The Effect of Freshwater Influxes on *Pisaster ochraceus* Larvae in the Salish Sea

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Abstract

Global temperatures have been steadily increasing annually, causing increases in artic ice melting. The resulting freshwater from this artic ice retreat enters local river systems, which flow down to the Pacific Northwest and add freshwater to the Salish Sea. With significant melting occurring in the summer months from May to June, the Salish Sea receives multiple influxes of low-salinity water every summer that can persist for a couple of days. These freshwater events can lower surface water salinity from normal 31‰ to as low as 21‰. Understanding the impact of these low-salinity events is particularly important for the larvae of seastar *Pisaster ochraceus,* which are limited in their ability to swim out of low-salinity surface waters. Since *P. ochraceus* can take over 200 days to develop and metamorphose, larvae are bound to experience at least one low-salinity event during their development. This study looked at the effect of a constant low-salinity environment verses a fluctuating salinity environment on *P. ochraceus* survival, morphology, development, and protein expression. No significant differences in survival and body size were found between treatments. However, low-salinity reared larvae had significantly shorter posterolateral arms, which has implications for feeding and swimming behaviors. Osmoregulatory and mechanosensory protein expression was upregulated in fluctuating salinity treated larvae, while low-salinity reared larvae were not significantly different from the controls. This upregulation indicates that *P. ochraceus* larvae are changing their protein expression in response to the lower salinity environment.

Introduction

As global temperatures continue to rise, arctic ice melting has been increasing in the northern hemisphere annually (Shepherd *et al.* 2012). As a result, there has been an increase in freshwater runoff into the ocean and nearby river systems. Fourteen rivers feed into the Salish Sea of the Pacific Northwest, carrying freshwater from the Arctic into the Salish Sea with the most significant flow coming from the Fraser River in Canada (Khangaonkar *et al.* 2011). These freshwater influxes can drop the local salinity of the Salish Sea surface waters to as low as 21‰. These low salinity events often coincide with periods of planktonic larval development (Mercier and Hamel, 2010). These freshwater influxes create haloclines, typically composed of a 5-10m thick layer of brackish water (~20‰) above a layer of saltier water (~30‰) (Khangaonkar *et al.* 2011), which can potentially create a barrier for planktonic larval mobility (Bashevkin 2012, Metaxas 2009), causing larvae to experience extended periods of development within a low salinity environment.

In particular, low salinity water (~20‰) can impact the larvae of local keystone predator *Pisaster ochraceus* due to its lack of ability to ion- and osmo-regulate (Held and Harley 2009). The reproductive season of *P.* ochraceus occurs from May to August, with peak spawning occurring in May and June (Strathmann 1987). This coincides with the major low salinity events in the Salish Sea (Sutherland *et al.,* 2011). Since *P. ochraceus* larvae take 40 (George 1999) to over 200 (Strathmann 1987) days to develop in a laboratory environment and possibly longer in the natural environment, they are bound to experience at least one low-salinity event prior to metamorphosing. Recently, *P. ochraceus* adults experienced extreme population reductions throughout the west coast of

North America, including within the Salish Sea, due to the outbreaks of sea-star wasting disease (SSWD) (Bates *et al,* 2009, Hewson et al 2014). Thus, understanding how these low salinity events impact the larval forms of these echinoderms could be vital in understanding how to fully protect their populations.

Previous studies have shown that low salinity seawater can cause the larvae of *P. ochraceus* to undergo morphological changes, including the development of a shorter and wider shape, as well as reduced arm length during the brachiolaria stage of development (Pia *et al.,* 2012). Since larval morphology is closely related to larval behavior (Chan, 2012), these effects are hypothesized to impact the ability of the larvae to swim, possibly reducing the larvae's ability to find food (George, personal communication). Weakened ability to swim could cause the larvae to become stuck in the lower salinity environment until the brackish water from the Fraser River recedes. Although increases in width and body length also increases the ciliated band length in *P. ochraceus* larvae (Strathmann, 1971), this has not been shown to increase the ability of larvae to swim. Instead, the ability of low-salinity reared larvae to swim and find food effectively is actually reduced (Bashevkin, 2012). Moreover, the low-salinity environment may be having an effect on the larvae's ability to sense their environment, possibly limiting their ability to find food or move through the halocline towards higher salinity water.

Studies have also shown that fluctuating low-salinity environments cause changes in overall protein expression among *P. ochraceus* larvae, including proteins involved in osmoregulation, metabolism, and motor abilities (Bashevkin, 2013). However, no previous research has compared changes in larval protein expression when reared in constant low-salinity environments versus a fluctuating low-salinity environment.

Although low salinity events in the Salish Sea typically last only a few days, it is possibly that these larvae are getting stuck in the low-salinity water for extended periods of time by being carried away by the receding water. Therefore, it is important to fully understand the implications this low-salinity environment may have on *P. ochraceus* larval mortality, development, and protein expression to grasp how populations may be responding.

This study set to answer the following question: Does protein expression vary between larvae reared in normal salinity (30‰), those reared in a fluctuating salinity (21‰/30‰), and those reared in constant low-salinity (21‰)? If there are differences, which proteins are up- and down-regulated in the given environments? Do these different salinity treatments cause differences in developmental rates and mortality? Additionally, this study set to confirm the morphological changes seen in previous studies (Pia *et al.* 2012). To investigate these questions, we reared larvae for 5 weeks in 30‰, 21‰, and in a fluctuating salinity environment in which salinity was dropped to 21‰ for 24 hours once a week, but was otherwise kept at 30‰.

Methods

Collection and spawning of adults

Pisaster ochraceus adults were collected in May 2015 from Cantilever Point, Friday Harbor, WA. Adults were injected through the mouth with 4mL of 100μM 1-Methyl Adenine to induce spawning. Eggs were fertilized with 8-10 drops of dilute sperm.

Larval rearing

After fertilization, embryos were transferred into two jars with 2000mL of 0.45µm filtered seawater (FSW) at 30‰ salinity and were kept in a sea table with flowing seawater coming directly from the Puget Sound to maintain a constant temperature of 12-15°C within the jars. Embryo concentrations never exceeded 1 larva/mL. Jars were each stirred with a paddle that was gently moved by a rotating motor system. This created constant motion throughout the water column within each jar. Once the bipinnaria stage of development was reached, larvae were fed a mixed diet of *Isochrysis galbana, Dunaliella tertiolecta, and Rhodomonas spp.* every 2-3 days. *I. galbana* and *D. tertiolecta* were spun in 15mL conical tubes for 5-10minutes at 2,000rpm and most of the F/2 media was removed before being fed to the larvae. *Rhodomonas spp.* was fed directly to larvae without media removal because centrifugation at 2000rpm destroyed *Rhodomonas spp*. cells.

Experimental design

Two days after fertilization, *P. ochraceus* embryos were observed at the gastrula stage. At this point the embryos were counted using a sample volume of the stock solutions and a gridded petri dish. Based on the counts, we estimated that there were about $1.0 * 10^5$ embryos between the two jars. Twenty new jars were filled with 1500mL of 0.45µm FSW, and ~4000 embryos were added to each jar. These jars were split into 3 treatments, each with 5 replicates: Fluctuating salinity (FS), Low Salinity (LS), and Control. The FSW was changed in the jars once a week, beginning with 9 day old bipinnaria larvae. During these changes the Control jars received new 30‰ FSW and the

LS jars received new 21‰, while larvae in the FS treatment experienced a drop to21‰ salinity for 24h before being returned to 30‰ seawater. Water changes were conducted by draining down the water levels in the jars to ~350mL, and then refilling the jars back up with 2000mL of the appropriate salinity. High salinity seawater was obtained by filtering seawater from the local environment. Low salinity seawater was obtained by mixing high salinity FSW with reverse osmosis (RO) water. All seawater was confirmed to be at the correct salinity with a refractometer prior to water changes, as well as randomly throughout the week.

Sampling for protein analysis

During each low-salinity event, samples of ~1000 larvae were obtained from each jar for protein analysis. Protein analysis shown is all from larvae sampled at 34 days old. For FS treatment jars, 500 larvae were taken directly before and immediately after the changes in seawater. For LS and Control treatment jars, 1000 larvae were taken directly before the change in seawater because no second water change was conducted. All samples from jars within the same treatment were combined into one sample to ensure that the protein concentration obtained was high enough for accurate readings from the Bradford Assay. Larval samples were first centrifuged at 2000rpm for 15 minutes in 15mL conical tubes, and then again for 5 min at 6000rpm in eppendorf tubes. As much seawater as possible was removed without significant loss of larvae

Protein extraction

NP-40 lysis buffer was added to pelleted samples at a volume 60% of that of the pellet. While on ice, samples were treated with lysis buffer for 30 minutes, with ~5 seconds of vortexing every 5 minutes. Next, samples were centrifuged at 4°C for 30 minutes at 13,000 rpm. Lysate was then removed from the pellets and aliquoted into eppendorf tubes in 10µL portions. Pellets and lysate stocks were stored at -80°C.

Protein determination

Protein concentrations of lysate samples was determined using the Bradford protein assay (purchased from life technologies). 10µL of sample lysate was combined with 250 µL of Coomassie Blue Staining Reagent and allowed to incubate for 10 minutes. Samples were then transferred to cuvettes and absorbance readings were obtained on a HACH DR 5000 spectrophotometer. A standard curve was made through a series of dilutions using bovine serum albumin (BSA) as the known protein content source. Blanks for the standard curve had molecular water and Coomassie dye. Blanks for the larval samples had lysis buffer, molecular water, and Coomassie dye.

Gel electrophoresis

Using the protein content determined from the Bradford assays, 15-20µg of protein was used for each lane of the Bis-Tris gel. Each protein sample was fixed with SDS sample buffer at a volumetric ratio of 2:1. Samples were then heated at 70°C for 10 min and then vortexed for 10 min @ 13,000 @ 4°C. Each gel well was loaded with 8-

10µL of lysate and 2xSDS sample buffer. For each gel, 10µL of Benchmark pre-stained protein ladder was added to one of the wells. Gels were run @ 200V for 50 min, and then fixed with a solution composed of methanol, acetic acid, and molecular water for 10 min while being gently moved?? on a Belly Dancer® shaker. Next, gels were stained for 7 hours using a solution of methanol, molecular water, and Coomassie stainer A and B (Life Technologies Colloidal Blue Staining Kit). After staining, gels were kept in molecular water for up to 24 hours, and then stored in 20% ammonium sulfate. Gel images were taken using a Kodak imager.

Size measurements

Samples were collected during the water changes each week for measurements. Measurements were made with ImageJ software on pictures of larvae taken with a compound microscope at 20-40X and a dissecting microscope at 6.3X. Body length, body width, and posterolateral arm length were all measured. Connection of coelomic pouches was also noted to gauge developmental rates.

Statistical analysis

Statistical analysis of data was conducted using JMP. Gels were analyzed using Kodak analyzer software. Figures were prepared using DeltaGraph version 5.7.

Results

Larval Survival

There were minimal differences in survival of larvae between treatments (Figure 1). Data was calculated based on larval estimates made for each jar per treatment. Estimates were made when larvae were 34 days old.

Size Measurements

Larvae length and width measurements did not differ significantly between treatments throughout development (Figures 2 and 3, ANOVA $p=0.985$)

Development

Stage of development, based on percentage of larvae with connected coelomic pouches, was different in the LS reared larvae compared to the C and FS treatments when larvae were 34 days old, however these differences were not statistically significant (ANOVA, $p=0.678$) By 42 days of development, all larvae across treatments had 100% of their coelomic pouches connected (Figure 4).

Protein Expression

For each protein analyzed, band intensity was measured to compare relative amounts of protein between treatments. Function of proteins based on band size was determined using mass spec data from Bashevkin 2013 (unpublished data). All data were from proteins extracted when larvae were 34 days old.

1. Osmoregulatory Proteins

, Larvae from the FS treatment showed upregulation of Na/K ATPase, calcium-transporting ATPase, and the canalicular multi-specific organic anion transporter protein compared to the control (Figures 5 and 6). Expression of Na/K ATPase and calcium-transporting ATPase in larvae from the LS treatment did not differ from those from the control, but the expression of the canalicular multi-specific organic anion transporter protein did. This latter protein was downregulated in LS treated larvae (Figure 6).

2. Mechanosensory Protein

The mechanosensory protein was upregulated in the FS treated larvae compared to the control treatment, while the LS treated larvae showed no significant differences in protein expression (Figure 7).

Discussion

The high rate of survival and lack of significant differences between treatments indicates that low salinity environments do not directly increase mortality of *P. ochraceus* larvae. Additionally, this high rate of survival indicates that the water changes themselves were not having a significant effect on the larvae. This data differs from that found in Pia et al. (2012), which instead found that larvae exposed to a low salinity environment were wider and longer than control larvae. These differences may be due to differences in methods used to measure larvae, such as differences due to the use of a cover glass during microscope imaging

Although the percentage of larvae surviving was high in all treatments it is possible that in the natural environment the low salinity environment would have a significant impact on survival. Since low salinity treated larvae were observed to have reduced posterolateral arm length, which impacts the ability of larvae to swim and orient themselves in the water column, low salinity would likely make larvae more susceptible to predation and make food capture more difficult. The slowed development also indicates that although the low salinity larvae were the same size as fluctuating salinity and control larvae, there are changes in energy investment occurring due to the low-salinity environment. Since development takes longer in low salinity larvae, it is likely that these larvae spend more time in the water column before metamorphosing. Larval stages are highly vulnerable to predation, so an increase in time prior to metamorphosis increases the likelihood of mortality.

The upregulation of protein expression in FS treated larvae in most of the osmoregulatory proteins indicates that brief exposure of larvae to low salinity water causes them to require more of certain proteins to deal with the change in saline environment. This upregulation, or increased protein expression, could help larvae compensate for changes in osmotic pressure caused by environmental salinity changes by increasing cellular protein pump activity, thereby preventing too much water from entering cells.

On the other hand, low salinity treated larvae mostly did not differ in expression of osmoregulatory proteins compared to control treatments. Since protein expression data was taken from when the larvae were 34 days old and had been exposed to low salinity

for 25 consecutive days, it is possible that the larvae had adjusted to this lower salinity environment and no longer needed to upregulate various osmoregulatory proteins.

This same trend was seen in the mechanosensory protein, in which the FS treated larvae displayed upregulation of protein expression, while LS protein expression was not significantly different from that of the controls. The lack of change in mechanosensory and osmoregulatory protein expression in LS treated larvae could be related to a previous study that found that LS reared larvae stay in low salinity surface waters when exposed to a halocline instead of moving into the saltier, more favorable water even if food patches are placed below the halocline (Bashevkin 2012). Mechanosensory proteins are used to help organisms sense their environment, such as by detecting changes in osmotic pressures due to changes in salinity, locating food, and orienting themselves in the water column. It is possible that the mechanosensory protein was upregulated in FS treated larvae to improve their ability to sense their surroundings in the stressful environment, such as by helping them better orient themselves in the water column for feeding , while the LS treated larvae were in the low salinity environment long enough to have made adjustments to the conditions, and no longer required the upregulation. It is also possible that the lack of mechanosensory protein upregulation in LS treated larvae signifies that the larvae struggle to sense their environment when they are reared in LS larvae for an extended period of time.

Conclusions

These results express the importance of understanding how the freshening events of the Salish Sea impact vulnerable species like *Pisaster ochraceus* larvae. Rearing of larvae in a constant low-salinity environment clearly slows larval development, likely increasing mortality of *P. ochraceus* prior to metamorphosing. Additionally, reduced posterolateral arm lengths in low-salinity reared larvae likely decreases swimming ability, which can have indirect effects on food capture and the ability of larvae to move through a halocline and into saltier water and escape predators. Unsurprisingly, these morphological and behavioral differences in low-salinity treated larvae caused differences in protein expression. However, our data shows that for some of the osmoregulatory proteins and the mechanosensory protein, protein expression in low-salinity treated larvae was not significantly different from that of the controls, while the fluctuating salinity environment showed short-term upregulation of these proteins. This could show that the larvae that experience low-salinity for extended periods of time make permanent changes that no longer require the upregulation of proteins, while larvae experiencing a brief low-salinity event temporarily compensate with the environment through upregulation of various proteins.

Works Cited

Bates, Ae, Bj Hilton, and Cdg Harley. "Effects of Temperature, Season and Locality on Wasting Disease in the Keystone Predatory Sea Star Pisaster Ochraceus." *Diseases of Aquatic Organisms Dis. Aquat. Org.* 86 (2009): 245-51. Web.

Bashevkin S, George S. 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.

Chan, K. Y. K. "Biomechanics of Larval Morphology Affect Swimming: Insights from the Sand Dollars Dendraster Excentricus." *Integrative and Comparative Biology* 52.4 (2012): 458-69. Web.

George, Sophie B. "Egg Quality, Larval Growth and Phenotypic Plasticity in a Forcipulate Seastar." *Journal of Experimental Marine Biology and Ecology* 237.2 (1999): 203-24. Web.

Held, Mirjam B.e., and Christopher D.g. Harley. "Responses to Low Salinity by the Sea Star Pisaster Ochraceus from High- and Low-salinity Populations." *Invertebrate Biology* 128.4 (2009): 381-90. Web.

Hewson, Ian, Jason B. Button, Brent M. Gudenkauf, Benjamin Miner, Alisa L. Newton, Joseph K. Gaydos, Janna Wynne, Cathy L. Groves, Gordon Hendler, Michael Murray, Steven Fradkin, Mya Breitbart, Elizabeth Fahsbender, Kevin D. Lafferty, A. Marm Kilpatrick, C. Melissa Miner, Peter Raimondi, Lesanna Lahner, Carolyn S. Friedman, Stephen Daniels, Martin Haulena, Jeffrey Marliave, Colleen A. Burge, Morgan E. Eisenlord, and C. Drew Harvell. "Densovirus Associated with Sea-star Wasting Disease and Mass Mortality." *Proceedings of the National Academy of Sciences Proc Natl Acad Sci USA* 111.48 (2014): 17278-7283. Web.

Khangaonkar, Tarang, Zhaoqing Yang, Taeyun Kim, and Mindy Roberts. "Tidally Averaged Circulation in Puget Sound Sub-basins: Comparison of Historical Data, Analytical

Model, and Numerical Model." *Estuarine, Coastal and Shelf Science* 93.4 (2011): 305-19. Web.

Mercier, Annie, and Jean-François Hamel. "Synchronized Breeding Events in Sympatric Marine Invertebrates: Role of Behavior and Fine Temporal Windows in Maintaining Reproductive Isolation." *Behavioral Ecology and Sociobiology Behav Ecol Sociobiol* 64.11 (2010): 1749-765. Web.

Metaxas, A., Ls Mullineaux, and J. Sisson. "Distribution of Echinoderm Larvae Relative to the Halocline of a Salt Wedge." *Marine Ecology Progress Series Mar. Ecol. Prog. Ser.* 377 (2009): 157-68. Web.

Pia, T. S., T. Johnson, and S. B. George. "Salinity-induced Morphological Changes in Pisaster Ochraceus (Echinodermata: Asteroidea) Larvae." *Journal of Plankton Research* 34.7 (2012): 590-601. Web. 7 Aug. 2015.

Shepherd, A., E. R. Ivins, G. A, V. R. Barletta, M. J. Bentley, S. Bettadpur, K. H. Briggs, D. H. Bromwich, R. Forsberg, N. Galin, M. Horwath, S. Jacobs, I. Joughin, M. A. King, J. T. M. Lenaerts, J. Li, S. R. M. Ligtenberg, A. Luckman, S. B. Luthcke, M. Mcmillan, R. Meister, G. Milne, J. Mouginot, A. Muir, J. P. Nicolas, J. Paden, A. J. Payne, H. Pritchard, E. Rignot, H. Rott, L. S. Sorensen, T. A. Scambos, B. Scheuchl, E. J. O. Schrama, B. Smith, A. V. Sundal, J. H. Van Angelen, W. J. Van De Berg, M. R. Van Den Broeke, D. G. Vaughan, I. Velicogna, J. Wahr, P. L. Whitehouse, D. J. Wingham, D. Yi, D. Young, and H. J. Zwally. "A Reconciled Estimate of Ice-Sheet Mass Balance." *Science* 338.6111 (2012): 1183-189. Web. 7 Aug. 2015.

Strathmann, RR (1971) The feeding behavior of planktotrophic echinoderm larvae: mechanisms, regulation, and rates of suspension feeding. J. Exp. Mar. Biol. Ecol. 6: 109-160.

Strathmann M. F. "Reproduction and Development of Marine Invertebrates of the North Pacific Coast" Data and Methods for the Study of Eggs, Embryos, and Larvae.Seattle: University of Washington Press; 1987. p. 1-670.

Sutherland, David A., Parker Maccready, Neil S. Banas, and Lucy F. Smedstad. "A Model Study of the Salish Sea Estuarine Circulation *." *J. Phys. Oceanogr. Journal of Physical Oceanography*41.6 (2011): 1125-143. Web.

Figures

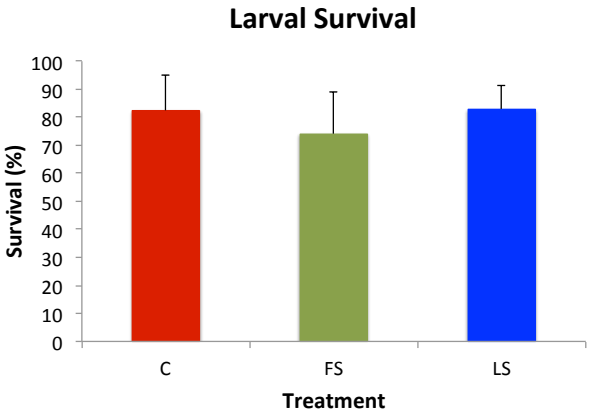

Figure 1: Percentage of *P.* ochracues larvae that survived. Data taken when larvae were 34 days old and had undergone four water changes. Survival was consistently high, with no significant differences between treatments. Values are means ± one standard deviation.

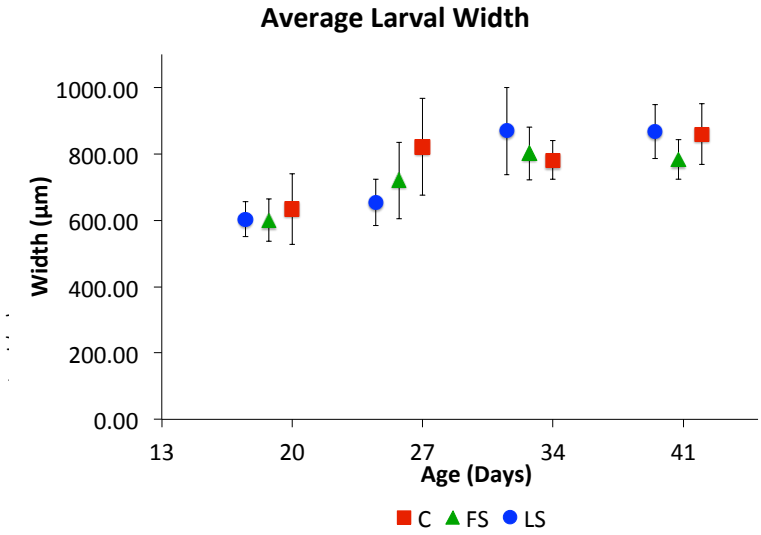

Figure 2: Mean body width of *P. ochraceus* larvae over time. Measurements were taken beginning when larvae were 20 days old and in the bipinnaria stage, and the last measurements were taken when larvae were 42 days old and in the brachiolaria stage of development. No significant differences in body width were found between treatments (n=120, p>0.05). Values are means ± one standard deviation.

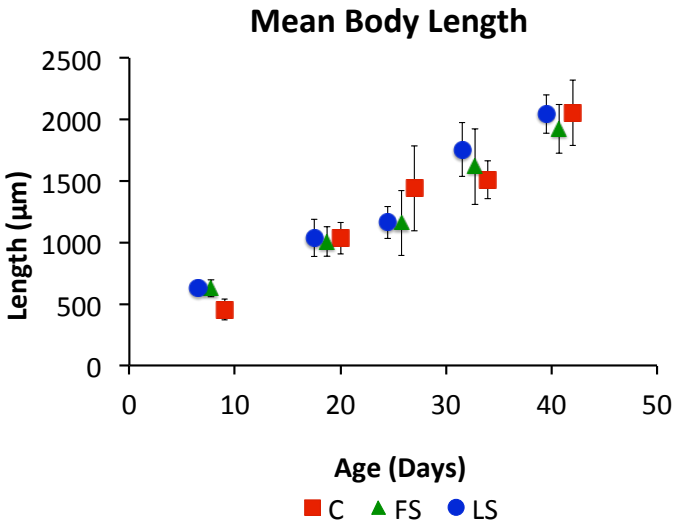

Figure 3: Mean body length of *P. ochraceus* larvae over time. Measurements were taken beginning when larvae were 9 days old and in the gastrula stage of development. The last measurements were taken when larvae were 42 days old and in the brachiolaria stage of development. No significant differences in body length were found between treatments. (n=120, p>0.05). Values are mean body length ± one standard deviation.

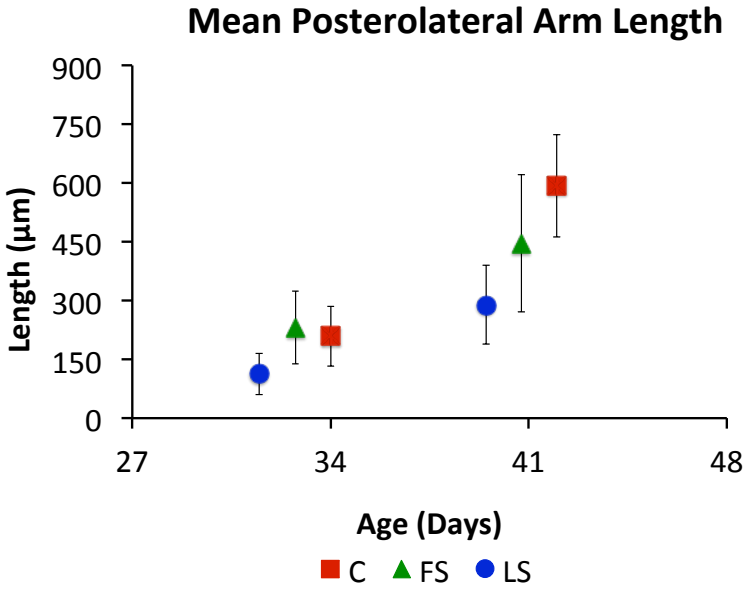

Figure 4. Mean posterolateral arm length of *P. ochraceus* larvae over time. Measurements were taken when larvae were 34 and 42 days old and in the brachiolaria stage of development. At 42 days old, differences in arm length between treatments was significantly different (p<0.05, n=120). Values are means ± one standard deviation.

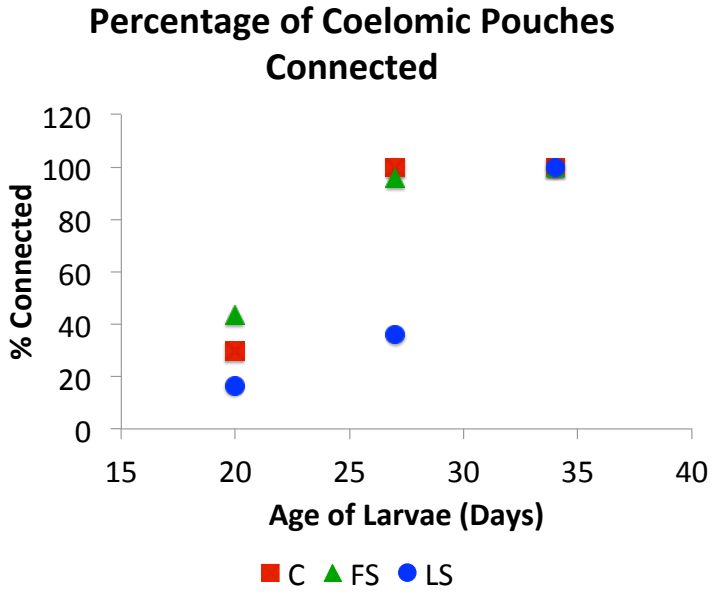

Figure 5. Percentage of coelomic pouches connected in *P. ochraceus* larvae. Counts taken when larvae were 27, 34, and 42 days old.

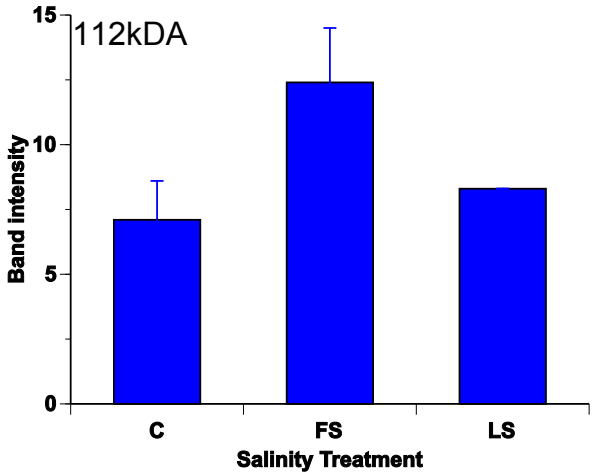

Figure 6: Band intensity of Na/K ATPase protein between treatments for 34 day-old larvae. Error bars are ± one standard deviation.

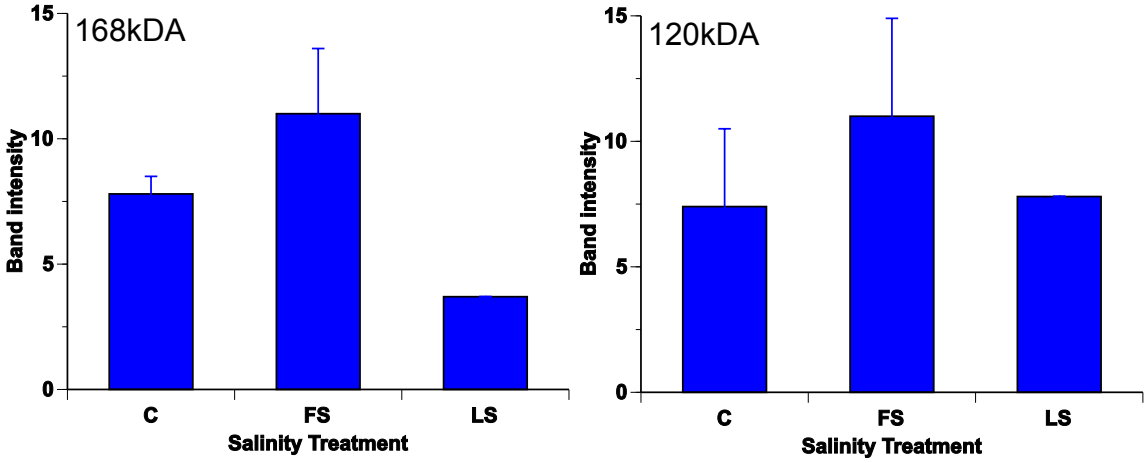

Figure 6: Band intensity of canalicular multi-specific organic anion transporter protein between treatments (left). Band intensity of calcium-transporting ATPase protein between treatments (right). All data are for 34 day-old larvae. Error bars are ± one standard deviation.

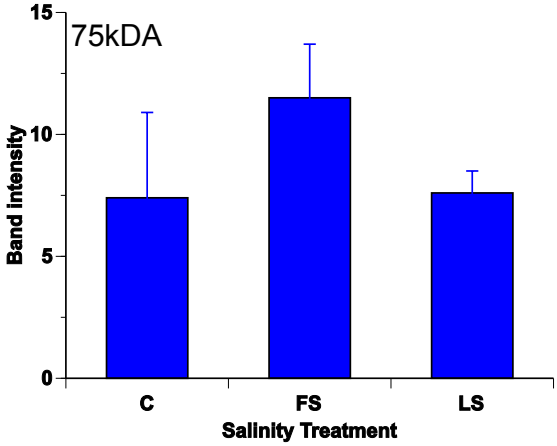

Figure 7: Band intensities of possible mechanosensory protein between treatments. Data are from 34 day-old larvae. Error bars are ± one standard deviation.