Protein Expression in *Pisaster ochraceus* Eggs and Larvae Exposed to Multiple Salinity Fluctuations

Eli Isael Maciel[1,2], Sophie George[1,3]

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[1] Friday Harbor Laboratories, University of Washington, Friday Harbor, WA 98250

[2]School of Natural Sciences, University of California, Merced, CA 95343

[3]Department of Biology, Georgia Southern University, Statesboro, GA 30458

Contact Information:
Eli Isael Maciel
School of Natural Sciences
University of California, Merced
5200 North Lake Rd.
Merced, CA 95343

Abstract

Climate Change affects the physical conditions of many environments. Increased temperature causes ice extent to decrease worldwide (IPCC, 2013). Glacier runoff during the spring and summer months increases the amount of freshwater pouring into the ocean creating salinity fluctuations. Surface salinity in the ocean can drop from~30ppt to ~21ppt. These fluctuations affect many marine organisms such as the sea star *Pisaster ochraceus* that cannot osmoregulate. *P. orchraceus* produces planktotrophic larvae that will also endure the fluctuations of salinity. The present study investigated whether differences in occurrence and intensity of salinity fluctuations at two locations in the Pacific Northwest cause sea stars to produce eggs and larvae with different protein profiles. Eggs and larvae from Snug Harbor with less influx of fresh water from the Fraser River and Cantilever Point with more, were exposed in the laboratory to two and four salinity fluctuations respectively during their development. Low salinity varied between 20 and 22 ppt and high salinity between 30 and 32 ppt. The length, width, and stomach width were noted and protein profiles obtained for larvae in control and fluctuating salinity treatments. Significant differences were observed between treatments for larvae from Snug Harbor, a site less influenced by the Fraser River. Larvae in the controls were significantly bigger than those reared in fluctuating salinity. No significant differences in larval size were observed for larvae from Cantilever Point, a site that experiences a significant influx of low salinity waters from the Fraser River several times during the summer months. Greater expression of a high molecular weight protein (>300kDa) was observed for eggs produced by females from Cantilever Point than for eggs produced by females from Snug Harbor. Larvae from both locations expressed this high molecular weight protein. Larvae from the two locations differed in mid-range to low molecular weight proteins. The High molecular weight proteins might be a kind of ATPase

pump, while the low to intermediate size proteins might be used for cell signaling or heat shock proteins that will help protein folding during stressful events of low salinity

Introduction

Globally, climate change affects and alters the physical conditions of many environments. Temperature, one of the biggest altered physical conditions, causes snow cover and ice extent to decrease; this in turn has led to a drastic shrinkage of ice cover worldwide (IPCC 2013). The glacier run off increases the amount of freshwater in the ocean which leads to lower levels of salinity. Consequently, much of the northern oceans close to the pole have become fresher (Curry et al, 2003). For example, in the Pacific Northwest salinity can drop from an average of 30 ppt to as low as 21 ppt (FHL weather station 2014, Sutherland 2011). This drop is in part due to the Fraser River that dumps a large amount of fresh water into the Puget Sound in Washington and lowers the salinity around the San Juan Islands during the summer months (Johannessen et al., 2003). However, the effects of salinity might differ with location around the San Juan Islands depending on their exposure to the Fraser River (UW Coastal Modeling Group, 2014).

Fluctuations of salinity can have a negative impact to all marine wild life in the Puget Sound. Low salinity can alter an organism's growth, reproduction, morphology and their survival (Campbell, 2003). Some species such as mollusks and crustaceans can tolerate and respond to fluctuations in salinity, but it has significantly lowered than usual, that even species with high tolerance are having trouble (NOAA 2008). Echinoderms like *Pisaster ochraceus* are vulnerable to low salinity because their larval stages have no ability to regulate osmotic pressure due to the lack of an excretory organ (Stickle, 1987). Adults move away from the intertidal zone to deeper waters to avoid low surface waters taking them away from their main food source, mussels

(Garza and Robles 2010). This leads to decrease foraging and lower reproductive output (Held and Harley, 2009). *P. ochraceus* is a keystone predator in the Pacific Northwest. In its absence, biodiversity would decrease (Paine 1966) as mussels would become the dominant space holder (Power, 1996). This issue can affect the biodiversity and is thus important to study the effects of changes in climate on *P. ochraceus* populations.

Although the reproductive cycle of *P. ochraceus* consist of both male and female, they show no sexual dimorphism. Spawning occurs around May to July when fluctuations of salinity increase ((Strathmann, 1987; Sutherland 2011). The decrease in salinity during spawning may affect offspring quality (Roller and Stickle, 1985). Low salinity leads to morphological changes in *P. ochraceus* larvae; Brachiolariae from embryos kept in 20ppt for 3 days were wider and shorter while those kept in 30 ppt were longer and slender (Pia et al. 2012). The behavior of these larvae is also affected. Compared to larvae reared in high salinity, those reared in low salinity avoid the halocline even when food is present (Bashevkin and George, 2012).

Further understanding of how decreased salinity levels affect *Pisaster* larvae is crucial, because of the impact it can have on the intertidal ecosystem of the Pacific Northwest. It is important to understand which proteins are being influenced when larvae are enduring lower salinity levels. Fluctuations in salinity might alter protein expression which in turn might affect larval size of this species during development. Results from the protein expression might shed light on how this keystone species adapts and maintains itself as salinity levels worsen. Preliminary data has shown the expression of heavy molecular weight proteins by larvae exposed to fluctuating salinity (Bashevkin, 2013). Identifying the molecular weight of some proteins can give us insight to which types of proteins are being expressed and the functions that help the larvae survive in low salinity.

The research was conducted in the San Juan Islands. The island has different bodies of water surrounding it; the strait of Juan De Fuca brings seawater (~31ppt salinity) coming from the south and west of San Juan island, and the Fraser River, which dumps freshwater from the northeast of the island (UW Coastal Modeling Group, 2014). The west and northeast side of the island are not equally exposed to the Fraser River and therefore the intensity and magnitude of salinity fluctuations might differ (UW Coastal Modeling Group, 2014). The present study will reveal whether offspring from different locations differ in their response to salinity fluctuations. The following questions will be addressed:

1. Will larval size differ with location in response to fluctuating salinity?
2. Will sea stars at Cantilever Point and Snug Harbor spawn eggs with different protein profiles?
3. Will protein expression differ for larvae reared in fluctuating salinity and controls?
4. Will larvae from eggs with different protein profiles vary in proteins expressed during development?

Methods

Spawning and fertilization of *Pisaster ochraceus*

In June 2014, *Pisaster ochraceus* were collected from two locations on the San Juan Islands: Cantilever Point (48˚32’57.6”N; 123˚00’21.6”W) and Snug Harbor (48˚34’19.7”N; 123˚10’21.5”W) on June 2014.

To induce spawning 100µL of 1mM 1-methyladenine was injected into the sea stars from Cantilever Point and 9mL of 100µM of 1-methyladenine into the sea stars from Snug Harbor. Two females and two males spawned from Cantilever Point and two females and three males

from Snug Harbor. Eggs were collected and fertilized with 3 drops of dilute sperm from both males.

Measurements of eggs from females

Collected eggs had there diameters measured using imageJ; the following equation was used to convert the pixels to micrometers; $8mm\ x\ \frac{1000\mu L}{mm} x\ \frac{dimeter\ of\ egg\ pixels}{674\ pixels}$. A total of 30 eggs were measured for each female per location.

Samples of eggs from each female were taken and placed in -80˚C for protein content determination and gel electrophoresis. The fertilized eggs were placed in 3.2 liter jars filled with 2 liters of 30ppt sea water. There were a total of 16 jars separated into two sets: set A: 1-8 and set B: 9-16. A large number of embryos were needed for protein profiles, jars were initially set up with approximately 30,000 embryos by counting the number of embryos in .5ml of seawater. Sea water was added to give an equal volume to each jar (3,812 ml of seawater). Experimental temperature was around 11-13˚C, with the salinity within the jars at 32‰.

Experimental design

In each set there were two groups: controls and fluctuating salinity. Each group was compromised of 4 control jars and 4 jars with larvae exposed to fluctuating salinity. The fluctuating group had their seawater of 32‰ salinity reduce to 21‰ for 24 hours at specific dates (06/26, 7/07, 07/14, and 07/28) corresponding to 20, 28, 35, and 41 day-old larvae respectively. The eggs from Snug Harbor were fertilized a day later than those from Cantilever Point. For treatments (control and fluctuating salinity), a sample of an estimated 10,000 or 5,000 larvae were taken from each of the 16 jars before and after 24 hours at low salinity (fluctuating

salinity). Samples were taken every week before fluctuation and once when no fluctuation occurred for the week. The numbers of larvae sampled decreased over time. For the last two fluctuations, larvae from all jars in a treatment were combined to ensure that the amount of protein obtained was sufficient for gel electrophoresis. The samples were analyzed for protein content and protein profile. To extract the larvae from the jars: 1000mL of seawater containing the larvae were removed and water was siphoned to a smaller volume. The sample was then centrifuged down (4,000 rpm) in order to separate the larvae from the sea water and washed three times with distilled water. After washing they were kept at -80˚C.

After each sampling pictures of the larvae were taken for measurement. ImageJ was used to measure the width, length, and stomach width of 5 larvae per jar.

Protein Content

Protein content was analyzed with the Bradford Protein Assay. Protein standards were diluted from a concentration of 2,000µg/mL to 125µg/ml. Before triplicates of samples were taken, the samples were washed twice and then mixed via vortex and centrifuged at 6,000rpm for 10 minutes 2 to 3 times. Next, 30µL of lysis buffer was added to samples that were approximately 50-100µL or 10µL to samples with less than 50µL. Higher volumes were added to samples greater than 100µL. Samples were vortexed for 30 minutes in 5 minute intervals and centrifuged at 13,200rpm at 4˚C for 10 minutes. The supernatant was kept at -80˚C until ready for processing. Triplicates were prepared of each supernatant. Aliquots consisted of 2.5µL of sample, 7.5µL of distilled water, and 500µL of Bradford dye. Each triplicate was run through a spectrophotometer and absorbance recorded at 595nm at three time intervals 25min, 30min, and 35min. The averages were taken for each time and the triplicates then compared to the standard's

curve to find the concentration in 2.5µL sample (the amount taken from the supernatant to run the assay).

Gel Electrophoresis under reducing conditions

Based on the amount of protein in the supernatant, a specific amount (12µg, 18µg, 20µg, and 25µg) of the sample was extracted and aliquot with 6.25-7 µL of LDS with the addition of 2.5 µL of reducing agent DTT; purified water was added to give the final volume of 25µL. Incubation of the sample proceeded for ten minutes in a water bath adjusted to 70˚C with vortexing before and after incubation. Each sample was then pipetted into a well and run at 200 volts for 35 minutes. NuPage Bis-Tris gels (4-12%) or Tris-Acetate (3-8%) (Life Technologies) that separate proteins after denaturing were used. MES SDS running buffer was used to resolve small molecular weight proteins for Bis-Tris gels, and TA running buffer for the Tris-Acetate gels. Gels were analyzed with Kodak molecular imaging software.

Statistical Analysis:

All data had equal variances and approximately 90% of the data were normally distributed. An analysis of variance (Anova) was used to determine whether the first and second set of 8 jars from the two Cantilever Point stocks differed significantly. No significant differences were noted for 27 and 35 day old larvae thus all data from these two sets were pooled for further analysis. Significant differences were observed between the two sets in length for 20 day old larvae from Cantilever Point. Anova's were done separately for these latter 2 sets. To determine whether larval size differed between treatments and sites a nested analysis of variance with jars nested within salinity treatment and one-way analyses of variance were run. Salinity treatment was a major factor and jar was a random factor. JMP 09 was used to analyze all data.

Results

Egg size

The average diameter of the eggs from cantilever point were 162µm for female 1 and 165.1µm for female 2. Snug Harbor had similar averages in their diameter, 163.5µm for female 1 and 162.4µm for female two (n = 30 for each female).

Larval size

Larvae were measured to see if there were any size differences between those exposed to fluctuating salinity and those in the control. Larvae from Snug Harbor exposed to fluctuating salinity differed significantly from the controls while those from Cantilever Point did not. This was even though Snug Harbor larvae were exposed to low salinity twice and Cantilever Point larvae 4 times.

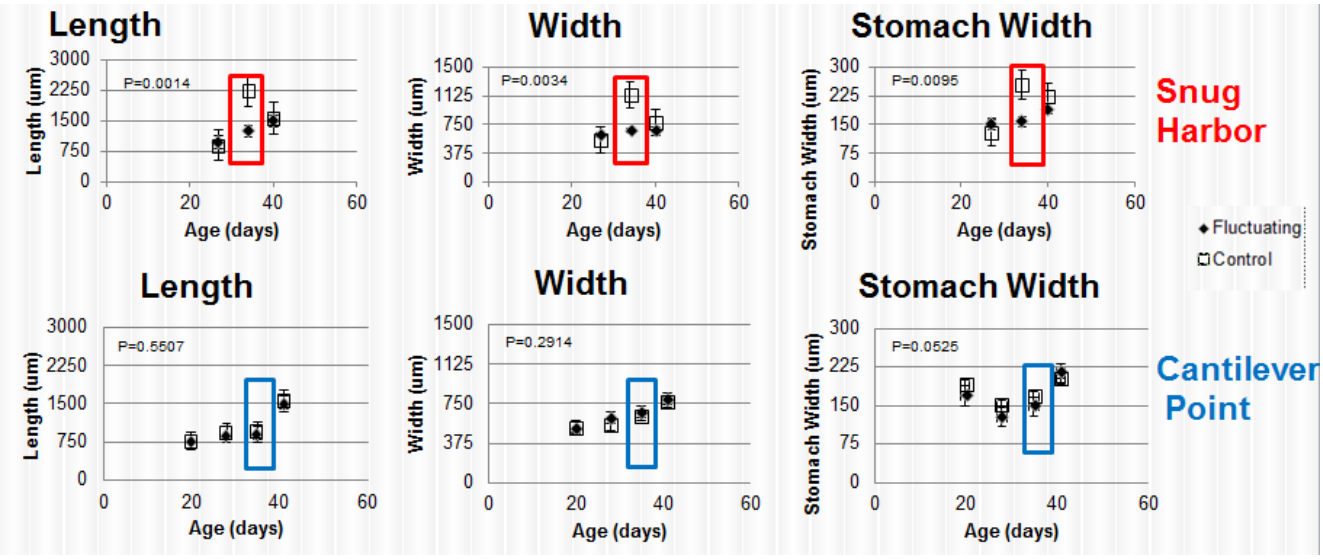

Figure 1: For Snug Harbor (SH) : total length, width and stomach width of 34 day-old larvae exposed to low salinity differed from those in the control (length P=0.0014, width p=0.0034, and stomach width p=0.0095). Total length, width, and stomach width did not differ significantly between treatments for 27 day-old and 40 day-old larvae (27 day: p= 0.2318, p= 0.2298, and p= 0.1819; 40 day: p= 0.2570, p= 0.1443, and p= 0.1841). Note that 41 day-old larvae were from a different stock and had only been exposed to one salinity fluctuation while 20 and 35 day-old larvae hade been exposed to two salinity fluctuations. For Cantilever Point (CP) no significant differences were observed in larval length, width, and stomach width for larvae in fluctuating salinity and control (20 day length 1st set: p= 0.6907, 20 day length 2nd set: p= 0.4147, 20 day width and stomach width: p= 0.1624 and p= 0.6576). (28 day: p=0.2318, p= 0.2298, and p= 0.1819; 35 day: p=0.5507, p= 0.2914, and p= 0.0525; 41 day: p= 0.1602, p= 0.1925, and p= 0.3034). Data points within the red and blue boxes indicate larvae of the same age from Cantilever Point and Snug Harbor.

Cantilever Point: Larvae in control or fluctuating salinity did not differ significantly in length, width, and stomach width (Figure 1).

Snug Harbor: Twenty day and 41 day old larvae did not vary in length, width or, stomach width (twenty day: p=.2318, p=.2298, p=.1819 and 41 day: p=.2570, p=.1443, p= .1841 respectively). However, 34 day old larvae in the controls were significantly longer, wider, with larger stomachs than those in fluctuating salinity (p=.0014, p=.0034, and p=.0095 respectively).

Protein profiles

The protein profiles of eggs from both sites differed (figure 2). Protein levels in larvae from the control and fluctuating groups differed after a second exposure to low salinity levels for both sites. The difference in expression of certain proteins continued as the larvae grew older.

Egg Protein Profiles

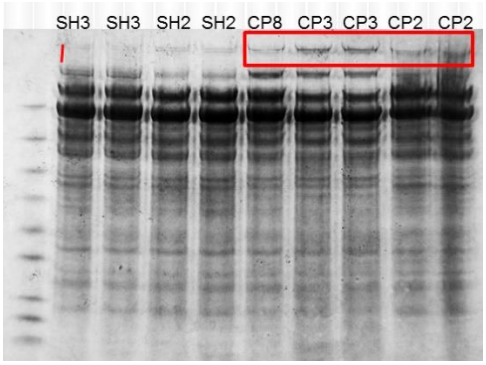

Figure 2: Cantilever Point (CP) females had eggs that expressed more of a heavy molecular weight protein (~300kDa) than Snug Harbor (SH) females. The number associated with the acronym stands for a different female.

Both locations showed strong similarities in the expression of most proteins with the exception of the heavy molecular weight proteins. Eggs from females at Cantilever point expressed a heavy molecular weight proteins (~300- 500 kDa); Very little of these proteins were expressed in eggs produced by females from Snug Harbor. There were also variations among females from the same location (figure 2).

Before Fluctuation and After Fluctuation 28 day-old Cantilever Point

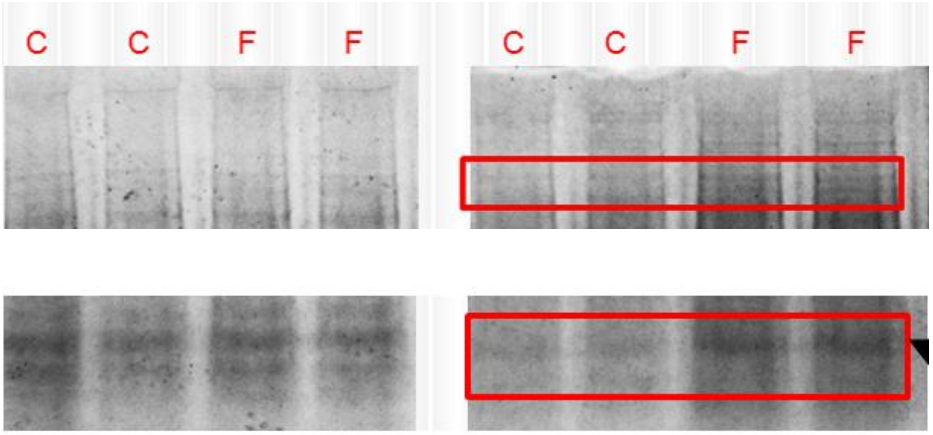

Figure 3: Protein expression in 28 day old larvae. Differences between larvae from Cantilever Point before (left) and after (right) fluctuation showed that larvae in fluctuating salinity expressed a heavy molecular weight protein, top, (~200kDa) and a lighter molecular weight protein, bottom, (~26 kDa). Controls (C) and fluctuations (F).

Before any fluctuation occurred, 28 day old Cantilever Point larvae in the control group and fluctuating group had similar heavy molecular weight proteins. After fluctuation when the salinity was dropped greater expression of a heavy molecular protein doublet (~240 kDa) in the fluctuating group. In addition a light molecular weight protein (~26 kDa) was expressed by larvae in the fluctuating group that was not clearly evident for larvae from the control group.

Before Fluctuation and After Fluctuation for 27 Day-old Snug Harbor larvae

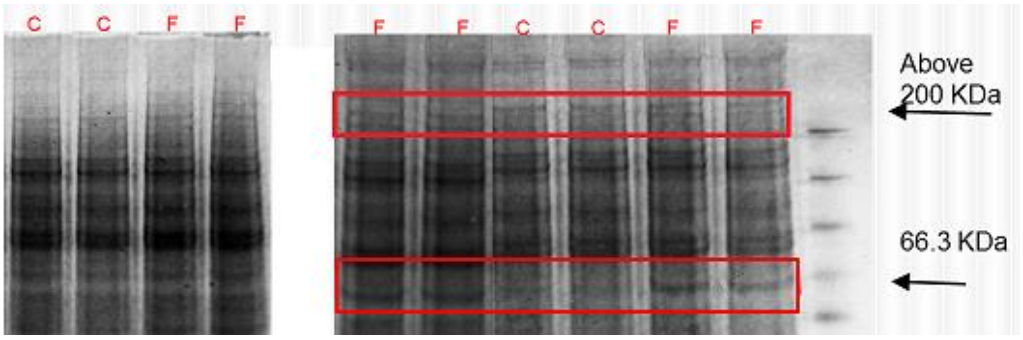

Figure 4: Protein expression in 27 day old larvae. Before fluctuation on the left and after fluctuation on the right. The fluctuating group had more expression of a heavy molecular weight (~240 kDa) and mid-range molecular weight protein (~66.3 kDa). Controls (C) and fluctuations (F).

Snug Harbor: Before fluctuation, protein profiles were similar for larvae in the controls and those exposed to fluctuating salinity (Figure 4). After fluctuation, a change in protein expression was observed between treatments (Figure 4). There was more of a 240 kDa protein expression in the fluctuating group compared to the control. When present in the control this band was hardly noticeable. Larvae in fluctuation salinity from Snug Harbor also expressed a mid-range molecular weight protein (~66.3 kDa) that was only faintly expressed by larvae from the controls. Before any fluctuations, protein profiles were similar for larvae in the controls and those exposed to salinity fluctuations.

Cantilever Point 41 day-old larvae

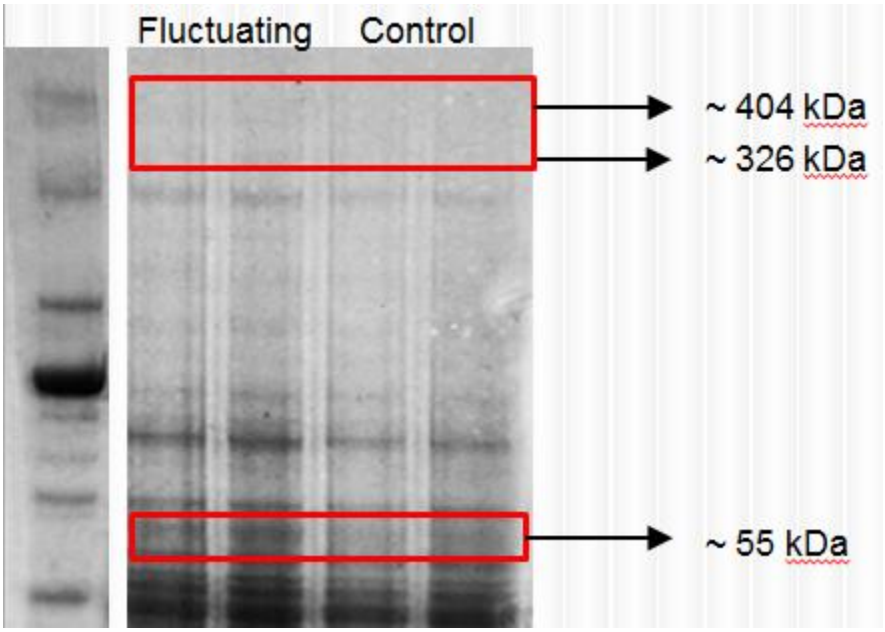

Figure 5: Protein expression in 41 day old Larvae. The gel type is a tris-acetate used to see a wide range of proteins from very light to heavy molecular weight proteins. Forty-one day old larvae from Cantilever Point expressed a heavy and mid–range molecular weight protein in the fluctuating group.

Forty-one-day-old larvae expressed similar proteins when exposed to fluctuating salinity (figure 5), as seen for 27 and 28 day-old larvae (figure 3 and 4). Forty-one-day-old larvae from Cantilever Point also expressed a mid-range protein not seen in 28 day-old larvae (figure 3).

Discussion

Larval size

Larvae from the fluctuating group of Snug Harbor were significantly smaller than those in the controls after been exposed to fluctuating salinity. Interestingly, those in the controls and fluctuating salinity from Cantilever Point did not show any significant difference in larval size. Snug Harbor is located on the west side of San Juan Island and might be less exposed to fresh water from the Fraser River. Cantilever Point is exposed to multiple fluctuations during the summer months (FHL Weather Station) and may be better acclimated to these fluctuations. Cantilever Point larvae in the controls were a lot smaller than Snug Harbor larvae. The larvae from Cantilever Point may be smaller due to its better acclimation to the fluctuations of salinity. More energy might be invested towards proteins that aid in acclimation and less in growth. Variation in larval densities could also account for size differences between locations. Although larvae from both locations received the same amount of food, larval densities were lower for Snug Harbor than Cantilever Point. As a result, Snug Harbor larvae might have received more food.

Protein Profiles

The eggs of sea stars from Cantilever Point and Snug Harbor did not have similar protein profiles. The eggs produced by females from Cantilever Point had a greater expression of heavy molecular weight proteins (300-500 kDa) that were only faintly expressed in the eggs of females from Snug Harbor. This could be the reason for the size difference between the controls of Snug Harbor and Cantilever Point. This heavy molecular weight protein may help Cantilever Point adapt to the fluctuating salinity but with the cost of energy that would have been invested in growth. Adaptions will sometimes demand energy expenditure and result in relocating resources

to meet the demand (Willmer et al., 2005). Females from Snug Harbor on the other hand produced eggs that are not using energy to make this high molecular weight protein. They might have invested more energy in growth and produced larger larvae in the controls. The production of this heavy molecular weight protein may depend on the fluctuating events in salinity that females are experiencing. Thus the females in Cantilever Point might be experiencing more drops in salinity- the eggs will then be packaged with more proteins that help with possible osmotic stress.

Larvae exposed to fluctuating salinity from both locations, expressed the ~ 240 kDa proteins a lot more compared to controls. This big protein may be some sort of sodium potassium pump or ATPase that will help move positive ions around to help find an osmotic equilibrium in the cells to avoid bursting the cell during a hyposalinity event. The protein sodium-potassium ATPase being a tetramer contains two α-subunits and two β-subunits which respectively have molecular weights of 100 kDA and 40 kDA, but contain a holoenzyme that has a molecular weight of 274 kDa (Peterson and Hokin, 1982). The ~240 molecular weight protein may be a holoenzyme of the Sodium-potassium ATPase which can influence the activation of this enzyme.

The lighter molecular weight protein produced by larvae from Cantilever Point may be a small protein that is used for cell signaling. The interactions of this protein may ultimately lead to PKA protein repression. PKA proteins such as a tyrosine kinase are responsible for cell growth and actin filament rearrangement (Park et. al, 2002). A signaling protein that can lead to inhibition of cell growth and actin filament rearrangement can explain why larvae in low salinity had trouble swimming through haloclines as shown by Bashevkin and George (2012) and even the reason why larvae in the controls from Cantilever point were smaller than larvae from Snug

Harbor. The ~66.3 kDa protein expressed more in fluctuating conditions may also be a part of some anion or cation transporter that might aid in osmoregulation during stressful events of low salinity. Also one of these proteins expressed would most likely be a type of heat shock protein, which are a type of chaperone proteins that help fold proteins during stress events. To further this experiment, it will be interesting to see if other locations around the Salish Sea had females that produced eggs with different variations of expression of the high molecular protein (300 kDa). With tools such as mass spectrophotometry, the specific proteins can be identified and the role they play during development of these larvae can be determined: Whether it helps adapt to lower salinity fluctuations could be investigated. This study is the first to show that there are specific proteins being expressed during salinity fluctuations. This added stress might also be contributing to the decimation of sea stars populations. It is important to understand what these larvae and adults are doing to increase their chance of survival in the face of climate change.

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Works Cited

Bashevkin S, George S. B. (2012). The ups and downs of life in a halocline: The response of *Pisaster ochraceus* larvae to food patches and prior exposure to low salinity. Friday Harbor Labs Library.

Bashevkin S, George S. B. (2013). The effects of fluctuating salinity on protein profiles in echinoderm larvae of the species *Dendraster excentricus* and *Pisaster ochraceus*. Friday Harbor Labs Library

Campbell J, Russell M. P. (2003). Acclimation and growth response of the green sea urchin *Strongylocentrotus droebachiensis* to fluctuating salinity. DEStech. 110-117

Curry R, Dickson B, Yashayaev I. (2003). A Change in the freshwater balance of the Atlantic Ocean over the past four decades. Nature 426: 826-829

FHL weather Station. (2014). Underwater. http://wx.fhl.washington.edu/vdv/VV_Frame.php

Garza, C, Robles, C. (2010). Effects of brackish water incursions and diel phasing of tides on vertical excursions of the keystone predator *Pisaster ochraceus*. Marine biology, 157: 673-682.

Held, M. B, Harley, C. D. (2009). Responses to low salinity by the sea star *Pisaster ochraceus* from high-and low-salinity populations. Invertebrate Biology, 128: 381-390.

IPCC. (2003). Climate Change (2013) The Physical Science Basis. http://www.ipcc.ch/report/ar5/wg1/docs/WGIAR5_SPM_brochure_en.pdf.

Johannessen, S. C, Macdonald, R. W, Paton, D. W. (2003). A sediment and organic carbon budget for the greater Strait of Georgia. Estuarine, Coastal and Shelf Science, 56: 845-860.

Noaa Ocean Service Education. (2008). Salinity. http://oceanservice.noaa.gov/education/kits/estuaries/media/supp_estuar10c_salinity.html

Paine, R. T. (1966). Food web complexity and species diversity. American Naturalist, 65-75.

Park Ek, Warner N, Mood K, Pawson T, Daar IO. (2012). Low-molecular-weight protein tyrosine phosphatase is a positive component of the firbroblast growth factor signaling pathway. Molecular and Cellular Biology. 22: 3404-14

Peterson G. L, Hokin L. E. (1981). Molecular Weight and Stoichiometry of the sodium- and Potassium-activated Adenosine Triphosphatase subunits. Biological Chemistry 256: 3751-3761

Pia, T.S, Johnson, T, George, S.B. (2012). Salinity-induced morphological changes in *Pisaster ochraceus* (Echinodermata: Asteroidea) larvae. J. Plankton Res. 34: 590-601.

Power, M. E. Tilman, D. Estes, J. A. Menge, B. A. Bond, W. J. Mills, L. Scott, Daily G. Castilla, J. C. Lubchenco, J and Paine, R. T., (1996). Challenges in the Quest for Keystones. Wildlife Biology Faculty Publications. Paper 1.

Roller, R. A, Stickle, W. B. (1985). Effects of salinity on larval tolerance and early developmental rates of four species of echinoderms. Canadian Journal of Zoology, 63: 1531-1538.

Strathmann. (1987). Echinoderms: Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast: Data and Methods for the Study of Eggs, Embryos, and Larvae. 535-555.

Stickle, W. B, Diehl, W. J. (1987). Effects of salinity on echinoderms. Echinoderm Studies, 2, 235-285.

Sutherland, D. A, MacCready, P, Banas, N. S, Smedstad, L. F. (2011). A Model Study of the Salish Sea Estuarine Circulation. Journal of Physical Oceanography, 41, 1125-1143.

UW Coastal Modeling Group. (2014). http://faculty.washington.edu/pmacc/cmg/cmg.html.

Willmer, P, G. Stone, I. Johnston. (2005). Environmental physiology of animals 2nd edition. MA: Blackwell.