The effects of fluctuating salinity on protein profiles in echinoderm larvae of the species *Dendraster excentricus* and *Pisaster ochraceus*

Sam Bashevkin[1,2], Sophie George[1,3]

Blinks/REU/Beacon Fellowship 2013
Summer 2013

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

[2]Department of Biology, Tufts University, Medford, MA 02155

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

Contact Information:
Sam Bashevkin
Biology Department
Tufts University
Medford, MA 02155

Keywords: Pisaster ochraceus, Dendraster excentricus, larvae, fluctuating salinity, protein expression.

Abstract

Salinity fluctuations are common in the Salish Sea of the Pacific Northwest and are expected to become more common as global warming continues to increase the rate of glacial melting. During these fluctuations, the salinity can drop down to as low as 20‰ from the normal level of 30‰. This usually occurs during the summer months when many marine invertebrates are reproducing. Especially affected are echinoderms since they lack the ability to osmo- or ion-regulate. In this study we investigated the effects of fluctuating salinity on protein expression in larvae of the sand dollar *Dendraster excentricus* and the seastar *Pisaster ochraceus*. Adults were spawned and larvae were reared in 2 treatments: control (constant salinity of 29-30‰) and fluctuating salinity (FS, salinity dropped to 21‰ for 2 day periods every ~7 days). Samples were taken periodically, analyzed for protein content by the Bradford Assay, and separated by SDS PAGE to look for differences in banding patterns. A number of differences in protein expression were discovered in both *P. ochraceus* and *D. excentricus* larvae exposed to fluctuating salinity but the exact identity of these proteins are not known until mass spectrometry is completed. For 25 day old *P. ochraceus*, larvae in the fluctuating salinity treatment expressed 301-310 and 240 kDa proteins much more than the controls. Thirty-eight day old *D. excentricus* larvae in the fluctuating salinity treatment expressed much more of proteins that were 48, 74, 34, and 15 kDa, while the controls expressed much more of a 97 kDa protein. Proteins too important to be altered were also discovered, including 180, 98, and 81-82 kDa proteins in 37 day old *P. ochraceus* larvae and 46-47 kDa protein in 5 day old *D. excentricus* larvae. The proteins that differed likely function in metabolism, skeletogenesis, ion transport, or muscle development and function.

Introduction

Global warming has increased the rate of glacial melting, especially in the northern hemisphere (Shepherd *et al.* 2012). This has huge implications for organisms living in near-shore marine environments as increased glacial runoff causes dramatic salinity fluctuations. In the Salish Sea of the Pacific North West, salinity fluctuations are normal in summer months but have become even more frequent in recent years. During these fluctuations the salinity can drop from the normal level of ~30‰ to as low as 20‰ (Khangaonkar *et al.* 2011). These dramatic drops in salinity occur just as many species are spawning and the fragile planktonic larvae are dispersing. If salinity fluctuations prove detrimental to larval settlement rates, the results would be catastrophic for near-shore ecosystems. Adult populations of many of the most important species would decline and the ecosystems would be greatly altered, perhaps permanently.

Especially affected by these salinity fluctuations are echinoderm larvae since echinoderms lack the ability to osmo- or ion-regulate and therefore must be able to tolerate this wide salinity range as they disperse to their adult habitat. The sea star *Pisaster ochraceus* and the sand dollar *Dendraster excentricus* are both ecologically important species of the Pacific Northwest. *P. ochraceus* is a keystone predator of the rocky intertidal zone while *D. excentricus* is among the most abundant macro-organism found on sandy beaches in the Pacific Northwest (Merrill and Hobson 1970). Therefore, it is vitally important that we understand how these salinity fluctuations are affecting *P. ochraceus* and *D. excentricus* larvae since any disturbances to the dispersion of these species will have huge consequences for the ecosystems of this region.

Previous research has shown that sea star and sand dollar larvae exhibit morphological and behavioral changes in response to low salinity. A study on *P. ochraceus* found that larvae develop a wider and shorter morphology after exposure to low salinity for 7 or 14 days (Pia *et al.* 2012). Further research has found that larvae raised in constant low salinity (22‰) are slower

swimmers and have more trouble penetrating a halocline (Bashevkin and George 2012). George and Walker (2007) found that *D. excentricus* larvae developed an alternative morphology when reared in low salinity. Overall size, arm length, rudiment growth, and juvenile production were all affected.

Clearly, echinoderm larvae are greatly affected by changes in salinity. The next step is to discover the specific mechanisms by which fluctuations in salinity are altering the morphology and behavior of these larvae. Any adaptations of the larvae to low salinity conditions are likely mediated by differential protein production. Previous research has shown that reduced salinity can alter gene expression in oysters (Green and Barnes 2010). However, no studies to date have investigated the effect of fluctuating salinity on protein expression in marine organisms. In this study we investigated the effects of fluctuating salinity on protein profiles in *D. excentricus* and *P. ochraceus* larvae. We set out to answer the following question: 1) Do protein profiles differ between larvae reared in constant salinity and those reared in fluctuating salinity, and if so, which proteins differ? To answer this question, we compared the protein profiles of *D. excentricus* and *P. ochraceus* larvae reared in constant and fluctuating salinity treatments.

Methods

Collection and spawning of adults

Dendraster excentricus

Dendraster excentricus adults were collected in May and June 2013 during low tide at East Sound, WA (48.6969° N, 122.9042° W). For experiment 1, 7 adults were injected with 1ml 0.55M KCl on June 17th, 2013 to induce spawning. Five females and 1 male spawned and the eggs from all 5 females were combined and fertilized with 5 drops of concentrated sperm. For

experiment 2, 8 adults were injected with 1ml 0.55M KCl on July 22, 2013. Three females and 1 male spawned and eggs from all females were combined and fertilized with sperm.

Pisaster ochraceus

Pisaster ochraceus adults were collected in June 2013 at low tide in front of Friday Harbor Laboratories in Friday Harbor, WA (48.5344° N, 123.0158° W). On June 18, 6 individuals were injected with 1.5ml of 10^{-4}M 1-Methyl Adenine to induce spawning. One female and 4 males spawned and eggs were fertilized with 6-8 drops of sperm from only 1 male since the other sperm samples were contaminated with ciliates.

Larval rearing

After fertilization, *D. excentricus* and *P. ochraceus* embryos were transferred to 1-gallon glass jars filled with 0.45µm filtered seawater (FSW) at 30‰ that were kept in a sea table with flowing seawater to maintain a temperature of 13-15°C. Larval concentrations never exceeded 1 larva/ml. Larvae were periodically fed the microalgae *Isochrysis galbana*, *Dunaliella tertiolecta*, and *Rhodomonas spp.*

Experimental design

Experiment 1

Two days after fertilization, (when *D. excentricus* were in the 2-arm pluteus stage and *P. ochraceus* were in the gastrula stage) larvae of each species were split into 2 treatments: fluctuating salinity (FS) and control with 4 replicate jars/treatment. Larvae in the fluctuating salinity treatment were periodically exposed to low salinity for 2 day periods while larvae in the control treatment were constantly maintained at 30‰ (Fig. 1).

Experiment 2

Experiment 2 was only conducted on *D. excentricus* larvae and consisted of a single salinity fluctuation. Four days after fertilization (when larvae were at the 4-arm stage), larvae were split into FS and control treatments with 4 replicate jars/treatment and the salinity of FS jars was dropped to 21‰. The experiment was terminated 20 hours later.

Water was changed in all larval jars each day the salinity was changed in the FS treatment. The controls were treated exactly the same as the FS treatment except water of the same salinity (29-30‰) was added after draining. Water changes were accomplished by draining water levels down to about 500ml and then filling the jars back up with seawater of the appropriate salinity. Low salinity seawater was made by mixing reverse osmosis (RO) water with FSW.

Sampling for protein analysis

Eggs, embryos, and larvae were sampled periodically for protein profiling. For experiment 1, *D. excentricus* eggs (from all 5 females) were sampled on day 0, 2-arm plutei on day 2, 4-arm plutei on day 4, and 8-arm plutei on days 12 and 14. *P. ochraceus* eggs were sampled on day 0, gastrulae on day 2 and bipinnariae on day 4. For experiment 2, *D. excentricus* eggs were sampled from 2 females on day 0, 4-arm plutei were sampled on day 4 right before the salinity drop, and 4-arm plutei were sampled 20 hours later when the experiment was terminated (Table 1). Samples were concentrated via centrifugation at 6000 rpm then stored at -80°C.

Size measurements

All measurements were made with ImageJ software on pictures taken with a compound microscope at 40-100X. For every spawned female of each species, the diameters of at least 20 eggs were measured. In addition, total body lengths of 5 *P. ochraceus* larvae in each treatment

and longest post-oral arm lengths of 5 *D. excentricus* larvae in each treatment were measured before and after each salinity fluctuation.

Protein extraction

Samples were removed from the -80°C freezer and kept on ice throughout the process. For *P. ochraceus* eggs and gastrulae and *D. excentricus* eggs, the samples were vortexed thoroughly and then subsamples of 30-50 µl were removed for protein extraction. These subsamples were then washed 2 times with ultrapure water. Each wash involved adding 1 ml ultrapure water to the sample, vortexing, centrifuging at 6000 rpm and 4°C for 1.5-2 minutes, and then removing the supernatant. To lyse cells and extract protein, NP-40 lysis buffer was added to samples at a ratio of 30 µl lysis buffer per 50 µl of egg or gastrulae removed from the original sample tube. Samples were then lysed on ice for 30 minutes and vortexed for 25 second periods every 5-7 minutes. Next samples were centrifuged for 30 minutes at 13,200 rpm and 4°C. The resulting supernatant was collected in a separate tube and stored at -80°C. Later samples were treated the same way except 30µl of lysis buffer was added to entire samples.

Protein determination

Protein concentration of each sample was determined in triplicate with the Bradford protein assay (purchased from Bio-Rad). The standard curve ranged from 120-1000 µg/ml BSA. A 2.5µl aliquot of each sample was mixed with 7.5 µl ultrapure water and 500 µl Bradford reagent. Absorbance at 595nm of the resulting solution was measured with a spectrophotometer.

Gel electrophoresis

Proteins were separated by SDS-Page using pre-cast NuPage 4-12% Bis-Tris Gels and the MES buffer system. All reagents and supplies were purchased from Invitrogen. For all gels, 10-20 µg protein were run through each lane. Samples were prepared by mixing 10-20 µg of

protein with 6.25µl LDS, 2.5 µl DTT, and ultrapure water up to a final volume of 25 µl. Each sample was then heated for 10 minutes at 70°C and loaded onto the gel. Gels were run at 200V for 35 minutes.

Statistical analysis

Gels were analyzed with Kodak molecular imaging software.

Statistical analyses for larval size and protein content were conducted with JMP 10. The data were found to violate the assumptions of the ANOVA so the non-parametric Friedman test was run on data from each sample date to determine if larval size or protein content differed between treatments. For *D. excentricus*, the data from experiments 1 and 2 were combined and analyzed together in order to determine the overall effect of fluctuating salinity on larval size and protein content throughout the larval lifespan.

Results

Length

For *D. excentricus* larvae, there were no significant differences in mean arm length between treatments at 4, 5, 12, 14, 21, and 23 days old (repeated Friedman tests, $p>0.7290$). However, at 26 days old, 8-arm plutei in the FS treatment had significantly longer arms than the controls (Friedman test, $p=0.0066$) (Fig. 2A).

For *P. ochraceus* larvae there were no significant differences in mean length between treatments at any measurement time (repeated Friedman tests, $p>0.0978$) (Fig. 2B).

Protein content

For both *P. ochraceus* and *D. excentricus* larvae, protein content did not differ significantly between treatments (repeated Friedman tests, $p>0.6336$) (Fig. 3).

Protein profiles

Until mass spectrometry is completed the identity of protein bands is uncertain but estimates can be made from the bands' molecular masses.

P. ochraceus

The greatest differences in band intensity were observed in 25 day old *P. ochraceus* larvae at the late bipinnaria stage (Fig. 4). Larvae in the fluctuating salinity (FS) treatment expressed proteins with molecular weights of 301-310 and 240 kDa that were almost completely absent in control larvae. The 301-310 kDa band was also present in unfertilized eggs (Fig. 5).

Some bands were found to be similar across treatments. In 37 day old larvae, protein bands at 180, 98, and 81-82 kDa were present in both treatments (Fig. 6).

D. excentricus

Thirty-eight day old *D. excentricus* larvae exhibited the most noticeable differences in protein expression. Control larvae expressed much more of a 97 kDa protein while FS larvae expressed 4 proteins much more than the controls; 48, 74, 34, and 15 kDa (Fig. 7). The 97 and 74 kDa bands appear to also be present in unfertilized *D. excentricus* eggs (Fig. 8).

Five day-old *D. excentricus* larvae all expressed a 46-47 kDa protein (Fig. 9).

Discussion

Although overall protein content and larval length (except in one case) were not affected by salinity treatment, protein expression was, as evidenced by clear differences in band intensity after SDS-PAGE.

Twenty-six day old, 8-arm *D. excentricus* plutei in the FS treatment had significantly longer arms than those in the control treatment but there was no significant difference at other measurement dates. This contrasts with the results of George and Walker (2007) who found that *D. excentricus* larvae raised in constant, normal salinity were always larger than those raised in

constant low or FS conditions. However, George and Walker noted that salinity had less of an effect on 8-arm stages, so it is possible that the FS larvae were bigger by the 8-arm stage because they were no longer strongly affected by the salinity. It is also possible that the FS larvae in this experiment invested more energy into growing longer larval arms for feeding while the control larvae invested more energy into growing the rudiment (see Strathmann et al. 1992).

Our values for protein content of 8-arm, 25 day old *D. excentricus* plutei agree well with the results of a study by McEdward (1984), although our values (~100ng/larva) are slightly lower than McEdwards (~180ng/larva). This difference could very well be a reflection of the different methods used to extract protein rather than a real difference in protein contents. While we used NP-40 lysis buffer to extract proteins, McEdward used ETS-B buffer. These differences in protein content could indicate that ETS-B buffer is more efficient in extracting protein from these larvae than NP-40 lysis buffer.

From comparing the molecular weights of proteins of interest to values in the literature (Anderson *et al.* 1993, Guttman and Nolan 1994), we came up with candidate identities for a number of these proteins. For 37 day old *P. ochraceus* larvae, one of the proteins that was conserved (unchanged) between treatments could be phosphorylase b or amyloglucosidase. For 37 day old *D. excentricus* the protein that was present more in controls than in FS larvae may also be either phosphorylase b or amyloglucosidase. The 4 proteins present more in FS than controls of this sample might represent fumarase, carboxypeptidase N, glycerophosphate dehydrogenase, glucokinase, pepsin, lysozyme, or myoglobin. In 5 day old *D. excentricus* larvae, the protein that was conserved between treatments could be a carboxypeptidase.

The most prominent group of proteins affected by fluctuating salinity seemed to be those involved in metabolism. Previous studies have demonstrated that *P. ochraceus* larvae reared in

low salinity are slower swimmers and have more trouble penetrating a halocline (Bashevkin and George 2012). If this altered swimming ability is due in part to lower energy levels it would make sense that larvae exposed to low salinity would have different metabolic demands and therefore express different metabolic proteins. Both O'Donnell *et al.* (2010) and Stumpp *et al.* (2011) found that sea urchin plutei reared in acidified seawater altered their expression of metabolic genes. Ocean acidification presents similar challenges to plutei as low salinity (increased difficulty of calcification) so a similar molecular response would be expected.

As previously mentioned, past studies have demonstrated that *P. ochraceus* larvae reared in low salinity are weaker swimmers. Therefore, we could expect changes in expression of muscle proteins after larvae are exposed to fluctuating salinity. It is possible that FS larvae expend so much energy on ion regulation and damage repair that they cannot afford to grow fully developed muscles. A study by Dyachuck *et al.* 2013 has identified many of the proteins present in larval muscle development, so future studies could investigate whether these proteins are up or down regulated in larvae exposed to fluctuating salinity.

Skeletons composed of calcitic spicules are vital for the success of *D. excentricus* plutei and spicules that develop in the rudiment of seastar brachiolariae are important for juvenile success in *P. ochraceus* (Hamanaka 2011). Low salinity presents problems to calcifying organisms because of the lower calcium concentration in low salinity seawater which both increases the energy required to incorporate calcium into their skeleton or shell and increases the dissolution rate of calcified elements. So these organisms in low salinity conditions must expend more energy to calcify and must calcify at a greater rate to outpace the rate of dissolution. Therefore, it would be expected that the expression of proteins involved in calcification and skeletogenesis would be affected by fluctuating salinity. While we do not know for sure whether

the protein differences we observed are related to skeletogenesis, previous studies on the effects of ocean acidification (which poses similar problems to calcification as low salinity) on echinoderm larvae have found differential expression of skeletal matrix and calcification proteins in echinoderm larvae (O'Donnell *et al.* 2010, Stumpp *et* al 2011). Since these proteins were affected in acidified conditions, it is very likely that the protein differences we observed are in proteins related to skeletogenesis or calcification.

Another class of proteins that were very likely altered by fluctuating salinity conditions is ion transport proteins. Due to the drastically different ionic conditions of 21 vs 30‰ seawater, we would expect proteins involved in ion transport to be affected so that the many cellular processes which rely on ionic concentrations can still proceed as normal. As described above, ocean acidification conditions result in similar stresses on echinoderm larvae as low salinity. Much like low salinity seawater, acidified seawater will have a different ionic balance than normal seawater, resulting in ionic stresses on the cellular level. In fact, previous studies have documented changes in the expression of ion transport genes in sea urchin plutei exposed to acidified seawater (O'Donnell *et al.* 2010, Stumpp *et al.* 2011).

More work is needed to identify the specific proteins that were differentially expressed in FS larvae in this species. This will be accomplished with HPLC and mass spectrometry. In addition, future studies should investigate the changes in gene expression and genetic mechanisms that underlie the observed differences in protein profiles. Other studies could investigate how the molecular response is affected by the severity and frequency of salinity fluctuations.

This study is the first of its kind to investigate the effects of fluctuating salinity on protein expression in marine invertebrate larvae. We have discovered a number of clear differences in

protein expression that will be investigated further with mass spectrometry. Salinity fluctuations are common in the Salish Sea and many estuarine systems during summer months and are expected to become even more common in the future as global warming continues to cause glaciers to melt at an increasing rate. It is vitally important to understand how local species of this very biodiverse region react to salinity fluctuations so we can better predict how these ecosystems will be affected by the threat of anthropogenic global warming.

Acknowledgements

We would like to thank Friday Harbor Laboratories, the University of Washington, and Billie Swalla for providing us with lab space and support, and Gary Wessel, Steve Stricker, and Michelle Herko for providing technical support. This study was funded by an NSF REU grant (DBI 1262239) to Adam Summers and the Anne Hoff Blinks endowment.

Literature Cited

Anderson BL, Berry RW, Telser A. 1983. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis system that separates peptides and proteins in the molecular weight range of 2500 to 90,000. Anal. Biochem. 132: 365-375.

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.

Dyachuk V, Odintsova N. 2013. Larval myogenesis in Echinodermata: conserved features and morphological diversity between class-specific larval forms of Echinoidae, Asteroidea, and Holothuroidea. Evolution and development. 15: 5-17.

George SB, Walker D. 2007. Short-term fluctuation in salinity promotes rapid larval development and metamorphosis in *Dendraster excentricus*. J. Exp. Mar. Biol. Ecol. 349:

113-130.

Green TJ, Barnes AC. 2010. Reduced salinity, but not estuarine acidification, is a cause of immune-suppression in the Sydney rock oyster *Saccostrea glomerata*. Mar. Ecol. Prog. Ser. 402: 161-170.

Guttman A, Nolan J. 1994. Comparison of the separation of proteins by sodium dodecyl sulfate-slab gel electrophoresis and capillary sodium dodecyl sulfate-gel electrophoresis. Anal. Biochem. 221: 285-289.

Hamanaka F, Hosaka E, Kuraishi R, Hosoya N, Matsumoto M, Kaneko H. 2011. Uneven distribution pattern and increasing numbers of mesenchyme cells during development in the starfish, *Asterina pectinifera*. Growth Differ. 53: 440-449.

Khangaonkar T, Zhaoqing Y, Taeyun K, Roberts M. 2011. Tidally averaged circulation in Puget Sound sub-basins: Comparison of historical data, analytical model, and numerical model. Estuar. Coast. Shelf Sci. 93: 305-319.

McEdward LR. 1984. Morphometric and metabolic analysis of the growth and form of an echinopluteus. J. Exp. Mar. Biol. Ecol. 82: 259-287.

Merrill RJ, Hobson ES. 1970. Field observations of *Dendraster excentricus* a sand dollar of Western North America. Amer. Mid. Nat. 83: 595-624.

O'Donnel MJ, Todgham AE, Sewell MA, Hammond LM, Ruggiero K, Fangue NA, Zippay ML, Hofmann GE. 2010. Ocean acidification alters skeletogenesis and gene expression in larval sea urchins. Mar. Ecol. Prog. Ser. 398: 157-171.

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

Shepherd A, *et al.* 2012. A reconciled estimate of ice-sheet mass balance. Science. 338: 1183-

1189.

Strathmann RR, Fenaux L, Strathmann MF. 1992. Heterochronic developmental plasticity in larval sea urchins and its implications for evolution of nonfeeding larvae. Evolution. 46: 972-986.

Stumpp M, Dupont S, Thorndyke MC, Melzner F. 2011. CO2 induced seawater acidification impacts sea urchin larval development II: Gene expression patterns in pluteus larvae. Comp. Biochem. Phys., A. 160: 320-330.

Figures

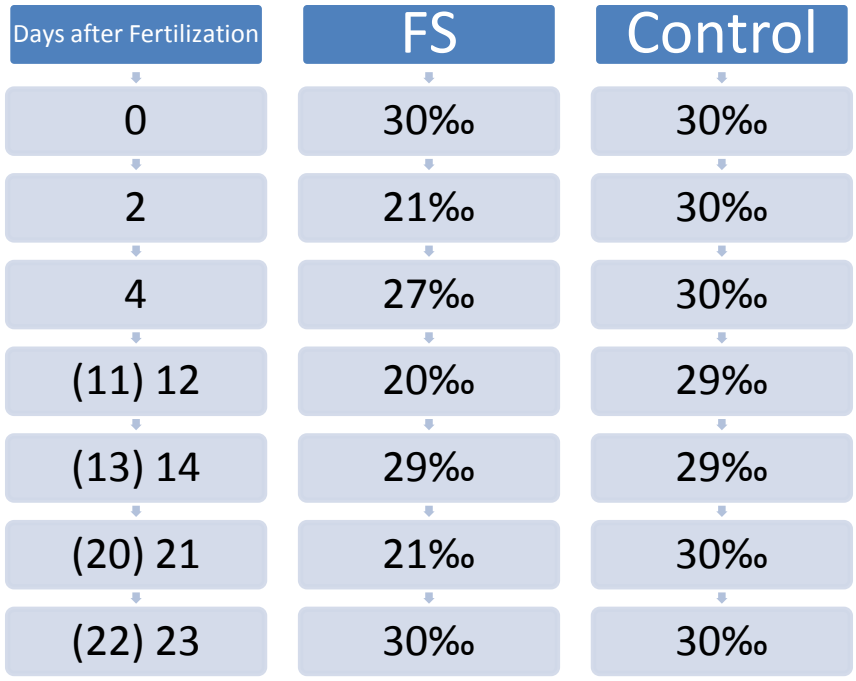

Figure 1. Experimental design of larvae salinity treatments. *D. excentricus* and *P. ochraceus* larvae were split into 2 treatments with 4 replicate jars/treatment: fluctuating salinity (FS) and control. Ages in parentheses represent the age of *P. ochraceus* larvae. FS larvae were exposed to low salinity for 2 day periods while control larvae were always in the same salinity.

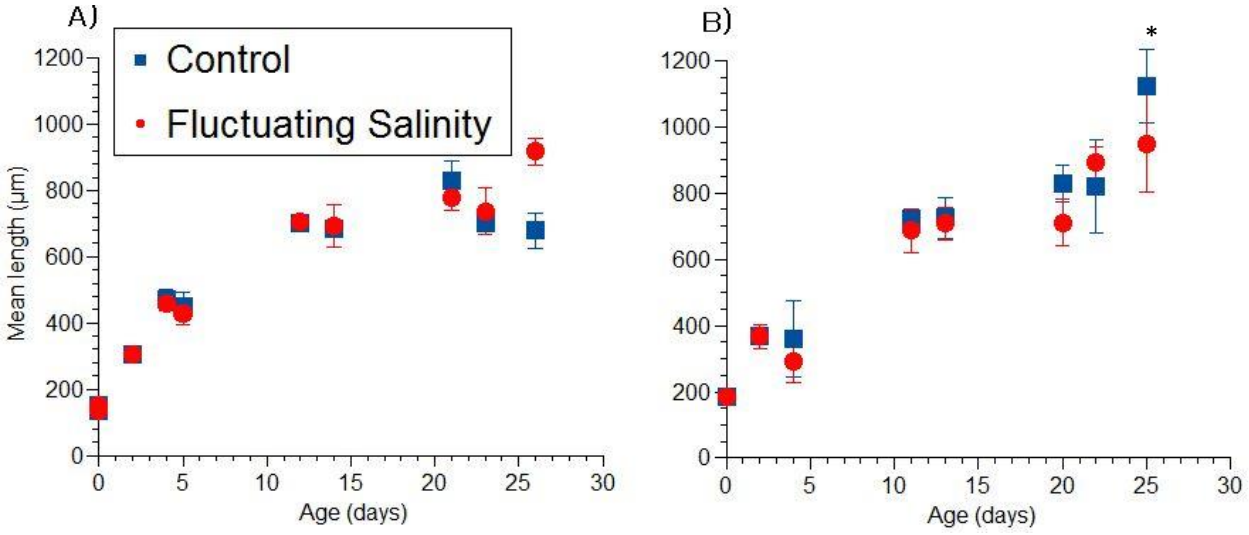

Figure 2. Mean length (µm) of developing A) *D. excentricus* and B) *P. ochraceus* in fluctuating salinity and control treatments. Measurements at age 0 represent egg diameters while the remaining measurements are total larval length for *P. ochraceus* and pre-oral arm length in *D. excentricus* larvae. Each data point represents the mean measurement of 20 eggs or 5 larvae and error bars represent 1 standard deviation. An asterix represents the only time point at which there were significant differences between treatments (repeated Friedman tests, bonferroni adjusted $p<0.05$).

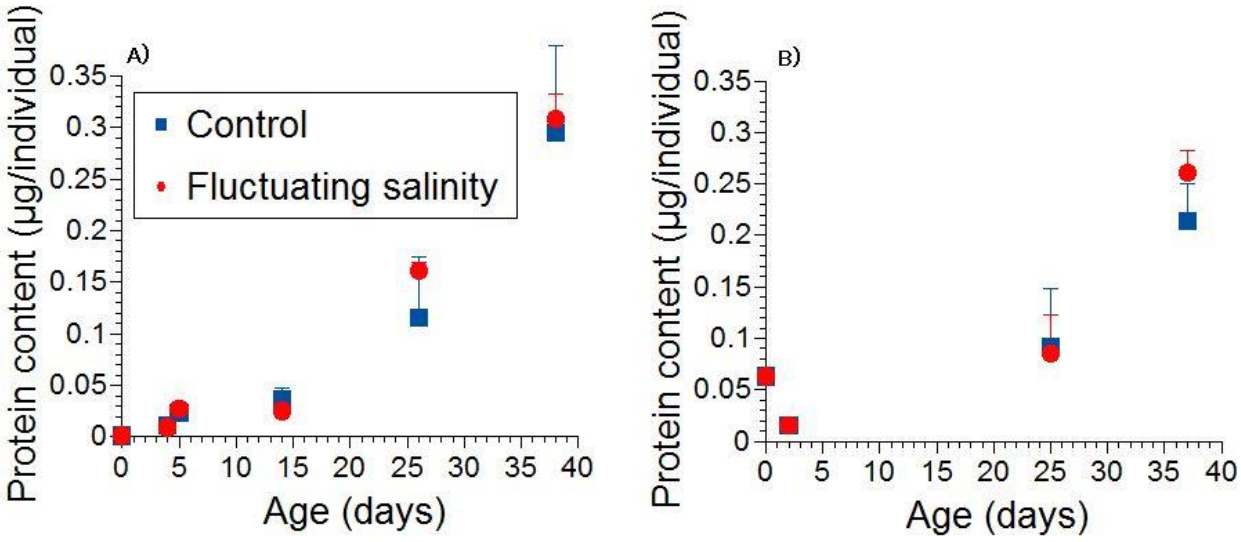

Figure 3. Mean protein content (µg) per individual in developing A) *D. excentricus* and B) *P. ochraceus* in fluctuating salinity and control treatments. For *P. ochraceus*, unfertilized eggs were collected at age 0, gastrulae at age 4 and all remaining data are from larvae. For *D. excentricus*, unfertilized eggs were collected at age 0, and larvae at every other sample date. There were no significant differences between treatments (repeated Friedman tests).

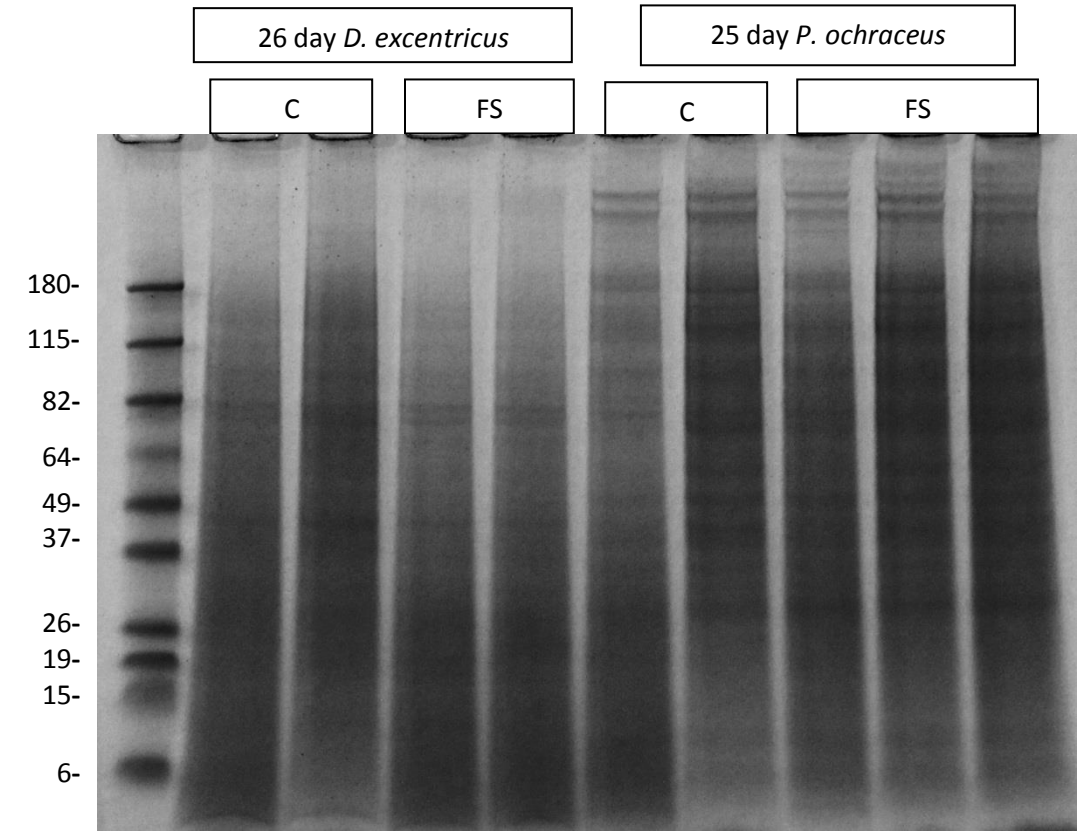

Figure 4. Gel with 26 day-old *D. excentricus* and 25 day-old *P. ochraceus* larvae from control (C) and fluctuating salinity (FS) treatments. The numbers on left represent molecular weight markers in kDa.

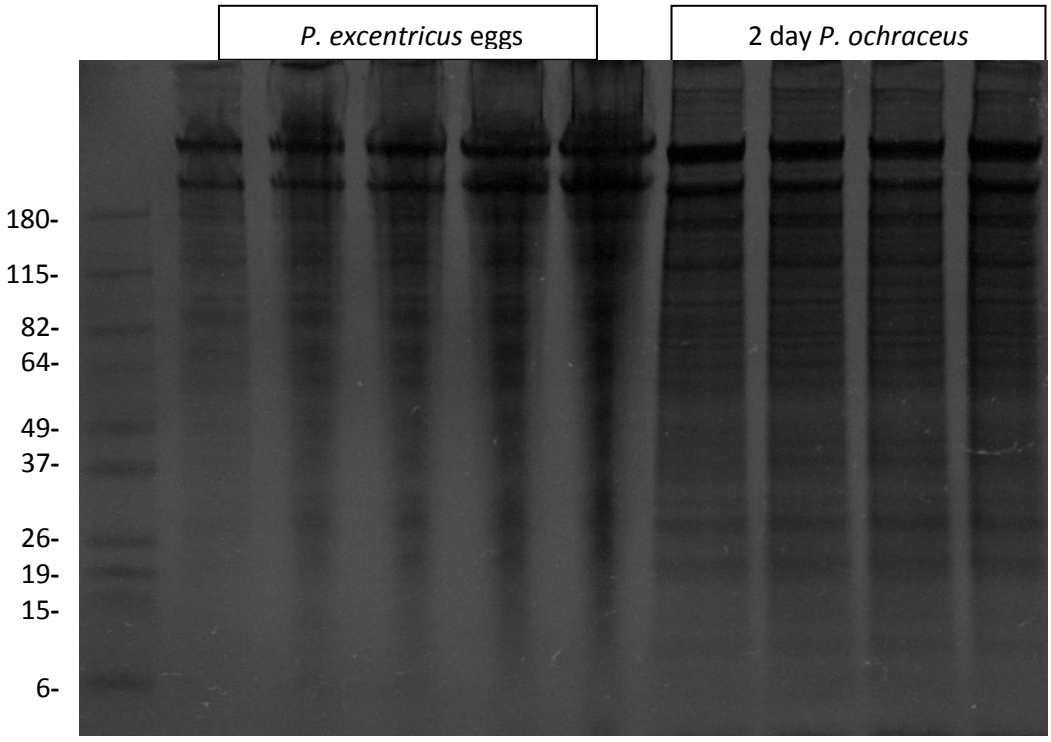

Figure 5. Gel with *P. ochraceus* eggs and 2 day old gastrulae. The numbers on left represent molecular weight markers in kDa.

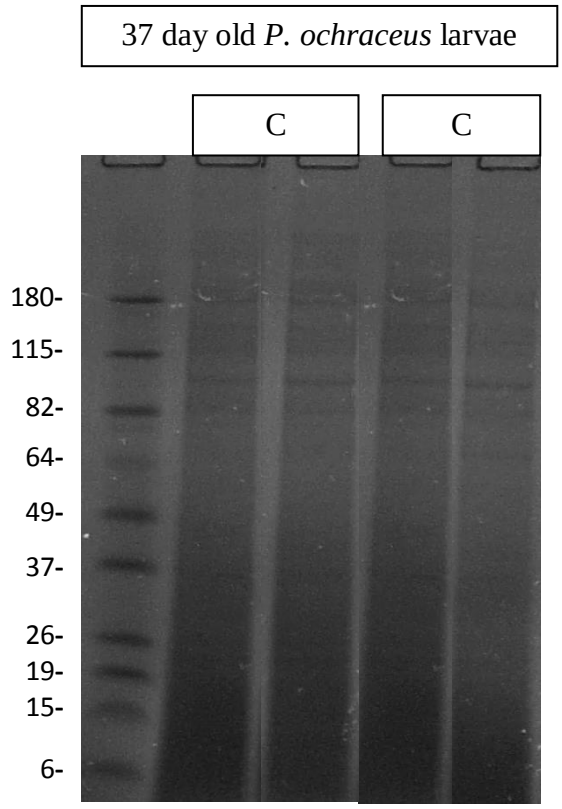

Figure 6. Gel showing 37 day old *P. ochraceus* larvae in control (C) and fluctuating salinity (FS) treatments. The numbers on left represent molecular weight markers in kDa.

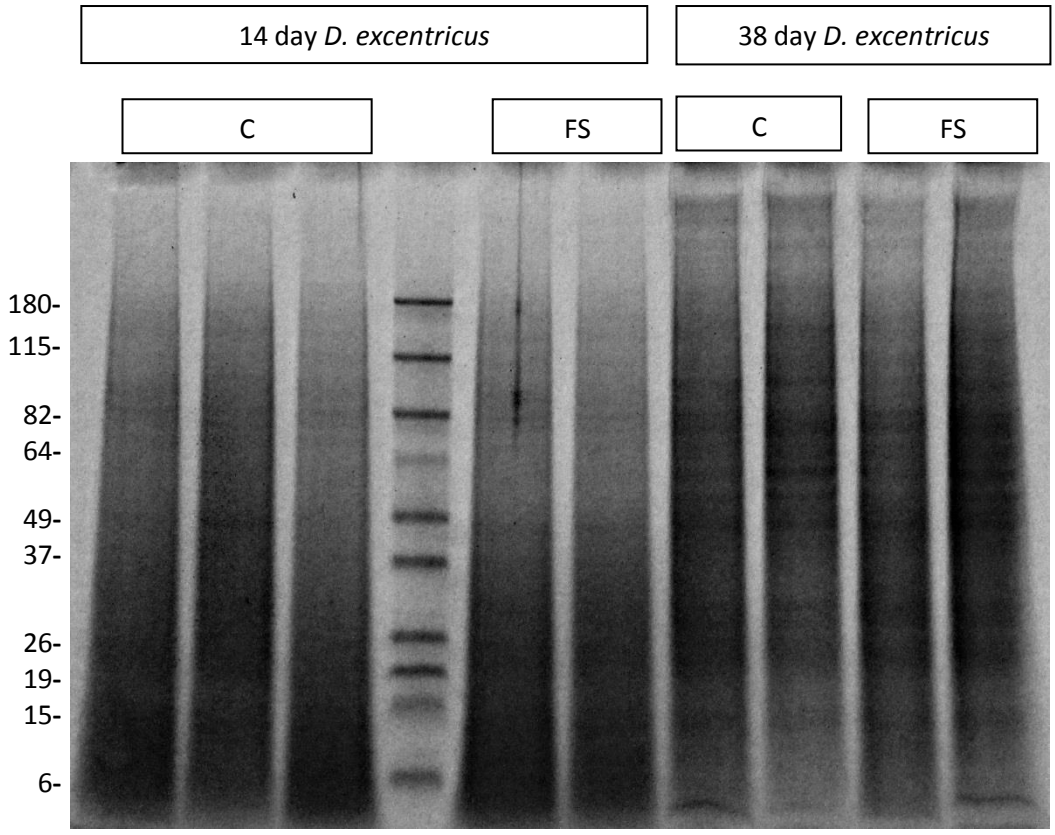

Figure 7. Gel showing 14 and 38 day *D. excentricus* from control (C) and fluctuating salinity (FS) treatments. The numbers on left represent molecular weight markers in kDa.

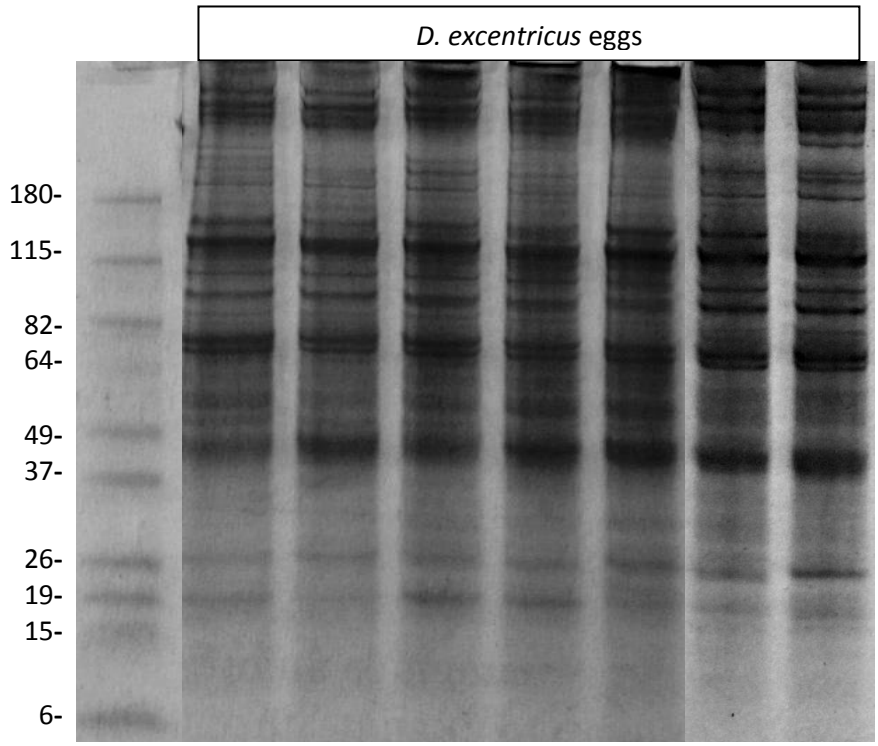

Figure 8. Composite image of 2 gels with *D. excentricus* eggs from 7 females. Numbers on the left represent molecular weight markers in kDa

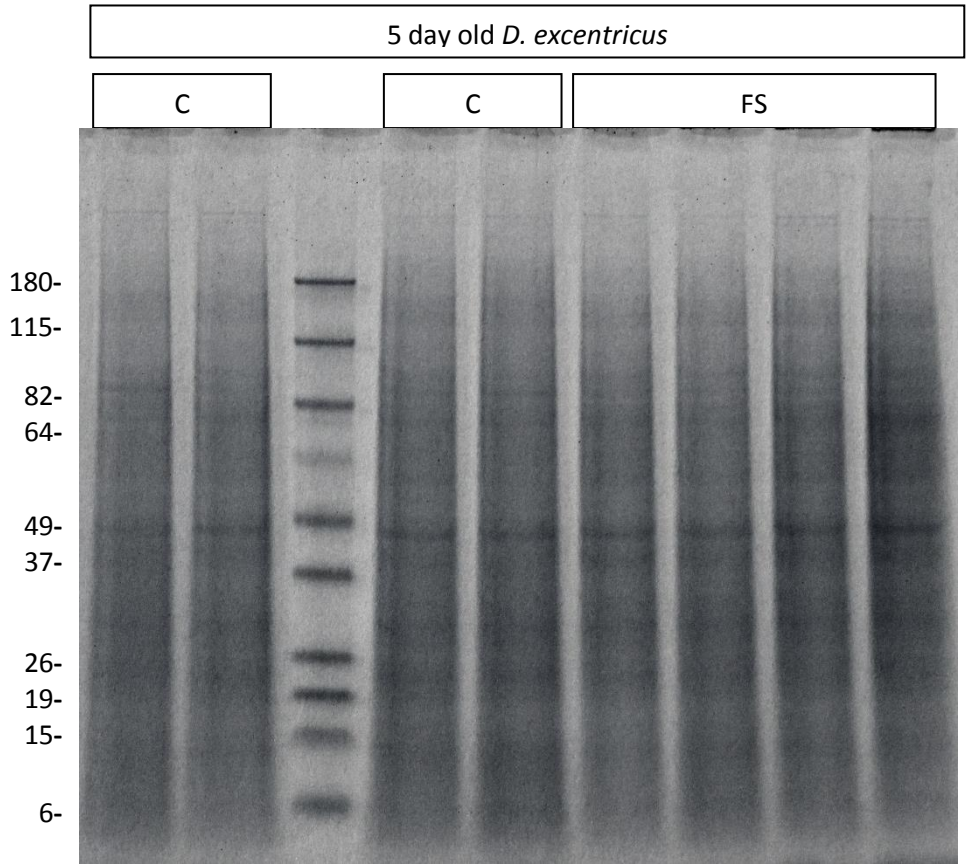

Figure 9. Gel showing 5 day old *D. excentricus* in control (C) and fluctuating salinity (FS) treatments. The numbers on left represent molecular weight markers in kDa.