, we generated three replicate bowls per dish. The concentration of eggs varied across species (~500–1000 eggs per bowl), but was held constant within an experiment to avoid the confounding effects of gamete concentrations on polyembryony. For all treatments in which polyembryony was assessed, percent fertilization was at least 80%; frequently, it was 95%–100%. When embryos reached the unhatched blastula stage, we scored each dish for polyembryony by counting the number of embryos out of 100 that were polyembryonic in each dish. This allowed us to confirm that embryos were within a single fertilization envelope (FE), and that twins or other types of multiples were forming viable embryos. In all four species, multiples that reached the blastula stage were able to hatch and form swimming larvae.

In one species, *Echinarachnius parma*, we measured the growth of larvae derived from polyembryony induction experiments in three independent male-female pairs. We isolated the sibling twin and single *E. parma* larvae used in these experiments, and individually reared them in 6-well plates containing 10 ml of FSW at 30 ppt. At the CSC, plates were floated in sea tables in order to maintain cultures at ambient seawater temperatures of 15–17 °C. All larvae were fed a diet of the unicellular alga *Dunaliella tertiolecta* at a concentration of 5.0 cells μl^{-1}. Water was changed using reverse filtration, and algal food was added every other day. Larval size was measured as post-oral arm length, and body length at a magnification of 100× using a compound microscope and ocular micrometer. Measurements were taken 8 d post-fertilization for two of the replicate experiments, and 9 d post-fertilization for the third experiment. To compare echinoid larval sizes, a mixed-model ANOVA was conducted with male-female pair modeled as a random effect, and larval type (twin or single) modeled as a fixed effect. Effects of age (8 *vs.* 9 d) were not detected, and thus were not included in the model.

For the species in which polyembryony was most commonly observed, *Eucidaris tribuloides*, we conducted a mixed-model ANOVA on the frequency of polyembryony. Data from three independent experiments—each with a separate male-female pair—were used in the analysis with temperature, salinity, and the interaction of temperature and salinity all modeled as fixed effects; male-female pair was modeled as a random effect. Prior to analysis, we used an arcsine square root transformation to account for the non-normal distribution of proportional data. We plotted the residuals from the model on a Quantile-Quantile (Q-Q) plot to allow visual inspection of the data, followed by a Kolmogorov-Smirnov test to confirm that the data approximated a normal distribution following transformation ($P = 0.68$). Where significant fixed effects were detected, the estimated marginal means were then subjected to multiple pairwise comparisons to test for significant differences between treatments using a Bonferroni correction.

We also conducted an ANOVA on the percentage of polyembryonic *E. parma* embryos, using arcsine square root transformed data. However, even after the transformation, the data still did not approximate a normal distribution, as revealed by visual inspection of the Q-Q plot and a Kolmogorov-Smirnov test ($P < 0.05$). Therefore, we also fit a binomial regression on the number of polyembryonic embryos out of the total number of embryos counted with temperature, salinity, and the interaction of temperature and salinity as fixed effects; cross was a random effect. We then used a chi-squared goodness of fit test to determine how each term affected the model.

Following the repeated induction of polyembryony in two species (*E. parma* and *E. tribuloides*), we tested the hypothesis that salinity treatments were correlated with osmotically induced swelling of the FE during and after fertilization. To do so, we manipulated salinity to induce changes in the FE, then measured the FE diameter following fertilization, but prior to first cleavage, for a single male-female pair from each species. For each treatment within this cross, we generated three replicate bowls; in each bowl, the diameters of 20 FEs were measured at a magnification of 100× using

Table 1

Percentage of polyembryony observed under all experimental conditions

Species	Trial	Holding temperature (°C)	Holding salinity (ppt)	Experimental temperature (°C)	Experimental salinity (ppt)										
					20	22	24	25	26	27	28	29	30	32	35
Echinarachnius parma	1	15-20	28-32	20	0.0 ± 0.0	–	0.0 ± 0.0	–	0.0 ± 0.0	–	–	–	0.0 ± 0.0	–	–
				21	0.0 ± 0.0	–	2.7 ± 1.8	–	0.0 ± 0.0	–	–	–	0.0 ± 0.0	–	–
				24	0.0 ± 0.0	–	3.3 ± 1.3	–	3.3 ± 2.4	–	–	–	1.3 ± 1.3	–	–
	2	15-20	28-32	19	–	0.0 ± 0.0	0.7 ± 0.7	–	4.0 ± 4.0	–	–	–	0.0 ± 0.0	–	–
				21	–	0.0 ± 0.0	0.0 ± 0.0	–	10.0 ± 2.7	–	–	–	0.0 ± 0.0	–	–
				24	–	0.0 ± 0.0	0.0 ± 0.0	–	0.7 ± 0.7	–	–	–	0.0 ± 0.0	–	–
Eucidaris tribuloides	1	22	32	22	–	–	–	–	16.0 ± 2.3	–	–	22.7 ± 3.3	–	23.3 ± 7.0	–
				26	–	–	–	–	33.3 ± 1.3	–	–	9.3 ± 2.9	–	5.3 ± 1.3	–
				28	–	–	–	–	38.7 ± 6.6	–	–	18.0 ± 2.3	–	4.0 ± 2.0	–
				30	–	–	–	–	42.0 ± 6.0	–	–	13.3 ± 4.6	–	1.3 ± 1.3	–
	2	22	32	22	–	–	–	–	0.0 ± 0.0	–	–	4.7 ± 0.7	–	10.0 ± 3.1	–
				26	–	–	–	–	15.3 ± 2.4	–	–	15.3 ± 4.1	–	5.3 ± 1.8	–
				28	–	–	–	–	22.0 ± 5.3	–	–	16.7 ± 2.9	–	10.0 ± 2.0	–
				30	–	–	–	–	42.0 ± 5.3	–	–	25.3 ± 0.7	–	9.3 ± 1.3	–
	3	22	32	22	–	–	–	–	0.0 ± 0.0	–	–	0.0 ± 0.0	–	0.0 ± 0.0	–
				26	–	–	–	–	0.0 ± 0.0	–	–	0.0 ± 0.0	–	0.0 ± 0.0	–
				28	–	–	–	–	2.0 ± 2.0	–	–	0.0 ± 0.0	–	1.3 ± 0.7	–
				30	–	–	–	–	10.0 ± 2.0	–	–	0.0 ± 0.0	–	0.0 ± 0.0	–
Lytechinus variegatus	1	22	32	24	–	–	–	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	–	–	0.0 ± 0.0	–	0.0 ± 0.0
				28	–	–	–	1.3 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	–	–	0.0 ± 0.0	–	0.0 ± 0.0
				32	–	–	–	2.0 ± 1.2	0.0 ± 0.0	2.7 ± 0.7	–	–	0.7 ± 0.7	–	0.0 ± 0.0
Strongylocentrotus droebachiensis	1	12	32	8	–	0.0 ± 0.0	0.0 ± 0.0	–	0.0 ± 0.0	–	0.0 ± 0.0	–	0.0 ± 0.0	0.0 ± 0.0	–
				11	–	3.3 ± 1.7	0.0 ± 0.0	–	0.0 ± 0.0	–	0.0 ± 0.0	–	0.0 ± 0.0	0.0 ± 0.0	–
	2	12	32	8	–	0.0 ± 0.0	0.7 ± 0.7	–	0.0 ± 0.0	–	0.0 ± 0.0	–	0.0 ± 0.0	0.0 ± 0.0	–
				11	–	3.3 ± 1.3	0.0 ± 0.0	–	0.0 ± 0.0	–	0.0 ± 0.0	–	0.0 ± 0.0	0.0 ± 0.0	–

Holding conditions reflect adult environment prior to spawning. Data are means ± SE.

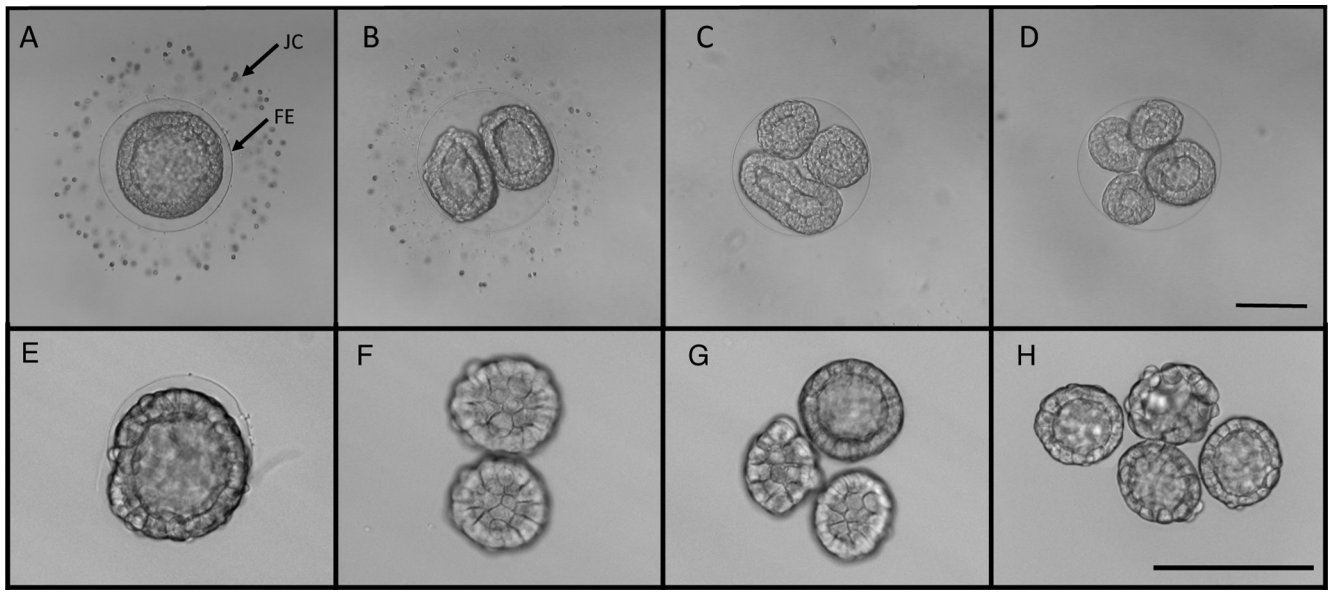

Figure 1. Multiple production in the sand dollar *Echinarachnius parma* (A–D) and in the pencil urchin *Eucidaris tribuloides* (E–H). Elevated temperature and reduced salinity most frequently resulted in polyembryony in *E. parma* and *E. tribuloides,* although the phenomenon was observed under a wide range of laboratory conditions. Figured for each species are four sibling embryos reared under identical conditions (21 °C and 26 ppt for *E. parma*, and 30 °C and 26 ppt for *E. tribuloides*). (A, E) Normal development of a single embryo to the blastula stage. (B, F) Development of twins, (C, G) triplets, and (D, H) quadruplets to the blastula stage, immediately prior to hatching. Image magnification is identical within each row. Scale bars represent 100 μm. FE, fertilization envelope, JC, jelly coat.

a compound microscope equipped with an ocular micrometer. We then tested the effect of salinity on the FE diameter using one-way ANOVA, after first testing for normality of the data with Kolmogorov-Smirnov tests of normality. When salinity was found to be significant, we used multiple pairwise comparisons to make post-hoc assessments of differences between treatments, using a Bonferroni correction for multiple tests. Statistical analyses were carried out using IBM SPSS version 20.0 software and R version 3.1.0.

Results

The frequency of polyembryony varied widely both across and within species. In the green urchin *Strongylocentrotus droebachiensis*, polyembryony was observed in embryos from both male-female pairs tested, but at very low frequencies. At 8 °C, only one of the 2000 embryos scored across both male-female pairs exhibited polyembryony (0.05%). At 11 °C, polyembryony was more frequent, but still rare, occurring in only 10 of 2000 embryos scored across both male-female pairs (0.5%). At 11 °C, all 10 cases of polyembryony were observed at the lowest salinity treatment (22 ppt). For this temperature (11 °C) and salinity (22 ppt) combination, the mean frequency of polyembryony was 3.3% for each of the two male-female pairs.

In the variegated urchin *Lytechinus variegatus*, polyembryony was observed in four of five male-female pairs tested, but, again, at low frequency ($< 1\%$). Across all temperature (32, 28, and 24 °C) and salinity (35, 30, 27, 26, and 25 ppt) treatments, polyembryony was observed in only 10 of 2250 embryos scored (0.44%). The highest frequency of polyembryony was observed at 32 °C and 27 ppt (2.67% $\pm$ 0.67%; mean $\pm$ SE). Polyembryony was also observed at 32 °C and 30 ppt (0.67% $\pm$ 0.67%), 32 °C and 25 ppt (2.00% $\pm$ 1.15%), and 28 °C and 25 ppt (1.33% $\pm$ 0.67%), but was not seen in any other treatments for this species.

Polyembryony was more frequently observed in the sand dollar *Echinarachnius parma* under conditions of increased temperature and reduced salinity (Fig. 1A–D). At hatching, these embryos separated from one another and swam apart as distinct individuals that proceeded to form normal pluteus larvae. *E. parma* exhibited polyembryony in 8 of 11 male-female pairs, although the frequency of multiples was highly variable between trials (1%–28%). For two male-female pairs, we conducted a detailed study of the frequency of polyembryony under combinations of four temperature (19, 20, 21, and 24 °C) and five salinity (20, 22, 24, 26, and 30 ppt) treatments (Table 1). Two-way ANOVA showed that salinity had a significant effect on the proportion of polyembryony ($F_{4,\ 55} = 2.929$; $P = 0.029$), but neither temperature ($F_{3,\ 55} = 1.306$; $P = 0.282$) nor the interaction between temperature and salinity did so ($F_{9,\ 55} = 0.607$;

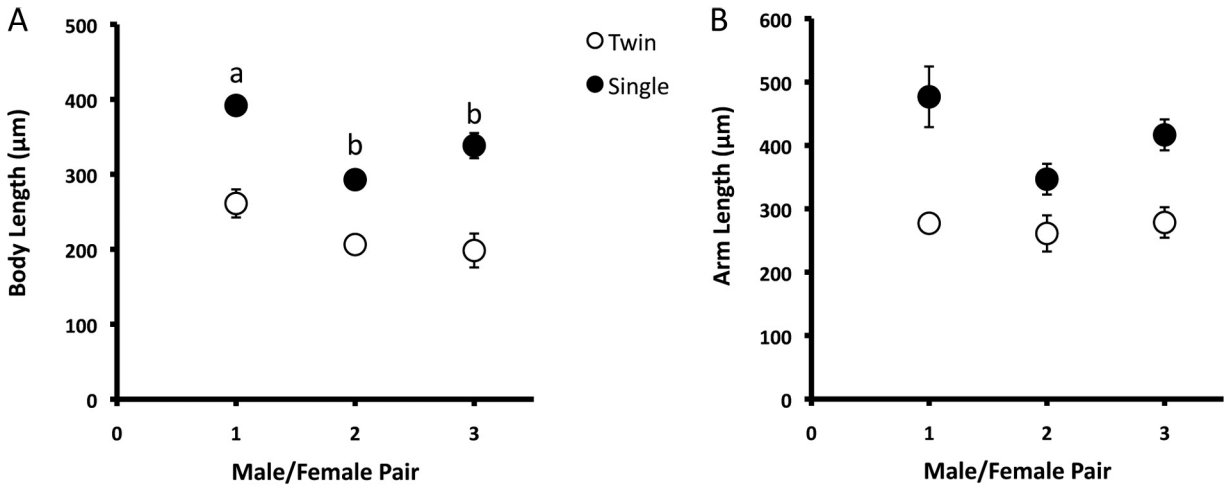

Figure 2. Mean (± SE) size of *Echinarachnius parma* larvae derived from single embryos (solid circles) and from twinned embryos (open circles). Larval cultures were generated from one of three unique male-female pairs, then measured 8–9 d post-fertilization. Both body length (A) and arm length (B) were significantly shorter in twin larvae than in single larvae ($P < 0.001$). However, male-female pair significantly affected larval body length ($P = 0.001$), but not arm length ($P = 0.065$; see text for details of statistical treatment). Letters above solid circles indicate significant differences among male-female pairs based on Bonferroni-corrected post-hoc tests.

$P = 0.785$). However, even after an arcsine-squareroot transformation, the residuals of the ANOVA were not normally distributed. Because ANOVA is known to be robust to the violation of the normality criterion (*e.g.,* Quinn and Keough, 2002; Gotelli and Ellison, 2012), we report the results above; however, we also analyzed the data using a binomial regression. After fitting the regression, a chi-squared goodness of fit test revealed that salinity, but no other terms, had a significant effect on the fit of the model: temperature ($\chi^2 = 0.0$; $P > 0.99$), salinity ($\chi^2 = 13.76$; $P = 0.008$), cross ($\chi^2 = 0.0$; $P > 0.99$), and the interaction of temperature and salinity ($\chi^2 = 7.39$; $P = 0.687$). Thus, both analyses yielded the same qualitative result.

For three *E. parma* male-female pairs, we followed the larvae that resulted from twinning events to document the consequences for larval growth and development. For larvae from all three pairs, twinned larvae developed normally, but were smaller than their siblings that were not polyembryonic. At 9 d post-fertilization, twins had shorter post-oral arms (ANOVA, $F_{1,\ 32} = 28.682$; $P < 0.001$) and reduced body length (ANOVA, $F_{1,\ 32} = 47.011$; $P < 0.001$) compared with normal larvae (Fig. 2). In all other ways, twins appeared to be normal.

Polyembryony was seen at the highest frequency (more than 40%) in the pencil urchin *Eucidaris tribuloides*. We observed polyembryony in all three male-female pairs tested, but, as in *E. parma*, there was significant variation in the frequency of polyembryony among pairs (Fig. 3). A mixed-model ANOVA found that there were significant effects of temperature ($F_{3,\ 94} = 5.996$; $P < 0.001$), salinity ($F_{2,\ 94} = 22.121$; $P < 0.001$), and the interaction between temperature and salinity ($F_{6,\ 94} = 9.702$; $P < 0.001$) on the frequency of polyembryony, even when accounting for the random effect of male-female pair. Post-hoc analysis of the estimated marginal means (with Bonferroni correction) showed that all three tested salinities (26, 29, and 32 ppt) were significantly different from one another ($P < 0.025$ for all comparisons). The same post-hoc analysis for temperature showed that the frequency of polyembryony at 30 °C was significantly greater than at 22 °C ($P = 0.002$) and 26 °C ($P = 0.030$), but not different from 28 °C ($P > 0.5$); the frequency of polyembryony at 28 °C was significantly greater than at 22 °C ($P = 0.027$), but no different from either 30 °C ($P > 0.5$) or 26 °C ($P = 0.234$); and the frequencies of polyembryony at 26 °C and at 22 °C were not different from one another ($P > 0.5$).

Following our discovery of polyembryony in *E. parma* and *E. tribuloides*, we tested the hypothesis that salinity treatments were correlated with osmotically induced swelling of the fertilization envelope (FE) following fertilization in each of these species. We found evidence to support this hypothesis in *E. parma* (Fig. 4A), but not in *E. tribuloides* (Fig. 4B). In *E. parma*, there was a highly significant effect of salinity on the FE diameter (one-way ANOVA, $F_{2,\ 6} = 52.665$; $P < 0.001$), while no such effect was noted in *E. tribuloides* (one-way ANOVA, $F_{2,\ 6} = 0.856$; $P = 0.522$).

Discussion

Environmentally induced polyembryony is an unusual developmental pattern in marine invertebrates. Our observations in *Echinarachnius parma* and *Eucidaris tribuloides*

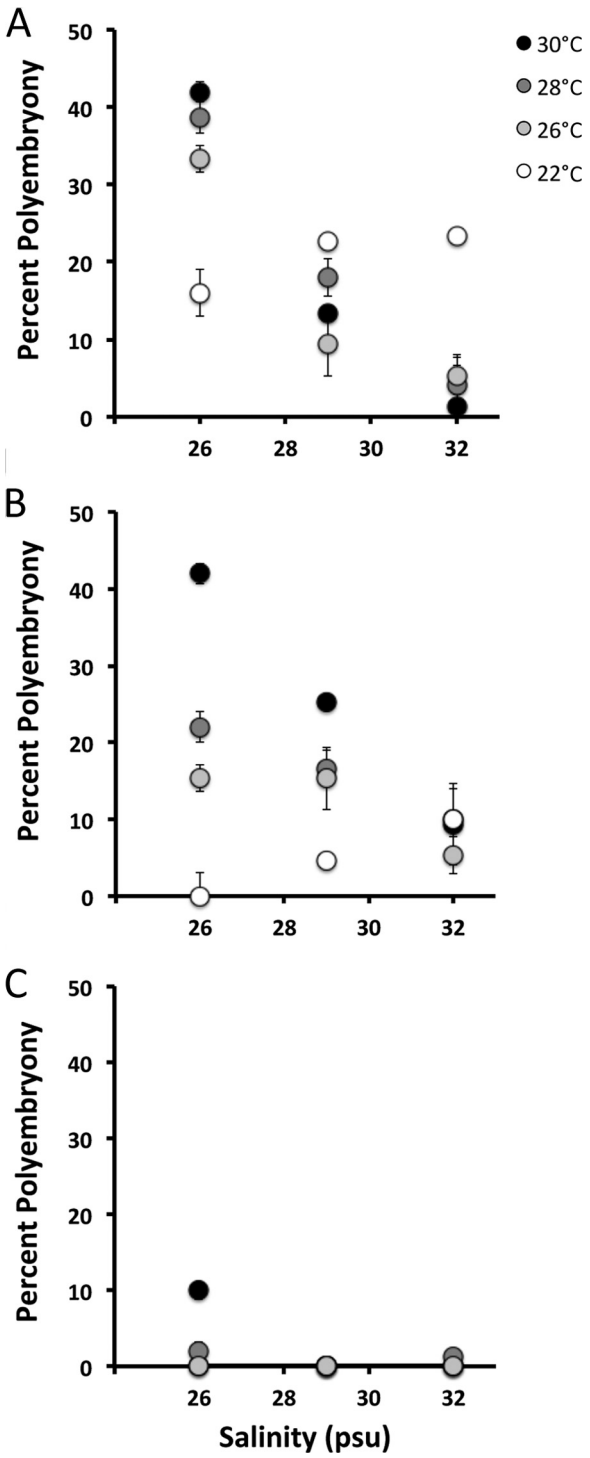

Figure 3. Mean (± SE) percentage of embryos exhibiting polyembryony under different combinations of temperature and salinity in three replicate male-female pairs of *Eucidaris tribuloides* (A–C). There were significant effects noted of the fixed main effects of salinity, temperature, and the interaction of salinity and temperature on the percentage of polyembryonic offspring, even when variance among females was taken into account as a random effect (see text for details).

are similar to the only existing account of this type of polyembryony in the literature: a brief description by Mortensen (1938) of another cidaroid urchin *Prionocidaris baculosa*. It is unclear whether polyembryony is, as Mortensen (1938) suggests, a normal part of development for *P. baculosa*. Two recent reports of development in *P. baculosa* make no mention of polyembryony in this species (Yamazaki *et al*., 2012, 2014), although the collection location of the more recent studies was Japan; Mortensen's (1938) specimens came from Egypt.

It remains unclear whether the production of multiples is an adaptive response to normal environmental fluctuations or a nonadaptive developmental phenotype induced by physiological stressors. The lack of an acclimatization period for embryos or adults in our experiments may have heightened the physiological stressors significantly, and thus affected the degree to which polyembryony occurred. It is similarly unclear to what degree polyembryony occurs in nature. In support of the inference that it does occur in nature, *E. parma* adults experience wide fluctuations in temperature and salinity in their natural habitat on a daily basis. At one of our sites of sand dollar collection, salinity varies by 10 to 15 ppt within a spawning season, and can vary by more than 3.5 ppt in a single day (Allen and Pechenik, 2010). Similarly, sea-surface temperature fluctuations of 10 °C are common over the course of the spawning season; sea-surface temperature can vary by more than 4 °C within a single day (Allen and Pechenik, 2010). While most echinoderms are considered to be stenohaline, coastal echinoids worldwide often encounter, and tolerate, very low salinities (Russell, 2013). For two of the species that we studied, Russell (2013) identified the lowest salinities tolerated by adults as considerably lower than the salinities that induce polyembryony (18 ppt and 14 ppt for *Lytechinus variegatus* and *Strongylocentrotus droebachiensis*, respectively). Russell also reported that the lowest salinities tolerated by larvae were at or below those salinities (26 ppt and 20 ppt, respectively). The lowest tolerable salinities are unknown for *E. parma* and *E. tribuloides*, but, at least for *E. parma*, are well below the threshold for polyembryony (Allen and Pechenik, 2010).

In the laboratory, physiological stress can induce polyembryony in echinoids. For example, Mazia (1958) showed that exposure of fertilized eggs of the sand dollar *Dendraster excentricus* to mercaptoethanol during early development yielded up to 90% twin or quadruplet embryos, depending on the timing of exposure. Similarly, Vacquier and Mazia (1968a) reported that the application of dithiothreitol to fertilized eggs of *D. excentricus* also resulted in a high proportion of polyembryony. However, the application of dithiothreitol failed to induce polyembryony in two species of sea urchins *Lytechinus pictus* and *Strongylocentrotus droebachiensis* (Vacquier and Mazia, 1968b). These results are in agreement with our own findings of consistent

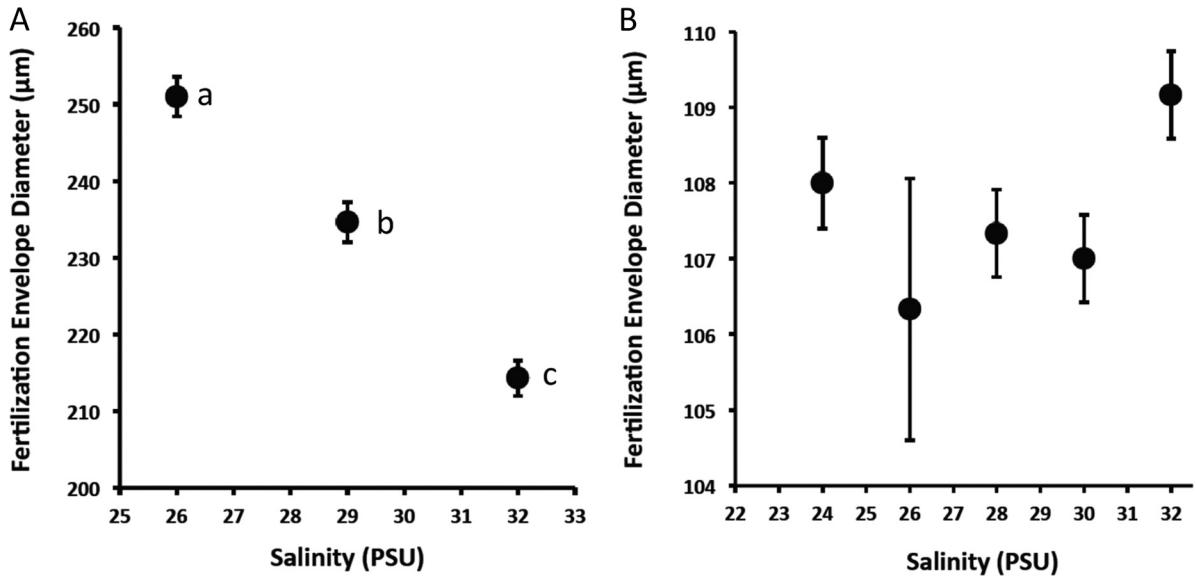

Figure 4. Effect of salinity on the fertilization envelope diameter (FED) of *Echinarachnius parma* (A) and *Eucidaris tribuloides* (B). Each point represents the mean ± SE of 20 fertilized eggs from a single male-female pair. In *E. parma*, there was a significant effect of salinity on FED (ANOVA, $F_{2, 6} = 52.665$; $P < 0.001$), while no such effect was seen in *E. tribuloides* (ANOVA, $F_{4, 10} = 0.856$; $P > 0.9$). Letters adjacent to data points represent significant differences between treatment levels, as determined by Bonferroni-corrected post-hoc tests.

polyembryony in a sand dollar species (*E. parma*), but only rarely in regular echinoids from the same genera studied by Vacquier and Mazia (1968b). Preliminary observations in *D. excentricus* confirm that polyembryony is induced under similar conditions of elevated temperature (20 °C) and reduced salinity (26 ppt), in similar fashion to our results for *E. parma* (Abdel-Raheem and Allen, unpubl. data).

Vacquier and Mazia (1968a, b) hypothesized that the hyaline layer plays a greater role in holding blastomeres together in regular sea urchins than in sand dollars. In support of this hypothesis, disruption of microvillar connections between blastomeres is sufficient to lead to polyembryony in sand dollars (Vacquier and Mazia, 1968a), while the same disruption of microvillar connections in regular sea urchins held together tightly by a hyaline layer results in relatively normal development (Vacquier and Mazia, 1968b). We suggest that, in our experiments, there are two potential mechanisms for breaking cell-cell connections. First, the reduction of Ca^{2+} from seawater at low salinity may, on its own, be sufficient to disrupt cell-cell connections, as has been shown in classic blastomere separation experiments (Horstadius, 1973). Second, the osmotic stress of fertilization in hypotonic seawater may cause the hyaline layer to swell, as has also been shown previously (Dan, 1960). This swelling of the hyaline layer, combined with the fact that cell-hyaline connections are stronger than cell-cell connections early in development (Dan and Ono, 1952), may cause blastomeres to be pulled apart from one another at low salinities. Later cleavage stages exhibit increased cell-cell affinity, suggesting that the window for swollen hyaline layers to affect cell connections may be limited to the early stages (McClay and Fink, 1982).

Our results from the cidaroid urchin *E. tribuloides* further support the role of the hyaline layer in promoting or preventing polyembryony. We chose to work with this urchin because it is in the same family (Cidaridae) as the only other echinoid species in which this type of embryonic polyembryony has been reported (Mortensen, 1938). One benefit of this selection is that *E. tribuloides* lacks a detectable hyaline layer (Schroeder, 1981). The high frequency of twin embryos in *E. tribuloides* suggests that the disruption of cell-cell connections is a sufficient mechanism for polyembryony in this species. While we originally speculated that swelling of the FE may promote polyembryony, it does not appear to be the case for *E. tribuloides* (though it may yet prove to occur in *E. parma*), as the FE diameter did not change with salinity. Interestingly, in one male-female pair, embryos developed naturally in the complete absence of a FE, but showed no greater propensity to twin than did those that remained tightly encased in the FE, as seen in Figure 5. Thus, we saw the frequent formation of both "D" twins (Fig. 5A), whose shape was constrained by the presence of a FE, and "O" twins (Fig. 5B), whose shape was unconstrained in the absence of a FE. This finding also lends credence to the hypothesis that the hyaline layer, not the FE, is the critical substrate on which polyembryony depends.

Regardless of the mechanism, if polyembryony occurs in nature, then the consequences are significant. Twin embryos developed into much smaller larvae, with shorter body and arm lengths. Reduced arm lengths reduce larval feeding

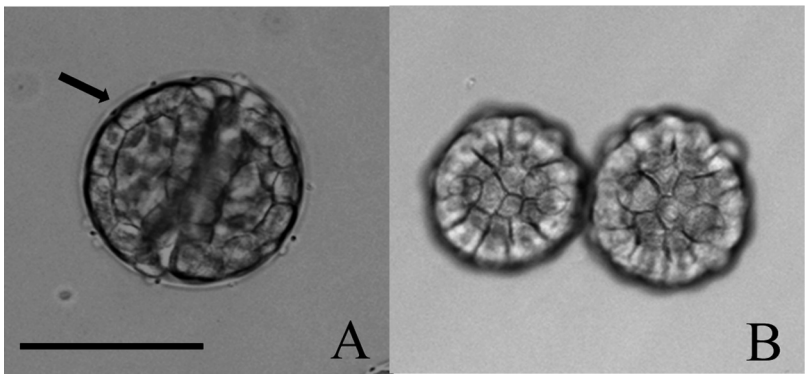

Figure 5. Twinning occurs in *Eucidaris tribuloides* in at least two ways. In A, there is a fertilization envelope (marked with arrow) that constrains the shape of the resulting blastulae to yield a "D"-shaped pair of embryos. In B, the apparent dissolution of the fertilization envelope at low salinity results in "O"-shaped twins that stay in contact with one another until swimming begins, at which point the embryos swim apart. Scale bar = 100 μm.

performance by reducing the length of the ciliary band (Hart and Strathmann, 1994). While we were unable to track larvae derived from multiples through metamorphosis, experimental manipulations of egg size in *Echinarachnius parma* and *Strongylocentrotus droebachiensis* provide strong evidence that changes in larval size and developmental timing will persist through settlement (Alcorn and Allen, 2009). In addition, in *Dendraster excentricus*, we have recently found that both twins derived from a single egg are capable of reaching metamorphosis; a more detailed report of the ecological consequences of natural twinning in that species is forthcoming (Abdel-Raheem and Allen, unpubl. data). More generally, environmentally induced polyembryony will affect both the number of offspring produced and their quality, and may influence future recruitment of benthic marine invertebrates. In particular, the production of clones and multiples is likely to extend larval developmental periods and increase variance in offspring size. The extension of larval development may be adaptive by increasing larval dispersal, but the evidence for extended dispersal as a fitness benefit is weak (Pechenik, 1999; Strathmann *et al*., 2002).

Several lines of evidence suggest that the production of multiples has the potential to be adaptive. First, there appears to be genetic variation in the frequency of polyembryony, as shown by inter-pair variability in polyembryony in the two species in which it was most frequently observed (*Echinarachnius* parma and *Eucidaris tribuloides*). Second, data on environmental variation for intertidal and shallow subtidal animals suggest that there may be strong selection for development to proceed under a wide range of temperature and salinity conditions. Yet polyembryony *per* se may not be the target of selection; rather, it is the continued progression of normal development that is the target, whether as one embryo, two embryos, or possibly more. Thus, even if polyembryony provided no benefit to normal development, it might still be favored over a failure to develop when the environment becomes stressful. Third, one *E. tribuloides* female produced embryos that were more than 20% polyembryonic, even under the presumably unstressful conditions of 22 °C and 32 ppt (Fig. 3A). Larvae of *E. tribuloides* have been successfully reared to metamorphosis at 28 °C (Emlet, 1988) and 25 °C (McPherson, 1968; but for the possibility of culture contamination, see Emlet, 1988). So it is possible that the cooler temperature of 22 °C is a stressful temperature for this warm-water species. However, previous embryological studies of development at 22 °C have not reported polyembryony (Wray and McClay, 1988). The observation of polyembryony in at least 5% to 10% of embryos under all rearing conditions suggests that polyembryony is a frequent developmental phenotype in *E. tribuloides*.

In general, flexible developmental patterns in animals increasingly have been recognized as potentially adaptive strategies rather than mere developmental noise (*e.g.,* Jacobs and Podolsky, 2010). Fluctuations in abiotic factors (temperature and salinity) on the scale of days and/or tidal cycles may be responsible for inducing the unusual developmental patterns reported here, as has been suggested for asexual reproduction in echinoderms more generally (Mladenov, 1996). These induced responses may signal the potential for adaptive responses to long-term environmental changes. Temperatures have been increasing and salinities have been decreasing for decades in the northern Atlantic Ocean (Curry *et al*., 2003), including the Gulf of Maine (Drinkwater *et al*., 2009), where two of the studied species are found. Similarly, there have been large, inter-annual fluctuations in salinity in Florida Bay resulting from variation in precipitation and runoff from mainland Florida, strongly affecting the salinity environment of *E. tribuloides* for at least the last 130 years (Wachnicka *et al*., 2013). The ability of marine organisms to respond to these types of

daily, seasonal, or annual environmental fluctuations is critical for their survival, but has not been well explored.

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