

Salinity Fluctuation's Effect on *Dendraster excentricus* Developmental Defects

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Abstract

The goal of this study was to understand how the environment affects the physics of morphogenesis. We wanted to know how salinity, a naturally fluctuating factor for all marine life, affects the sensitivity of sand dollar *Dendraster excentricus* embryos to inducers of exogastrulation, a developmental defect in which the archenteron evaginates instead of invaginates. We based our predictions on the fact that salinity either swells or shrinks cells. We hypothesized that if the apical extracellular matrix resists cell swelling, the pressure within the embryo will increase and so will the tendency to evaginate. However, if the blastocoel resists expansion of the embryo, the pressure will drop and promote invagination. If the blastocoel and the apical extracellular matrix accommodate the expanding embryo then the internal pressure will not change and therefore should not affect the sensitivity to exogastrulation. Exogastrulation can be induced by exposure to various conditions; a calcium deficient environment is one of them. We observed embryos in low calcium (10% of normal seawater) artificial seawater and its effects in normal salinity (32 ppt) and low (25 ppt) salinity, which is a range that these sand dollars are exposed to in nature. Preliminary studies suggest that low salinity conditions alone did not affect the sensitivity to exogastrulation, but further studies could reveal more about salinity's effect. The blastocoel to cell volume ratio among treatments were similar, which is consistent with the hypothesis that the blastocoel expanded freely with the cell layer.

Introduction

All organisms develop in a dynamic environment. Any environment, whether it's terrestrial or aquatic, is never constant and fluctuates in various manners. Salinity fluctuation is an important factor, which marine organisms must tolerate during development (George & Walker 2007). However, if the salinity fluctuation exceeds the organism's tolerance threshold, the success rate of development decreases and the chances of abnormal development increase (Roller & Stickle 1985). Many developmental defects are hard to overcome and can reduce their chance of survival and ultimately leaving offspring. Better understanding developmental abnormalities' response to environmental factors, such as salinity, can provide insight towards the underlying mechanism of the defect.

Echinoderms have been used to study many developmental processes because of many ideal characteristics. We used *Dendraster excentricus* to study the effects of fluctuations in salinity on development because they are found along the Puget Sound area where the salinity can vary between 25 ppt and 32 ppt (FHL weather station). Moreover, *D. excentricus* is found in the intertidal zone in which the salinity fluctuates due to a larger amount of freshwater runoff. Their embryos make them an ideal developmental model because they have a simple geometry making mathematical approximations possible. The embryos are also transparent so that one can monitor their developmental stages without difficulty. Lastly, their gametes are accessible in large quantities to allow for adequate sample sizes.

The developmental process known as gastrulation is common to both vertebrates and invertebrates and involves extensive tissue movement to achieve embryonic reorganization (Hardin & Cheng 1986). Gastrulation in echinoderms is a two step process.

The first step, *primary invagination*, is when the vegetal plate of the blastula dimples inward and begins the formation of the archenteron. The next step, *secondary invagination*, is when the pocket formed by the initial dimple turns into the archenteron tube which eventually connects to the other end of the embryo and creates the mouth and the larval gut (Hardin & Cheng 1986).

However, under certain environmental conditions, embryos can develop abnormally during gastrulation. One study showed that a developmental defect known as exogastrulation can be induced in a calcium deficient environment in the sea urchin, *Psuedocentrotus depressus* (Okazaki 1956). Exogastrulation is when the archenteron ends up on the outside as opposed to within the embryo. This developmental defect is also inducible by other environmental conditions such as exposure to lithium chloride, tar compounds, certain pesticides, and tobacco smoke, which makes this defect especially interesting (Child 1940; Child 1948; Mwatibo & Green 1998; Pillai et al. 2003; Vacquier 1971). With so many known pollutants that can stimulate this particular defect, it is likely that there are other pollutants in the environment that can induce this same defect. Understanding the mechanism of this abnormal developmental defect can shed light upon the mechanism of how successful gastrulation occurs. Many studies have already been done on hypothesized mechanisms for gastrulation (Kimberly & Hardin 1998; Davidson et al. 1995).

We based our first hypotheses on the change in blastocoel and cell layer volume (M. von Dassow, personal communication). When cells are placed in hypotonic solutions, they swell due to osmotic pressures. However, we did not know whether the blastocoel would react in the same manner in the hypotonic solution because it is not

known whether the epithelium is permeable or not. Thus, if the blastocoel is permeable to the salts and will not expand at the same rate as the cell layer we predicted that the embryo will buckle inward and invaginate successfully because the blastocoel is essentially sucking in the cell layer. If the blastocoel isn't permeable to salts and will expand at a similar rate as the cell layer we predicted that the apical extracellular matrix (ECM), which surrounds the entire embryo, will restrict expansion because it is significantly stiffer than the cells itself and therefore the embryo will want to buckle outward during gastrulation (Davidson 1999). Essentially, we predict that if the blastocoel is expanding in a hypotonic solution similar to the cell layer, then pressure within the embryo will build because the apical ECM will be restricting the expansion. Thus, successful invagination will be unlikely because doing so would increase the pressure and so buckling outward will be more likely, to alleviate the pressure buildup.

For the next hypothesis, a few mechanisms proposed in previous studies regarding gastrulation were taken into consideration. Davidson studied a model known as the apical constriction model (1995). This model states that the cell layer's apical end will constrict and the basal end will expand to form wedge-shaped cells. This non-uniform shape allows the vegetal plate to buckle inward. However, in a hypotonic solution the cells will swell and that will make it harder for cells to contract their apical ends because their ability to deform are inhibited. Imagine a basketball that has the maximum amount of air and one that is only filled with half its capacity. The ball that has the maximum amount of air is very difficult to deform, while the one that isn't maximized can deform more easily. Thus, we predict outward buckling to be more likely when placed in a low salinity environment and when the inducers of exogastrulation are present.

Davidson studied another model known as the apical ring contraction model where a ring composed of contracting cytoskeletal elements run through the apical end of a ring of cells on the vegetal plate. At the beginning of gastrulation, this ring contracts and compresses the cells in the center of the ring and causes the vegetal plate to buckle upward. For this model, we hypothesize that if the embryos are placed in a hypotonic solution and exposed to exogastrulation inducers the embryo will buckle outward. If the cells are swelling, the ring will require more force to contract the now enlarged central cells. The cells in the center provide resistance to the ring. If the cells were larger within the ring the resistance would increase and if that resistance is beyond the ring's capabilities to contract, then the vegetal plate will buckle in less and be more susceptible to exogastrulation. Thus, if the embryo is exposed to exogastrulation inducers, a low salinity environment will increase the sensitivity to exogastrulation.

Kimberly and Hardin proposed a similar mechanism in which a group of cells in the shape of a ring are found on the vegetal plate (1998). These cells, which were termed bottle cells, contract their apical ends, similar to the apical contraction model, and they claim that the shape changes of these cells in the shape of a ring cause primary invagination. Our hypothesis for this mechanism is that if placed in a hypotonic solution the cells will swell and that will prevent the bottle cells from contracting their apical ends and therefore prevent successful invagination. There are many other hypothesized models, however we were not able to make predictions for them.

To test how salinity affects the sensitivity to the exogastrulation defect, embryos were placed in treatments that varied calcium and salinity independently and analyzed

how the archenteron length changed. Calcium-deficient concentrations were used because they are known to induce this particular defect.

Methods

Animal collection and embryo handling

Dendraster excentricus were collected on East Sound, Orcas Island, WA between the months of June and July 2013. The methods of spawning and embryo handling used were similar to protocol from Strathmann (1987). Gametes were collected by injecting 1-2 ml of 0.5 M KCl near the mouth of a ripe *D. excentricus* to induce spawning. Sperm were collected via pipette from the top of the sand dollar and was then a drop of sperm was diluted into 50-80 ml of filtered sea water. Eggs were collected by inverting the female over a beaker or custard dish. Eggs were fertilized by adding about 10 drops of diluted sperm in a custard dish with a single layer of eggs. The eggs were then placed in a water bath set at 11-12° C for 20 hours until they hatched through their fertilization envelope.

Treatment solutions & Preliminary test

Preliminary tests were conducted to determine the desired calcium concentration for *Dendraster excentricus*, a concentration that could show an increase or decrease in successful invagination. Another preliminary test was used to ensure that the embryos did not prefer an orientation on a glass slide.

To determine if salinity had an effect on the sensitivity of the developmental defect: exogastrulation. Okazaki's (1956) findings suggested that the embryos survival rate were dramatically higher when they were placed in the calcium deficient treatments

after hatching. Therefore, we transferred embryos to their respective treatments after hatching (20 hours post-fertilization). The four treatments were: “100% Ca | 100% Na”, “10% Ca | 100% Na”, “100% Ca | 78% Na”, and “10% Ca | 78% Na”. All treatment values were percentages of normal concentrations found in normal seawater: “100% Na” is equivalent to 32‰ salinity and “78% Na” is about 25‰ salinity. Major ions other than Ca^{2+} were adjusted to maintain approximately constant proportions relative to the Na^+ concentration. Ca^{2+} was at either 100% or 10% of the concentration at 32‰. All treatments were concocted using Ca-free artificial seawater, general-purpose artificial seawater, Ca-solution, and reverse osmosis (RO) water (Tables 1 & 2).

Blastocoel & Cell Layer Volume

At 25 hours post-fertilization, images were taken using a light microscope (*Nikon Eclipse E600, Q camera*). All images were viewed under a 20X objective with a 1X camera adapter. Glass slides and coverslips were coated with a 1% protamine sulfate solution to allow for still images so accurate measurements could be taken. Protamine sulfate creates positively charged surfaces so that the negatively charged embryos are immobilized without adversely affecting the overall structure (Ettensohn et al. 2004). 130 μl of 1% protamine sulfate were placed on the glass slide and the coverslip and sat for about 1 minute. The slides were then wiped dry and washed with filtered seawater if necessary because sometimes the solution appeared to be too strong for the embryo and disrupted its hyaline layer which can have a strong impact on the size of the blastocoel and cell layer. All slides were prepared by placing clay-feet on two adjacent edges of the coverslip forming a wedge for the embryos to get trapped in. The embryos were dropped

next to the coverslip so that they would wick underneath the coverslip. For each clutch, images were taken of the first 6 embryos that were in the animal-vegetal plane.

Using *ImageJ*, the major and minor axis lengths were measured for both the blastocoel and the entire embryo (Figure 1). The measurements were taken at this specific time as it is right before the onset of gastrulation and the embryo is still ellipsoidal. This allows us to make a decent estimate of the blastocoel and cell layer volume by assuming that the embryo takes the shape of a prolate ellipsoid. The prolate ellipsoid volume equation is as follows: $4/3\pi a^2 b$, where a is the minor axis and b is the major axis. If the measurements were taken any later the shape would not fit this simple geometric shape and an accurate measurement could not be obtained easily.

Archenteron Length

At 42 hours post-fertilization, images were taken to measure the archenteron lengths and whether or not it invaginated or evaginated. The same protocol for taking images of embryos was used. To measure the archenteron lengths, the inner cell layer to inner cell layer length was measured and the outer cell layer to outer cell layer length was measured and then the average of the two measurements was used (Figure 2). The reason both lengths were measured was because certain embryos had one length that was harder to measure than the other. If we only chose to measure one length, this would put us at risk of losing valuable data. This way a decent representation of the archenteron length could be acquired for all embryos instead of having impeccable measurements on a few embryos. Positive measurements represent invaginated lengths and negative measurements represent evaginated lengths.

A total of four clutches were used in this study.

Data Analysis

Using the statistical program, *SPSS*, and *Matlab* a 3-way ANOVA was used for both the blastocoel and cell layer volumes and the archenteron lengths. Even though an ANOVA was used, the archenteron length data did not meet the assumptions that it was normally distributed and that there is a uniform variance among all groups, which could in turn weaken the test. A random effect was assigned to take into account clutch to clutch variation. Calcium ("Ca") and salinity ("Na") were assigned as fixed factors in the analysis.

Results

Cell layer and blastocoel volume

Preliminary tests showed that a 10% calcium treatment had the most even number of invaginated and exogastrulated embryos (results not shown). Another preliminary test showed that embryos, whether invaginated or exogastrulated, did not prefer a certain orientation on the glass slide (results now shown).

Salinity had a significant effect on the cell layer volume ($p=0.001$), and showed that cells of embryos placed in the 78% Na treatment had a 17% higher volume than those in the 100% Na treatment (Figure 3). The effect of calcium did not have a significant effect on cell layer volume ($p=0.269$; Table 3). Salinity did not have a significant effect on the blastocoel volume ($p=0.269$). Even though there was a 12% increase in blastocoel volume in the 78% Na treatment it was not statistically significant (Figure 4). Calcium also did not have a significant effect on blastocoel volume ($p=0.263$; Table 4).

To get a better assessment of whether the blastocoel was expanding with the cell layer in the hypotonic solution, the blastocoel to cell layer ratio was measured (blastocoel volume / cell layer volume). Each of the calcium treatments had similar ratios despite the fluctuating salinity (Figure 5). None of the factors were statistically significant (Table 5). For the 10% Ca conditions, the ratios were 0.605 (78% Na) and 0.628 (100% Na). For the 100% Ca conditions the ratios were 0.810 (78% Na) and 0.753 (100% Na).

Archenteron length

Calcium was the only factor that had a significant difference on archenteron length ($p=0.0008$) (Figure 6). Neither salinity ("Na"; $p=0.124$) nor the Ca*Na interaction factor had significant effects on the archenteron lengths after performing an ANOVA test ($p=0.093$; Table 6). However, this data set did violate some of the ANOVA assumptions such as being non-normal and having a non-uniform variance among all groups. We did try to transform the data but none of the transformations eliminated these violations.

Discussion

We hypothesized that if the embryos were exposed to low salinity, the cells would swell and in turn, alter the mechanical stresses placed upon the embryo and ultimately affect the sensitivity to exogastrulation. However, the embryo could act in two ways depending on how the blastocoel reacts in the low salinity condition.

The data collected makes it most reasonable for us to conclude that either low salinity makes embryos more sensitive to exogastrulation or low salinity has no effect on sensitivity to exogastrulation. Therefore, it is reasonable to reject the hypothesis that the

embryo will buckle inward in a hypotonic solution, making embryos less sensitive to exogastrulation in a low salinity solution.

Based off of the blastocoel to cell layer ratios in the different salinity conditions, it is apparent that the blastocoel does expand at a similar rate as the cell layer. This finding allows us to reject the hypothesis that the blastocoel does not expand with the cell layer and is consistent with the hypothesis that the blastocoel and the cell layer expand at the same rate. However, this doesn't necessarily indicate that there are no changes in mechanical stress.

Crudely looking at the archenteron lengths one can see that there is a considerable difference between the different salinity conditions. The fact that the difference was not statistically significant could be due to the fact that an ANOVA was not the ideal test for the type of data that was gathered because of the previously mentioned violations. Thus, if a suitable test is performed, the results might be more accurate. If the results prove to be significant, that would suggest that embryos exposed to exogastrulation inducers in a low salinity environment are more sensitive to exogastrulation. Therefore, it is possible that there are changes in mechanical stresses that the blastocoel to cell layer volume cannot recognize.

The "10% Ca | 100% Na" treatment showed a split between embryos that invaginated and ones that evaginated. This is interesting because it shows that a lower salinity environment could induce more exogastrulation defects when one compares both treatments with low calcium. This may suggest that a low salinity environment may make the embryo more prone to exogastrulation and a high salinity environment may rescue an embryo that has the potential to develop abnormally.

In the future, we'd like to look at embryo shape changes as that can highlight changes in mechanical stresses that volume measurements may not be able to identify. Volume could be staying constant but one of the axes may be elongating faster than the other, which can also cause changes in mechanical forces on the embryo. We'd also like to look at the hyaline layer and how its mechanical properties change in the varying calcium and salinity treatments. It is known that the hyaline layer is important in the gastrulation process and if the hyaline layer is not present gastrulation cannot occur (Lane et al. 1993). Calcium is also known to have a direct effect on the hyaline layer and can change its mechanical properties (Citkowitz 1971). This can be important in identifying the mechanism of gastrulation as Davidson showed that only certain mechanisms could work within a certain elastic modulus range (1995). Lastly, we'd also like to investigate a statistical test that better suits the data when more time permits.

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Tables and Figures

Table 1. Final concentrations of the components of the solutions used in the experiment.

Ca-free artificial seawater (Okazaki 1956)	General-purpose artificial seawater (Strathmann 1987)	Ca-solution (Strathmann 1987)
NaCl - 26.5 g/L KCl - 0.7 g/L MgSO ₄ · H ₂ O - 11.9 g/L NaHCO ₃ - 0.5 g/L	NaCl - 24.72 g/L KCl - 0.67 g/L CaCl ₂ · 2H ₂ O - 1.36 g/L MgCl ₂ · 6H ₂ O - 4.66 g/L MgSO ₄ · 7H ₂ O - 6.29 g/L NaHCO ₃ - 0.180 g/L	CaCl ₂ · 2H ₂ O - 1.36 g/L

Table 2. The amount of each solution used to make each 25 mL treatment.

Treatment name	Ca-free artificial SW (mL)	General-purpose artificial seawater (mL)	Ca-solution (mL)	RO Water (mL)
100% Ca, 100% Na	0	25	0	0
10% Ca, 100% Na	20.8	2.5	0	1.68
100% Ca, 78.1% Na	0	19.5	5.47	0
10% Ca, 78.1 % Na	15.8	2.50	0	6.74

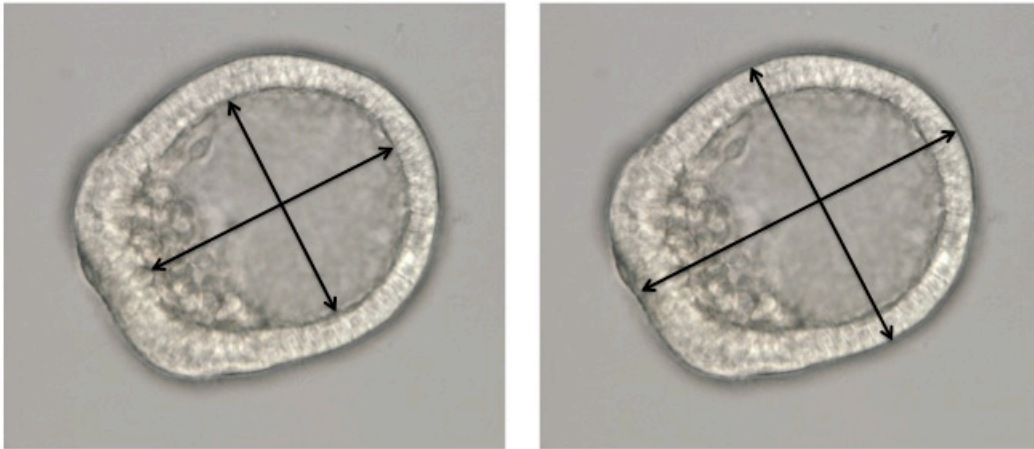


Figure 1. Measurements of the major and minor axes 25 hours post-fertilization for the blastocoele (left) and cell layer volume (right).

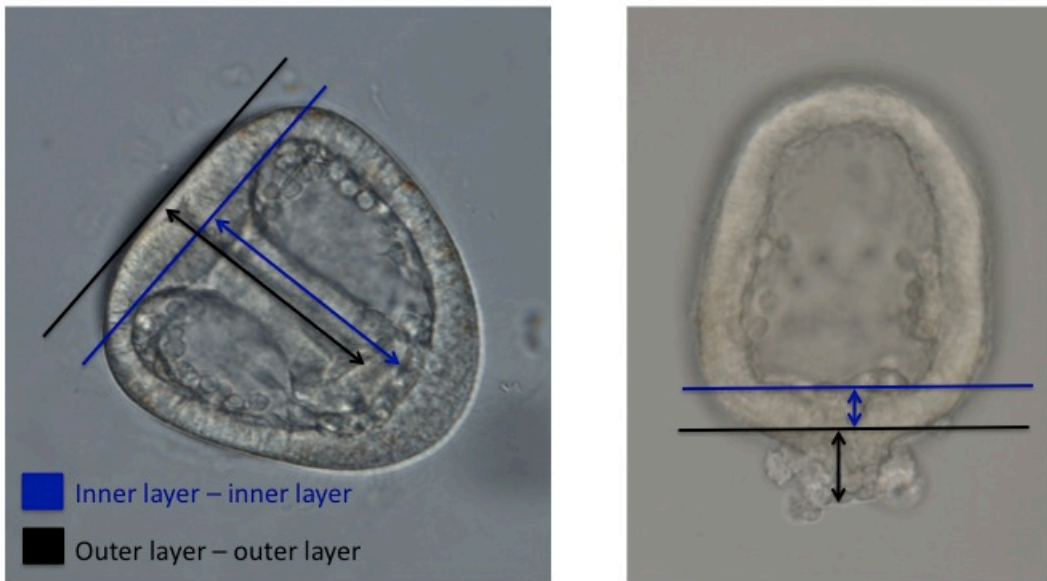


Figure 2. Measurements of the inner layer to inner layer archenteron length and outer layer to outer length 42 hours post-fertilization for both invaginated (left) and evaginated embryos (right).

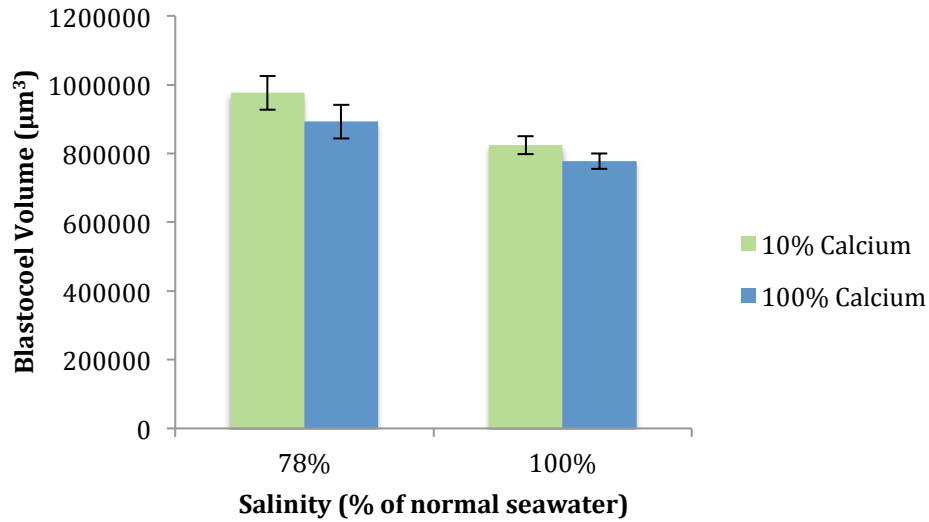


Figure 3. The mean cell layer volume in varying salinity and calcium treatments. Salinity did significantly affect the cell layer while calcium and the salinity calcium interaction did not. A 17% increase in volume was noticed in the treatments in 78% Na compared to those in the 100% Na treatments.. The error bars denote standard error.

Table 3. The effect of various factors on cell layer volume and their respective P and F values with their two degrees of freedom after running an ANOVA. Clutch was assigned as a random factor and Ca and Na were both fixed.

Clutch	P = 0.290 ($F_{3, 1.7} = 2.984$)
Ca	P = 0.269 ($F_{1, 3.0} = 1.835$)
Na	P = 0.001 ($F_{1, 3.0} = 186.8$)
Clutch*Ca	P = 0.151 ($F_{3, 3} = 3.809$)
Clutch*Na	P = 0.917 ($F_{3, 3} = 0.160$)
Ca*Na	P = 0.506 ($F_{1, 3.0} = 0.569$)
Clutch*Ca*Na	P = 0.738 ($F_{3, 80} = 0.422$)

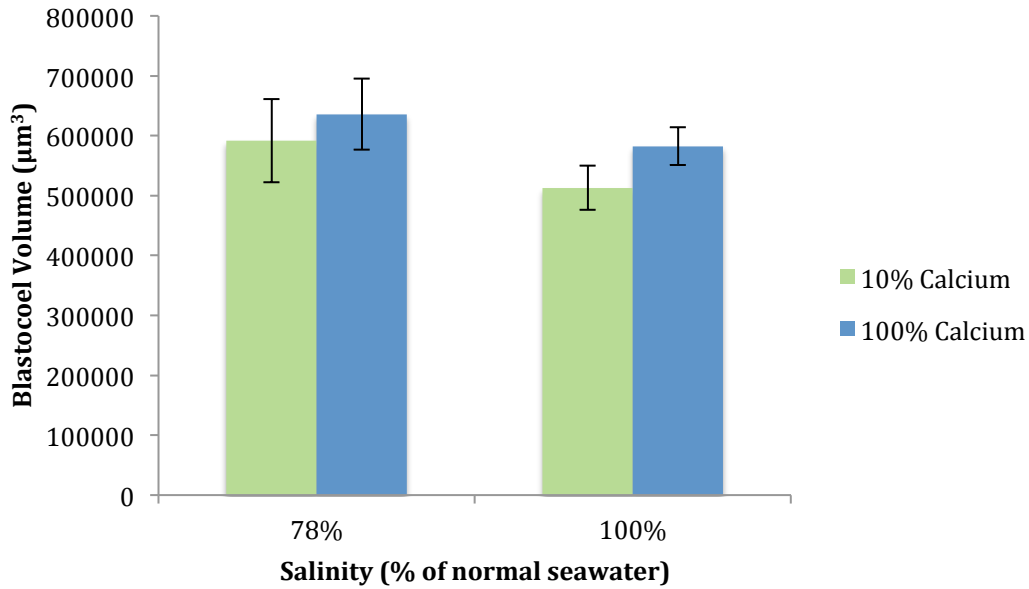


Figure 4. The mean blastocoel volume in varying salinity and calcium conditions. The blastocoel volume was not significantly affected by salinity, calcium, or the interaction of the two. However, there was a 12% increase in the blastocoel volume when placed in the low salinity conditions. The error bars denote standard error.

Table 4. The effect of various factors on blastocoel volume and their respective P and F values with their two degrees of freedom after running an ANOVA. Clutch was assigned as a random factor and Ca and Na were both fixed.

Clutch	P = 0.069 ($F_{3, 2.9} = 7.599$)
Ca	P = 0.263 ($F_{1, 3.0} = 1.886$)
Na	P = 0.341 ($F_{1, 3.0} = 1.274$)
Clutch*Ca	P = 0.388 ($F_{3, 3} = 1.428$)
Clutch*Na	P = 0.206 ($F_{3, 3} = 2.849$)
Ca*Na	P = 0.737 ($F_{1, 3.0} = 0.136$)
Clutch*Ca*Na	P = 0.553 ($F_{3, 80} = 0.703$)

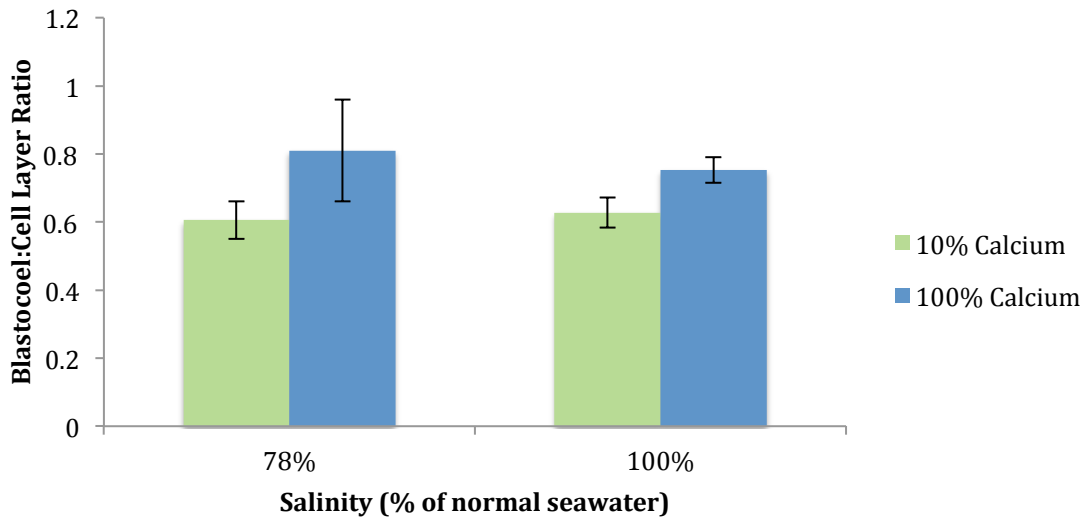


Figure 5. The mean blastocoel to cell layer ratio in varying salinity and calcium treatments. Salinity, calcium, nor the interaction factor of the two had a significant effect on the ratio. The two varying salinity conditions appeared to not have an effect on the ratio as they stayed relatively constant throughout (i.e. the blue bars were relatively similar and the green bars were relatively similar). The error bars denote standard error.

Table 5. The effect of various factors on the blastocoel : cell layer ratio and their respective P and F values with their two degrees of freedom after running an ANOVA. Clutch was assigned as a random factor and Ca and Na were both fixed.

Clutch	P = 0.362 ($F_{3, 1.8} = 2.001$)
Ca	P = 0.299 ($F_{1, 3.0} = 1.572$)
Na	P = 0.855 ($F_{1, 3.0} = 0.039$)
Clutch*Ca	P = 0.291 ($F_{3, 3} = 2.007$)
Clutch*Na	P = 0.539 ($F_{3, 3} = 0.883$)
Ca*Na	P = 0.698 ($F_{1, 3.0} = 0.182$)
Clutch*Ca*Na	P = 0.223 ($F_{3, 80} = 1.492$)

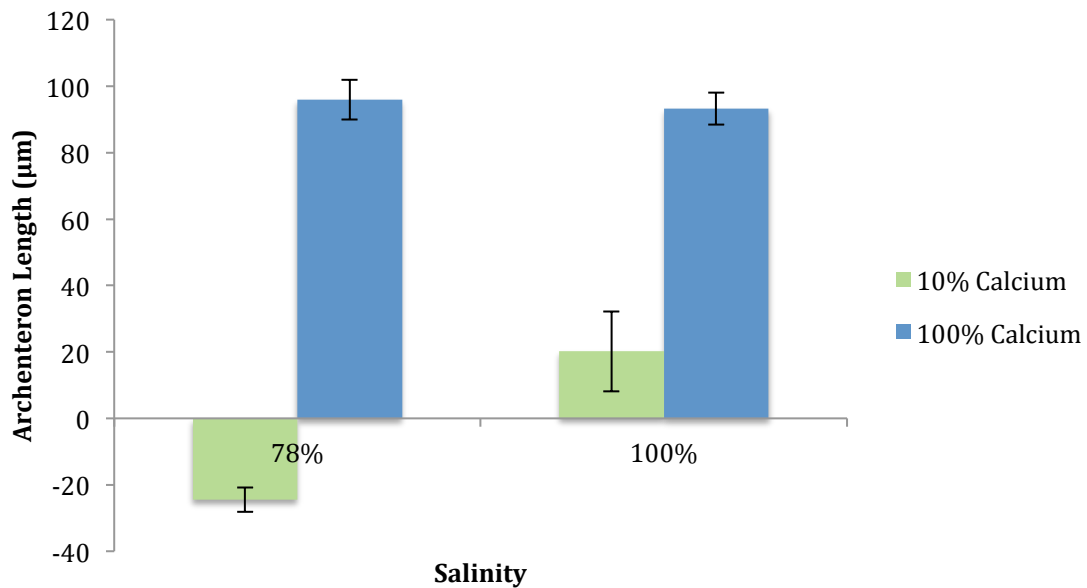


Figure 6. The effect of varying calcium and salinity treatments have on the archenteron lengths. Positive values are indicative of invagination lengths and negative values are indicative of evaginated lengths. The “10% Ca | 78% Na” treatment had majority of the embryos exogastrulate, displayed by the negative average. The “100% Ca | 78% Na” treatment had majority of the embryos invaginate. The “10% Ca | 100% Na had a split between embryos that invaginated and evaginated, thus the reason for a low positive average. The control group “100% Ca | 100% Na” showed that they all successfully invaginated.

Table 6. The effect of various factors on archenteron lengths and their respective P-values and F-values with their two degrees of freedom after running an ANOVA. Clutch was assigned as a random factor and Ca and Na were both fixed.

Clutch	P = 0.4340 ($F_{3, 0.55} = 6.34$)
Ca	P = 0.0008227 ($F_{1, 3.0} = 190$)
Na	P = 0.1238 ($F_{1, 3.0} = 4.51$)
Clutch*Ca	P = 0.6561 ($F_{3, 3} = 0.603$)
Clutch*Na	P = 0.4773 ($F_{3, 3} = 1.07$)
Ca*Na	P = 0.0926 ($F_{1, 3.0} = 5.94$)
Clutch*Ca*Na	P = 0.0908 ($F_{3, 80} = 2.23$)