How environment affects the mechanics of development in *Dendraster*

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Abstract

Organisms can develop normally and thrive in dynamic environments. The interplay between the environment and the biomechanics of morphogenesis can help us understand how development operates in a changing world. Effects of salinity on blastula expansion in the sand dollar *Dendraster excentricus* were used to investigate interactions among the environment, development, and mechanics. Salinity fluctuations, which are common in *Dendraster*'s habitat, affect embryonic cell size. The hyaline layer, an extracellular matrix layer closely associated with the outside of the blastula, is hypothesized to resist blastula expansion. Four models describing how the hyaline layer responds to blastula expansion were compared. In three models the hyaline resists expansion as either an elastic, plastic, or viscous layer. In these models, salinity-driven changes in cell size were predicted to cause changes in the ratio of blastocoel volume to cell volume. In a fourth model, the hyaline is a perfectly accommodating layer that allows the embryo to expand freely as cells divide in a single layer. In this model, salinity changes were predicted not to affect the blastocoel-to-cell-volume ratio. To test these hypotheses, *Dendraster* embryos were placed in either 25‰ or 32‰ seawater wells and switched to wells of either the same or the other salinity. In a second experiment, *Dendraster* embryos were raised in 32‰ seawater until the 16-cell stage, at which point a subset of embryos were moved to 25‰ seawater for one cleavage stage and then moved back to 32‰ for the duration of the experiment. In both experiments blastocoel-to-cell-volume ratios were not affected by the salinity treatment. These results suggest that the hyaline layer does not resist blastula expansion in *Dendraster*, thereby maintaining a constant blastocoel-to-cell-volume ratio, despite salinity variation.

Introduction

Early development represents a point in an organism's life where environmental perturbations may have disastrous effects. But early development is also flexible and significant changes are frequently overcome to yield a normal embryo (Kobayakawa & Satoh, 1978). The interplay between environment and mechanics can be used as a way to better understand these crucial developmental mechanisms. Blastula expansion is a process central to early development in many organisms (Gilbert, 2010). In order to survive a variable environment, one would expect the blastula to be able to physically and mechanically develop despite the changes it encounters. We aim to understand the mechanism behind this process by examining the changes in cell and blastocoel volume caused by variation in salinity.

Dendraster excentricus embryos are useful organisms with which to study questions regarding early development due to several features. *D. excentricus* is an abundant echinoid found along the Pacific coast of much of North America (Merrill 1970).. Echinoid embryonic development entails constant exposure to the external environment (Roller & Stickle, 1985; Stickle & Diehl, 1987). Organisms living in the Puget Sound in particular are exposed to variable salinity gradients due to glacial and freshwater runoff (George & Walker, 2007). Sand dollar embryos are spherical and simple in shape, thus the mathematical predictions regarding volume change are easy to make. Eggs are fairly large and transparent, thus cleavage can be easily observed through the embryo. Spawning can be carried out easily and gametes are obtained quickly in large quantities (Strathmann 1987).

Different mechanisms have been proposed to explain the mechanics of blastula expansion, but *Dendraster* demonstrates the same overall developmental processes as other echinoderms. Echinoderm embryos radially cleave leading to a blastula stage. The blastula can be described as a spherical ball of cells with an interior fluid filled space called the blastocoel (Fig. 1; Gilbert, 2010; Wolpert & Gustafon, 1961). An extracellular matrix called the hyaline layer is laid down in echinoderms following fertilization, before the first cleavage (Dan, 1952; Citkowitz, 1971, McClay & Fink, 1982). The hyaline layer can be observed during cleavages as the blastomeres divide and fluid collects in the middle of the embryo, forming the blastocoel (Dan, 1952; Larabell & Chandler, 1991). Dan (1952) described the expansion of the blastula as being osmotic in nature, hypothesizing that large molecules are secreted into the blastocoel leading to the blastocoel swelling osmotically. When blastula from the sea urchin *Strongylocentrotus intermedius* are placed in hypotonic seawater, blastomeres swell and the blastocoel shrinks as a result of a force resisting the expansion of the blastula, causing the blastomeres to swell into the blastocoel (Shinozaki, 1952). Alternatively, Wolpert and Gustafson's (1961) hypothesized that blastula expansion was a cell packing issue.

We proposed four models to describe how the hyaline may or may not be resisting blastula expansion (M. von Dassow, personal communication). Models based on the osmotic mechanism were broken into the elastic, plastic, and fluid models. A cell packing mechanism was also proposed, in which the hyaline does not resist expansion. They have been named as follows: cell packing, elastic, plastic, and fluid. Predications were made for each model for the effects of changing salinity and how this would swell or shrink the cells of the blastula. In three of the following models, it was predicted that a swelling or shrinking action would affect the blastocoel because the blastomeres and blastocoel are occupying the same restricted space.

Cell packing model

The cell-packing model predicts that the expansion of the blastula is the result of the space required to keep the dividing blastomeres in a single cell layer (Wolpert and Gustafson, 1961). The hyaline is totally accommodating and will allow the blastula to expand freely in order to maintain its structural integrity. The ratio of blastocoel to cell volume will not be affected by salinity treatment because the blastula expands or contracts if the cells expand or contract without shape change or rearrangement. Embryos returned to regular seawater will recover in total volume.

Elastic model

The elastic model proposes that the hyaline acts as a spring like layer that will provide some resistance as the blastula expands in a hypotonic seawater solution. The hyaline restricts blastula expansion, so the blastomeres will swell into the blastocoel space. Shinozaki (1952) described a similar mechanism in the sea urchin *Strongylocentrotus intermedius* where the blastomeres swell and the blastocoel shrinks when an embryo is placed in hypotonic seawater. Cell volume will increase as the blastocoel volume decreases. The ratio of blastocoel volume to cell volume will decrease as the embryo swells. If the embryo is raised in a hypotonic solution and dropped to regular seawater, the blastocoel:cell volume ratio will increase as the embryo shrinks. This ratio will not be affected if the embryo is initially raised in regular seawater, swelled in a hypotonic solution for a short time, and moved back to regular seawater again because an elastic material will return to its original size and shape once the force causing the deformation is removed.

Plastic model

In the plastic model, the hyaline is a layer that will provide some resistance on the expanding blastula. As the blastocoel swells osmotically, the force pushes on the hyaline until a threshold is reached and the blastula expands. The hyaline will continue to expand in this model until the osmotic pressure is no longer great enough to exert a stress greater than the yield stress. At a swollen state, the plastic model appears like the cell packing model and predicts a blastocoel:cell volume ratio that will not be affected by different salinity treatments. Once embryos are placed in regular seawater, the ratio between blastocoel and cell volume is expected to increase. As the cells shrink, the blastula will increase in volume because the hyaline does not exert a spring like force to contract the blastula.

Fluid model

The fluid model proposes that the hyaline is a viscous, honey-like layer that will provide resistance against the blastula as it expands when it is placed in hypotonic seawater. The hyaline will expand slowly, thus as the blastomeres swell, there will be a decrease in blastocoel volume. The blastocoel to cell volume ratio will decrease at a swollen state. When the embryo is moved to regular seawater, the ratio between the blastocoel and cell volumes will increase. The cell volume will recover but the hyaline will not provide a force back on the embryo shrinking it back to its original size, so the blastocoel volume will not recover.

Methods

Dendraster excentricus specimens were collected at low tide from Crescent Bay on the East Sound in Orcas Island, WA during May and June 2012. Sand dollars were transported to Friday Harbor Labs and housed in running seawater tanks.

Roller and Stickle (1985) found that at salinities lower than 25‰, developmental rates were slow enough to indicate significant future larval mortality in other echinoderm species native to San Juan Island. A brief experiment was conducted to verify the range of tolerable salinities. 25‰ was found to be the lowest salinity that yielded mostly healthy embryos.

Following the procedures described by Strathmann (1987), *D. excentricus* individuals were given 1-2 ml coelomic injections of 0.53M KCl to induce spawning. Females were inverted over a beaker filled with seawater to collect eggs. Eggs were then concentrated by transferring them to a conical tube and letting them settle. Males were placed upright in a dish with seawater and sperm was collected with a pipette. 50 μl of eggs were added to wells in twelve well plates. 100 μl of eggs were added in six well plates. A drop of sperm was diluted in 50 ml of filtered seawater. 40 or 80 μl of this solution was added to each well for 50 μl and 100 μl of eggs respectively.

Local seawater was measured with a refractometer to have a varying salinity of 27 to 32‰. Salinity was measured every day and always before making new solutions. When seawater was found to be below 32‰, NaCl was added until the desired 32‰ salinity was reached. This solution was diluted with reverse osmosis purified water to a salinity of 25‰. 5 ml of seawater solution was added to wells in twelve well plates. 10 ml of seawater solution was added in six well plates. Well plates were kept covered in a 20°C water bath for the duration of each experiment.

Previous authors have shown that salinity has an effect on developmental rate in other echinoderms (Roller and Stickle, 1984). Preliminary experiments suggested that up to a 10% difference in developmental rate existed between embryos raised in 32‰ versus 25‰ salinity. Therefore, embryos cultured for long periods of time in varying salinity were not compared to each other, but we were able to compare embryos cultured for short periods in different salinities.

Two experiments were performed to try and discern the mechanical processes at work in blastocoel formation. For both experiments, pictures were taken in brightfield with a Moticam 2500 5.0 megapixel camera on a Nikon TE2000 DIC scope with a 20x objective and 1.5x intermediate magnification. The widest section across the embryo was first measured, followed by another diameter measurement made perpendicular to the first at the midpoint (Fig. 1). For embryos at a 32 cell stage or later, the widest section across the blastocoel was measured followed by another perpendicular measurement at the midpoint. Diameter measurements were made with ImageJ software. A micrometer was photographed at the same power and intermediate magnification so that a scale in µm could be obtained. These diameters were converted to µm, averaged and used to find the entire embryo and blastocoel volume by approximating the embryo and blastocoel as spheres.

Experiment 1: Salinity switch

The first experiment performed was a simple salinity switch where embryos were moved once between 25‰ and 32‰. This experiment aimed to examine how salinity treatment affected embryo swelling and shrinking responses. 50 μl of embryos were taken from their 25‰ seawater well and switched to 32‰, four hours after fertilization. This was repeated with embryos in 32‰ seawater, which were switched to 25‰ water. As a control, 50 μl of embryos were kept in their starting salinities for the duration of the experiment. Pictures were taken of the embryos at three different time points: 15 minutes, four hours after fertilization, and two hours after the salinity treatment switch (six hours after fertilization).

Experiment 2: Salinity switch back

This was followed by another experiment in which the embryos were switched between salinities, and then switched back to test the ability of the embryos to recover. 100 μl of embryos were taken from 32‰ seawater wells and switched to 25‰ at the 16 cell stage and then switched back to their original salinities once at 32 cells. A control group of embryos were switched to 32‰ at the same cleavage points. Six embryos were photographed in each well. Pictures were taken at the 16 cell stage five minutes after the switch, 32 cell stage before the switch, 32 cell stage 10 minutes after the switch, at the 64 cell stage, and approximately 2 and a half hours after the switch back to 32‰ (six hours post fertilization).

Salinity and developmental rate

Salinity and temperature have been observed to cause detectable differences in developmental rate in echinoderms (Roller and Stickle, 1984). We tested whether culturing in 25‰ seawater affected the rate of development in *Dendraster*. 100 µl of embryos were pipetted into wells with either 32‰ or 25‰ seawater. Embryos were observed starting at 2nd cleavage every ten to fifteen minutes until the 5th cleavage was observed. Embryos were observed again approximately eight hours after fertilization for the presence of cilia. During each observation, the following were counted in a field of 15 embryos: dead/non-cleaving embryos, embryos before the cleavage stage, cleaving embryos, and embryos after the cleavage stage. This was repeated so that a total of 30 or more embryos were counted at each observation.

Data analysis

A two-way analysis of variance (ANOVA) was used to examine the difference between treatment groups. SPSS software was utilized to carry out these analyses. Cell volume and blastocoel:cell volume ratio were treated as the dependent variables. Salinity treatment was selected as a fixed factor and the egg clutch and egg clutch by salinity interaction were random factors.

Results

Salinity and developmental rate

Development in 32‰ seawater was found to be approximately 10% faster than in 25‰ seawater. This difference was considered to be great enough to treat the 25‰ and

32‰ embryos separately and perform switches at a certain cleavage point rather than a time point. Four clutches were tested in the developmental rate experiment. Clutches varied slightly from batch to batch in regards to overall timing of developmental events in the course of an experiment. But despite this timing variability, eggs raised in 32‰ were usually found to be developing at a faster rate.

Experiment 1; Salinity switch

The salinity switch aimed to examine how the blastula would swell or shrink in response to a salinity treatment in which embryos were moved between 25‰ and 32‰ seawater solution after four hours at an early blastula stage.

A two-way analysis of variance (ANOVA) was used to examine a difference between salinity treatments in the two treatment groups of embryos that either started in 32‰ seawater or embryos that started in 25‰ seawater (Tables 1 and 2). Embryos initially raised in 32‰ seawater were not compared to embryos initially raised in 25‰, due to a possible difference in developmental rate (Roller and Stickle, 1984)

For all blastula expansion models, a difference in cell volume was expected between treatments (Fig. 2). A statistically significant effect of salinity on cell volumes was found in the embryos initially raised in 25‰ (p=0.049) and embryos initially raised in 32‰ (p=0.026). In embryos initially placed in regular seawater, cell volume was found to be greater in embryos switched to 25‰ salinity than embryos that remained in 32‰ seawater. Similarly, embryos initially placed in 25‰ seawater and switched to 25‰ seawater were found to have a greater cell volume than embryos that were moved from

25‰ to 32‰ seawater. These results verify that the experimental procedure was effectively swelling cells placed in a hypotonic seawater solution.

No significant difference was found between the 32‰ and 25‰ embryos for the blastocoel to cell volume ratio (Fig. 3). A value of *p*=0.097 was found between treatment groups in embryos that were initially placed in a 32‰ well. A value of *p*=0.857 was found between treatment groups in embryos initially placed in a 25‰ well.

Experiment 2; Salinity switch back

The salinity switch back experiment examined the ability of the embryos to recover from swelling. Embryos were first raised in 32‰ seawater and then moved to 25‰seawater, and back to 32‰ seawater again.

Differences between treatment groups were analyzed with ANOVA for four of the data sets: 32 cell stage before the switch back to 32‰, 32 cell stage 10 minutes after the switch back to 32‰, 64 cell stage (next cleavage), and six hours after fertilization (Table 3 and 4).

A difference in cell volume was found between 25‰ seawater and 32‰ seawater treatment groups for embryos pictured at the 32 cell stage before they moved from 25‰ to 32‰ (*p*=0.025) (Fig.4). Differences in cell volume were not statistically significant at ten minutes after the switch back (*p*=0.961), one cleavage later at 64 cells (*p*=0.065), and at a later point six hours after fertilization or 2-3 hours after the last switch (*p*=0.217). This again showed that the hypotonic seawater solution of 25‰ causes the cells to swell and that cell volumes recover quickly after being returned to 32‰ seawater.

Differences in blastocoel to cell volume ratio were not statistically significant between 32‰ and 25‰ seawater treatment groups at the 32 cell stage before the switch back (p=0.844), ten minutes after the switch back (p=0.568), and at a later point six hours after fertilization or about 2-3 hours after the last switch (p=0.217) (Fig. 5). Embryos observed one cleavage after the switch back at 64 cells were found to have a difference between treatment groups with a value of p=0.039, where embryos that had been raised in only 32‰ were found to have a greater blastocoel to cell volume ratio than embryos that had been switched from 32‰ to 25‰ seawater.

Discussion

The world provides a constantly changing environment that impacts the mechanics and function of the organisms that live in it. It is evident that the mechanisms of development interact with the changing environment in some way because we see organisms continue to live.

Four models were proposed to explain how the hyaline resists blastula expansion when embryos are exposed to changes in salinity. All three models based on hyaline resistance were rejected, while the cell-packing model was not. In the three hyaline resistance models (elastic, plastic, and fluid) the blastocoel to cell volume ratio is expected to be affected by the salinity treatment in at least one, if not both, of the experiments showing shrinkage and recovery. However, this study shows that the blastocoel to cell volume ratio was unaffected by salinity treatments in both experiments. The maintenance of constant blastocoel to cell volume ratio despite swelling of

Dendraster excentricus blastula in a hypotonic solution is consistent with the cell packing model.

In the second experiment, the salinity switch back, embryos observed one cleavage after the switch back at 64 cells were found to have a significant difference in blastocoel to cell volume ratio between treatment groups. Embryos raised in only 32‰ were shown to have a greater blastocoel to cell volume ratio than embryos switched from 32‰ to 25‰ salinity. These results are not consistent with the plastic or viscous models, which would have predicted a difference in ratios for this experiment, but with embryos that had been switched to 25‰ having a greater blastocoel to cell volume ratio.

The expansion of the blastula is not necessarily due to osmotic pressure acting against mechanical resistances provided by the hyaline. Dan (1952) showed that blastomeres are adhered to the hyaline during development. Later, Wolpert and Gustafson (1961) hypothesized that as the cells divide, the blastula continues to expand in order to maintain a single cell layer and cell-cell adhesion.

The cell packing model was one of only two models that predicted total volume recovery. Given that the cell packing mechanism of blastula expansion maintains a constant blastocoel to cell volume ratio despite changes in salinity, this might help maintain consistent swimming performance. Supposing volume recovery is related to embryo recovery, the predictions made by this model are important to consider in terms of embryo survival and dispersal. Many factors affecting swimming and movement in water are related to the radius of the blastula, such as gravity, buoyancy, and stability (McDonald, 2012). Changes to the blastula radius due to swelling caused by fluctuating salinity have the potential to change any of these factors in a way that affects swimming and dispersal. Grunbaum and Strathmann (2003) showed that differences in larval morphology also affect swimming patterns and stability in planktonic larval forms. The capacity for embryo recovery and development of a normal larval form would presumably allow the organisms to swim and disperse normally in its habitat as well.

It would be worthwhile to follow development in embryos that were at some point swelled in hypotonic seawater solution. Though cell packing predicts a volume recovery, changing salinity can also have affects on embryo biochemistry. George and Walker (2007) investigated the effects of low and fluctuating salinity on *Dendraster* larvae. They showed that exposure to low salinity had an effect on developmental rate, growth, and metamorphosis. Larvae that were exposed to low or fluctuating salinity were able to develop to metamorphic competency, but only larvae that had been exposed to fluctuating salinity in the short term were able to metamorphose (Walker & George, 2007). Future morphological deformities could form as development proceeds with an even earlier exposure to low salinity.

This present study showed that the cell packing model is supported as the mechanism for expansion in *D. excentricus*. Blastula expansion has been described several other studies and in at least three species of echinoderm. These other echinoderm studies have shown different results and likely different mechanisms for blastula expansion. For example, Shinozaki (1952) examined blastula expansion in the sea urchin *Strongylocentrotus intermedius* and showed that the blastocoel decreased in volume when blastula swelled in response low salinity, indicating a change in blastocoel to cell volume ratio consistent with osmotic swelling against an elastic resistance. If this mechanism is related to embryo survival, *Dendraster* might have evolved to expand in this particular way so that embryos were able to normally develop in a changing environment and habitat. If so, a further study of related sand dollar and echinoderm species would be useful for studying how blastula expansion mechanisms evolved.

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Figures and Tables

Figure 1

Blastulae were measured four times. The black arrows show total blastula diameter measurements. Red arrows show blastocoel diameter measurements.

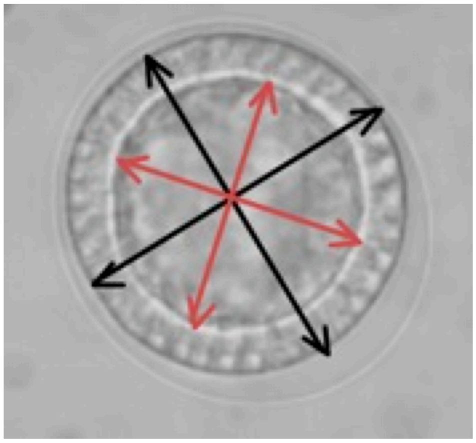


Figure 2
Embryos in 25‰ seawater were greater in volume than embryos treated in 32‰ seawater. Egg clutches from independent female *Dendraster* individuals are separated due to clutch variation. The cell volume in um^3 is shown on the y-axis. **(a)** Embryo*s* initially treated in a 25‰ salinity treatment. 25-25 embryos remained in 25had trou‰ salinity. 25-32 embryos were moved from 25‰ to 32‰. **(b)** Embryo*s* initially treated in a 32‰ salinity treatment. 32-32 embryos remained in 32‰ salinity. 32-25 embryos were moved from 32‰ to 25‰.

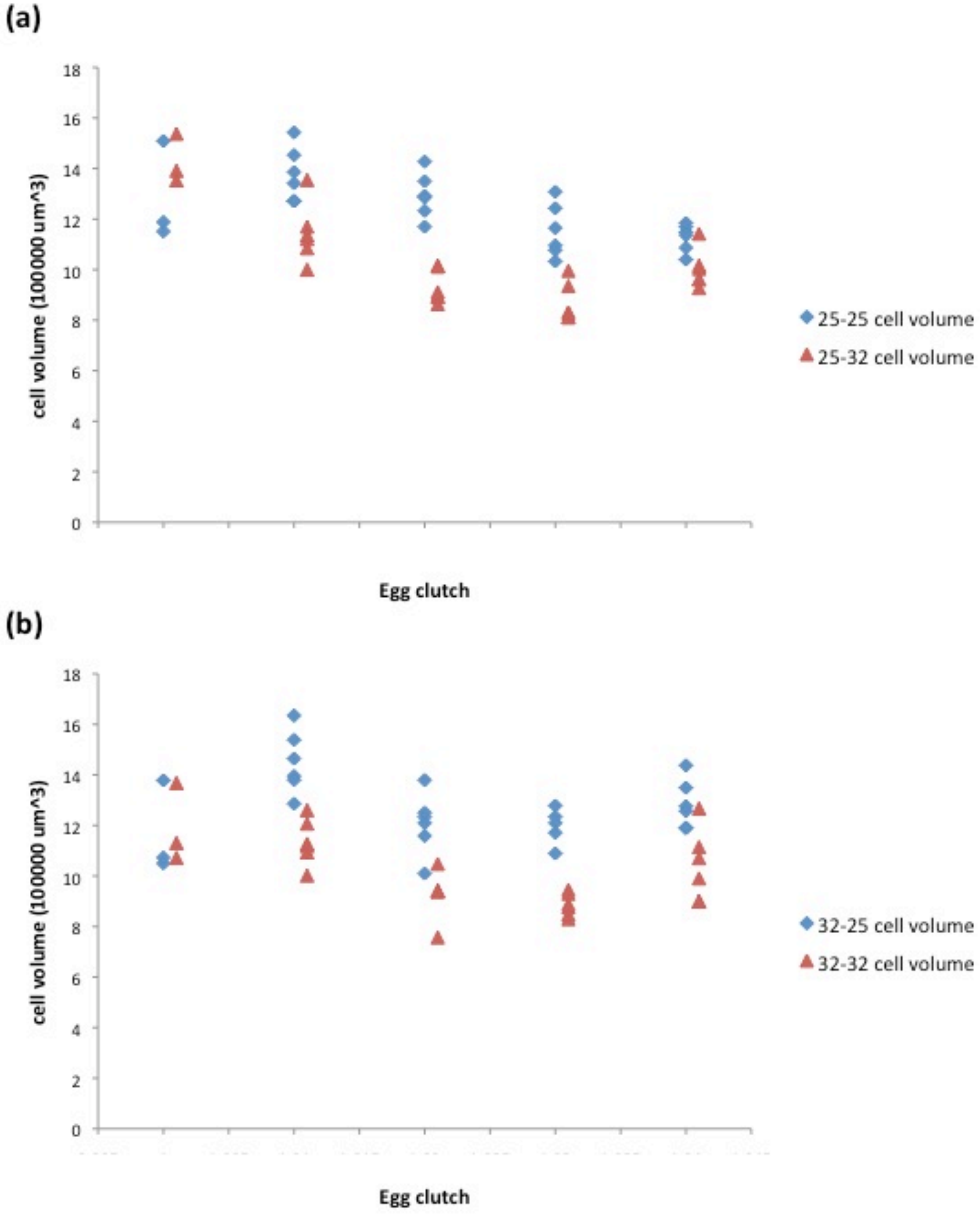


Figure 3

Blastocoel to cell volume ratio was not affected by salinity treatment. Egg clutches from independent female *Dendraster* individuals are separated due to clutch variation. The ratio of blastocoel to cell volume ratios is shown on the y-axis. **(a)** Embryo*s* initially treated in a 25‰ salinity treatment. 25-25 embryos remained in 25‰ salinity. 25-32 embryos were moved from 25‰ to 32‰. **(b)** Embryo*s* initially treated in a 32‰ salinity treatment. 32-32 embryos remained in 32‰ salinity. 32-25 embryos were moved from 32‰ to 25‰.

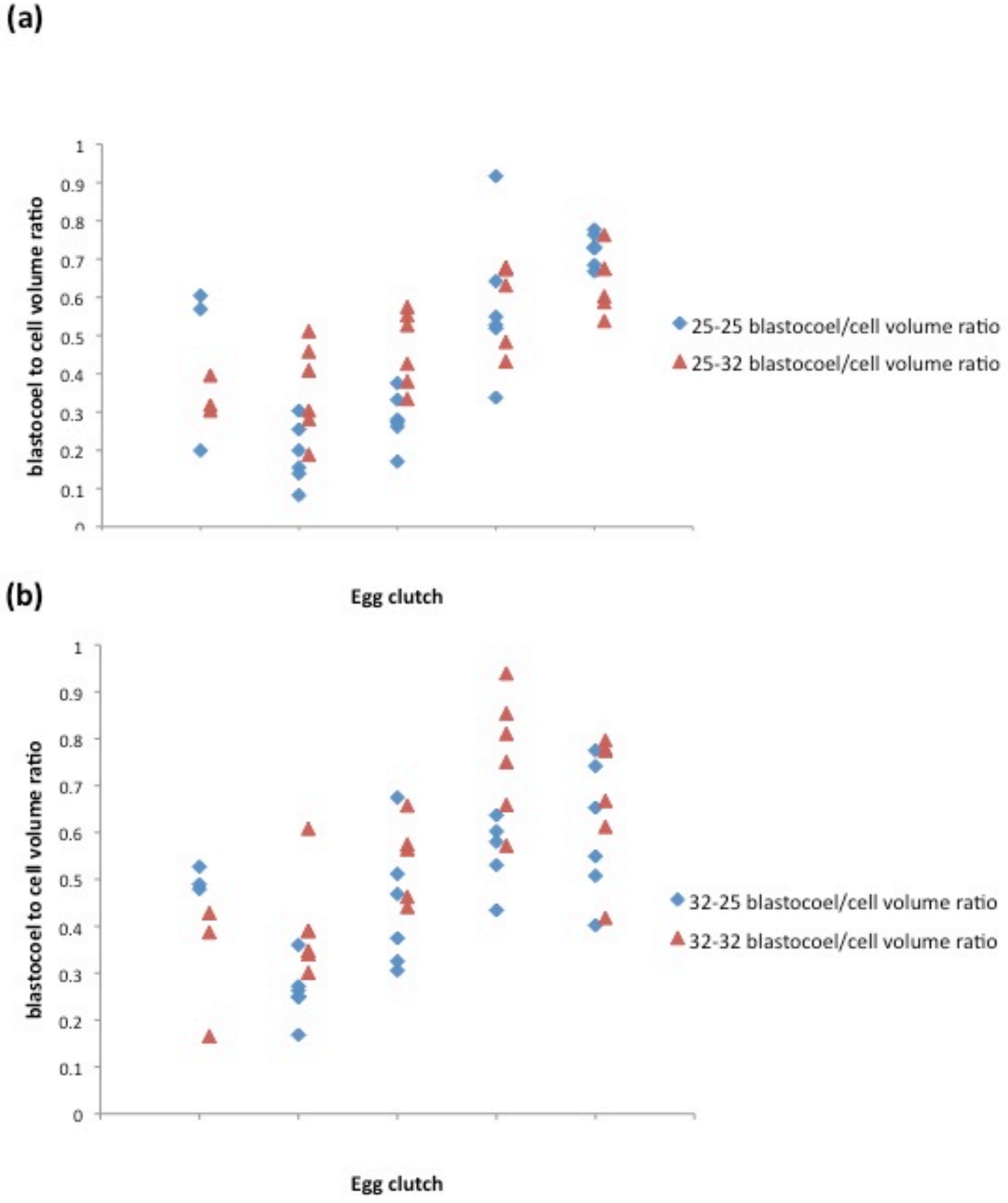


Figure 4

Cell volume recovers after embryos are switched back to 32‰. Egg clutches from independent female *Dendraster* individuals are separated due to clutch variation. The cell volume in um^3 is shown on the y-axis. 32-25-32 are embryos that were switched to 25‰ at the 16 cell stage. 32-32-32 embryos developed in only 32‰ for the duration of the experiment. **(a)** Cell volumes found at the 32 cell stage, before embryos were switched from 25‰ to the final 32‰ well. **(b)** Cell volumes found at a 32 cell stage, ten minutes following the switch to the final 32‰ well. **(c)** Cell volumes found at a 64 cell stage after the final switch **(d)** Cell volumes found at six hours after fertilization. This was approximately two and half hours after the switch back to 32‰.

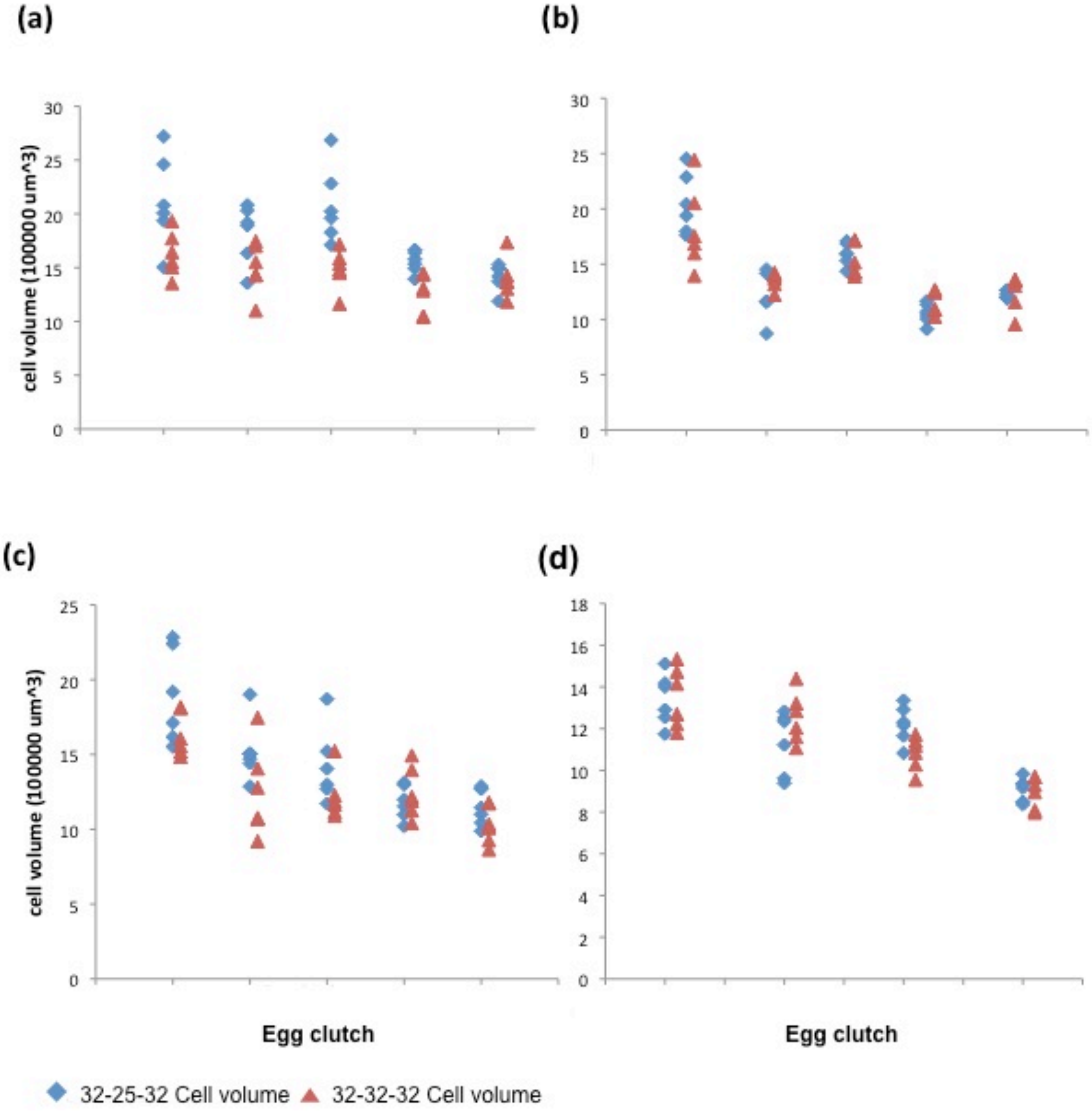
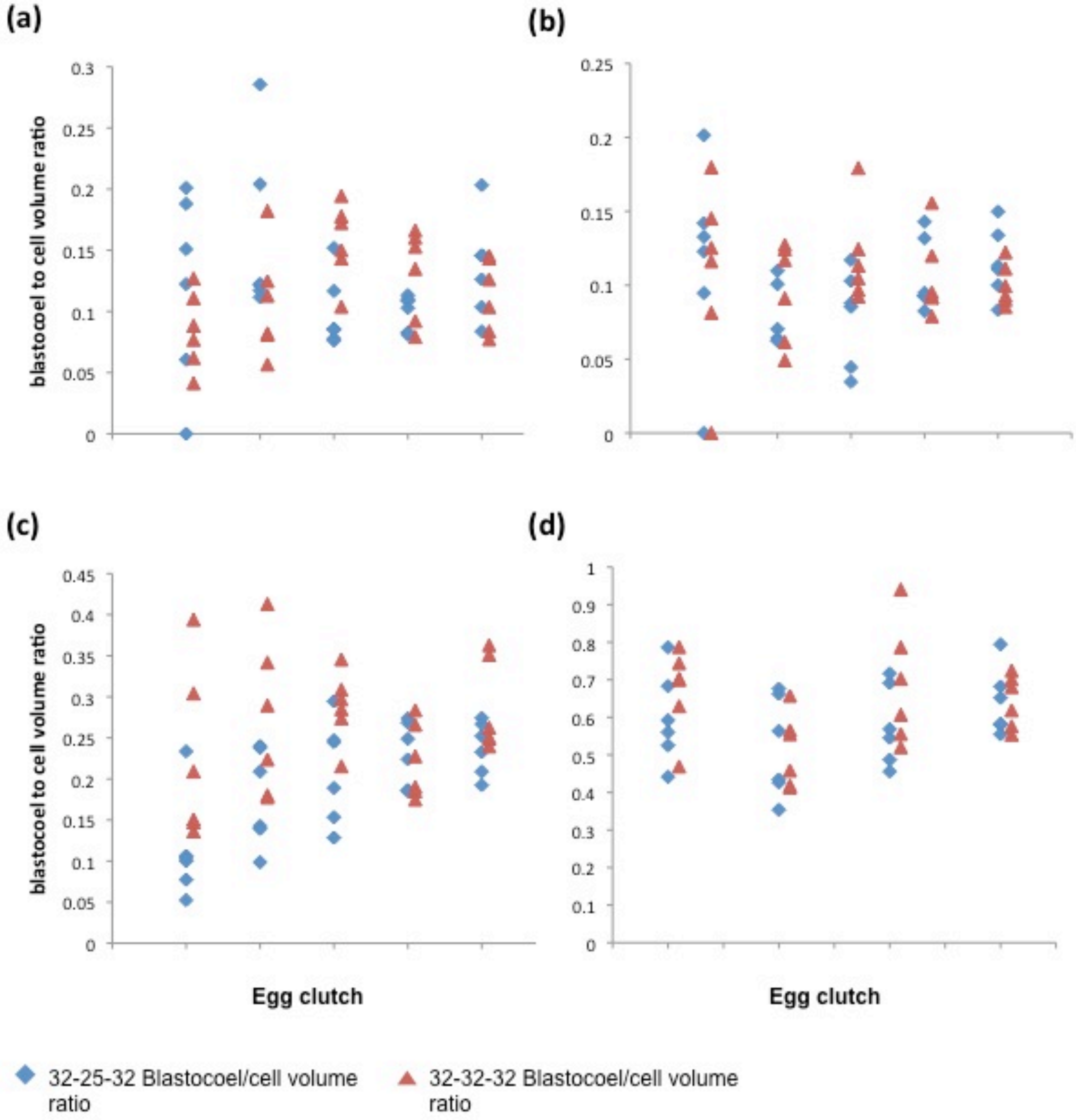


Figure 5

Blastocoel to cell volume ratio did not change with salinity treatments. Egg clutches from independent female *Dendraster* individuals are separated due to clutch variation. The blastocoel to cell volume ratio is shown on the y-axis. 32-25-32 are embryos that were switched to 25‰ seawater at the 16 cell stage. 32-32-32 embryos developed in only 32‰ seawater for the duration of the experiment. **(a)** Ratios found at the 32 cell stage, before embryos were switched from 25‰to the final 32‰well. **(b)** Ratios found at a 32 cell stage, ten minutes following the switch to the final 32‰well. **(c)** Ratios found at a 64 cell stage after the final switch **(d)** Ratios found were six hours after fertilization. This was approximately two and half hours after the switch back to 32‰.



Tables 1: ANOVA for experiment 1: the salinity switch. P and F values

Experiment 1 Salinity switch Initial salinity	Measurement	Salinity p value (F__.__=___)	Clutch p value (F__.__=___)	Salinity*Clutch p value (F__.__=___)
Embryos initially raised in 25‰	Cell volume	p = 0.049 $F_{1,4.108} = 7.658$	p = 0.128 $F_{4,4} = 3.471$	p = 0.000 $F_{4,44} = 6.434$
Embryos initially raised in 32‰	Cell volume	p = 0.026 $F_{1,4.083} = 11.735$	p = 0.220 $F_{4,4} = 2.302$	p = 0.005 $F_{4,44} = 4.341$

Tables 2: ANOVA for experiment 1: the salinity switch. P and F values

Experiment 1 Salinity switch Initial salinity	Measurement	Salinity p value (F__.__=___)	Clutch p value (F__.__=___)	Salinity*Clutch p value (F__.__=___)
Embryos initially raised in 25‰	Blastocoel to cell volume ratio	p = 0.857 $F_{1,4.342} = 0.037$	p = 0.075 $F_{4,4} = 4.965$	p = 0.001 $F_{4,44} = 5.990$
Embryos initially raised in 32‰	Blastocoel to cell volume ratio	p = 0.097 $F_{1,4.089} = 4.444$	p = 0.018 $F_{4,4} = 11.631$	p = 0.253 $F_{4,44} = 1.394$

Table 3 ANOVA for experiment 2: the salinity switch back. P and F values

Experiment 1 Salinity switch Initial salinity	Measurement	Salinity p value (F__.__=___)	Clutch p value (F__.__=___)	Salinity*Clutch p value (F__.__=___)
32 cell stage Before switch to 32‰	Cell volume	p = 0.025 $F_{1,4} = 12.133$	p = 0.113 $F_{4,4} = 3.780$	p = 0.061 $F_{4,50} = 2.418$
32 cell stage 10 minutes after switch back to 32‰	Cell volume	p = 0.961 $F_{1,4.005} = 0.003$	p = 0.006 $F_{4,4} = 20.536$	p = 0.143 $F_{4,49} =1.806$
64 cell stage Next cleavage stage	Cell volume	p = 0.065 $F_{1,4} = 6.350$	p = 0.013 $F_{4,4} = 13.700$	p = 0.254 $F_{4,50} = 1.380$
6 hours after fertilization (2-3 hours after switch)	Cell volume	p = 0.919 $F_{1,3} = 0.012$	p = 0.035 $F_{3,3} = 12.103$	p = 0.054 $F_{3,40} = 2.769$

Table 4 ANOVA for experiment 2: the salinity switch back. P and F values

Experiment 1 Salinity switch Initial salinity	Measurement	Salinity p value (F__.__=___)	Clutch p value (F__.__=___)	Salinity*Clutch p value (F__.__=___)
32 cell stage Before switch to 32‰	Blastocoel to cell volume ratio	p = 0.844 $F_{1,4} = 12.133$	p = 0.887 $F_{4,4} = 3.780$	p = 0.017 $F_{4,50} = 2.418$
32 cell stage 10 minutes after switch back to 32‰	Blastocoel to cell volume ratio	p = 0.568 $F_{1,4.009} = 0.003$	p = 0.634 $F_{4,4} = 20.536$	p = 0.430 $F_{4,49} = 1.806$
64 cell stage Next cleavage stage	Blastocoel to cell volume ratio	p = 0.039 $F_{1,4} = 6.350$	p = 0.220 $F_{4,4} = 13.700$	p = 0.177 $F_{4,50} = 1.380$
6 hours after fertilization (2-3 hours after switch)	Blastocoel to cell volume ratio	p = 0.217 $F_{1,3} = 0.012$	p = 0.120 $F_{3,3} = 12.103$	p = 0.540 $F_{3,40} = 2.769$