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Effects of photoperiod and mechanical stress on Olympia oyster physiology

This work is fullfills a course requirement for FISH495

Abstract

Once dominant along the North American west coast, Olympia oyster (*Ostrea lurida*) populations have declined significantly since the early 1900's. Restoration efforts have encountered many problems, one of which is the slow growth of *O. lurida*. This study aims to determine the effect of photoperiod and mechanical stress, environmental factors controllable in an aquaculture setting, on *O. lurida* stress and growth physiology. Natural photoperiod and absence of mechanical stress were expected to elicit a greater growth response. Oysters were separated into 12-hour light:12 dark or 24-hour light photoperiod treatments followed by mechanical stress or no mechanical stress. Tissue of oysters was analyzed using quantification of genes related to stress and growth. Results of stress genes indicated 12:12 photoperiod with mechanical stress induced greater stress. Growth genes implied 24-hour photoperiod with mechanical stress induced greater growth. Findings that 24-hour light was less stressful contradicted predictions that oysters would be better suited for conditions similar to natural lighting. The longer 24-hour light treatment could signal the growing season, which generally occurs in the summer months. Indications of growth in the 24-hour with stress treatment were also of interest. The results suggest that mechanical stress may play a role in stimulating growth in oysters. During the study, no growth was actually measured meaning quantification of genes only suggests possible physiological changes. Future work will aim to verify our results with real measured growth.

Introduction

Olympia oyster (*Ostrea lurida*) is the native oyster species of the Pacific Northwest with natural habitat ranging from the Californian to Canadian coast (White et al. 2009). In the past, *O. lurida* have been prized as a luxurious delicacy, but at the turn of the 20th century, introductions of the Eastern oyster (*Crassostrea virginicus*) and the Pacific oyster (*Crassostrea gigas*) replaced *O. lurida* as the dominant fisheries species (White et al. 2009; Beck et al. 2011; Dumbauld et al. 2011). This was largely due to the small size and slow growth of *O. lurida*, which made it less suitable for commercial aquaculture. Since this transition, *O. lurida* numbers have decreased sharply over the years. In more recent years, restoration of native species, such as *O. lurida,* has become a priority in many areas including the Pacific Northwest. Problems with this process have emerged, which include the lack of available habitat, proper nutrients and the relatively strenuous requirements for settlement (White et al. 2009; Wasson 2010). *O. lurida* require a much thicker and calmer culch bed than the dominant oyster species in the region, *C. gigas*. Additionally, the relatively slow growth and maturation have also slowed recovery efforts.

Unfortunately, due to the back seat *O. lurida* has taken to other oyster species over the last few decades, extensive research has not been invested in the species. While the restoration efforts have led to a slew of papers being published citing the ecological benefits of the Olympia oyster, research on physiological changes have been absent (White et al. 2009; Beck et al. 2011;

Dumbauld et al. 2011). Of the limitations on *O. lurida* success, slow growth can be considered one of the most prominent factors (Wasson 2010). Literature specific to Olympia oyster growth is lacking but growth is well studied across a wide range of animals. The simplest growth studies are done my measuring the animal before and after treatment. While effective for certain animals, this method does not suffice for bivalves due to lack of correlation between shell growth and tissue growth (Kimbro et al. 2009). Weighing of animals is also imprecise as volume of seawater found in the bivalve can vary at any time. Furthermore, measurements on bivalves can be skewed by inconsistent gamete production. Therefore, measuring growth in Olympia oyster will require an indirect method.

One popular method revolves around an energy budget model termed scope for growth (SFG), based on the idea that energy assimilated in the animal is allocated to specific functions (Widdows and Johnson 1988). Energy is first allocated towards essential body maintenance and then excess energy can be used towards growth, reproduction, and storage. Another similar energy budget model called dynamic energy budget (DEB) diverges from SFG in that it assumes energy is first stored and then allocated out (Pouvreau et al. 2006). This method is also noteworthy for assuming common processes in most animals allowing the DEB models to be easily applied different animals without modification of the model. Although SFG and DEB models differ in some assumptions, both share the fundamental goal of explaining growth due to environmental factors. Both of these models have been used successful in determining growth and energy expenditure in a variety of bivalves including Blue mussels (Mytilus edulis), Eastern oyster (*C. virginicus*), and Pacific oyster (*C. gigas*) (Widdows and Johnson 1988; Cherkasov et al. 2006; Pouvreau et al. 2006). These models also verify that growth can vary depending on the environment. Current research on factors impacting growth such as food availability and environment are extensive. Specific to marine species, environmental factors commonly studied include water temperature, osmolarity, and pH. For example, *O. lurida* has been found to spawn above temperatures of 16^{o}C and display maximal growth at around 12^{o}C (Seale and Zacherl 2009). Another commonly studied factor, particularly for bivalves, is water flow, because of its importance in the animal's ability to obtain food, oxygen, and remove waste as filter feeders (Jones et al. 2005). Due to their significant effects, each of these factors has been well studied.

Two factors that are not as prominent, photoperiod and mechanical stress, are examined in this study. While both environmental factors have been shown to regulate physiological changes in other species, including vertebrates and invertebrates, no research has been presented specific to *O. lurida*. Effects of photoperiod on growth have been well documented in other species of oyster, fish, and bivalves (Fabioux et al. 2005; Domínguez et al. 2010; Türker et al. 2011). Mechanical stress has also been shown to impact regulation of certain genes in the Pacific and Eastern oysters (Lacoste et al. 2002; Roberts et al. 2012). Beyond simply impacting physiological change, photoperiod and mechanical stress could be important factors in restoration efforts because they are controllable in aquaculture settings. Photoperiod may be particularly useful in hatchery settings as it can be easily controlled and have significant impact on vulnerable early life stages such as larvae and spat. Mechanical stress acts to mimic potential transportation movements in the hatchery setting or tidal flow during grow out. Grow out location may be of particular interest because there have been studies suggesting *O. lurida* do not survive well in strong tidal zones (Kimbro et al. 2009; White et al. 2009; Wasson 2011;

Dumbauld et al. 2011). This causes *O. lurida* to be generally found in protected and sheltered waters. Better understanding of the effect of mechanical stress may allow conservationists to better predict locations where *O. lurida* can flourish.

With technological advancements, new methods of indirect growth such as gene expression have been developed. Similar to SFG and DEP models, gene expression can be used to estimate growth in individuals due to environmental factors. The difference is that instead of using a model of energy budget to determine growth, we use the quantity of specific expressed genes to determine growth. In order to undergo physical changes, animals use processes and pathways within the individual that are mediated by proteins. The proteins are produced from mRNA, which are temporary strands of genetic information transcribed from genomic DNA. The gene expression method assumes that increased mRNA levels correspond to increased protein levels, and subsequent physiological changes. This method has been used successfully in studies of oyster species and relies on our knowledge of gene function (Meistertzheim et al. 2007; Roberts et al. 2012). If expression of genes related to growth can be targeted, then differences in expression of those genes will signify changes in growth. Unfortunately, gene function for *O. lurida* is not well studied. Therefore, we assumed that *O. lurida* genes sharing similar sequences with other species may also share a similar function with those species. Out of these conserved genes, six genes related to either grow or stress response were selected for this study.

Stress genes can be used as proxies for growth because studies have found increases in stress often slow growth (Calabrese et al. 1977; Berge et al. 2006). The SFG and DEB models affirm this finding because the increased energy expenditure to mitigate stressors will reduce the available energy for growth. This conclusion has been found with a variety of stressors and in a wide range of animals suggesting the universal nature of this correlation. One stress related gene, BCL2-associated athanogene (Bag) produces a range of proteins all characterized by their BAG domain near the C-terminus (Kabbage and Dickman 2008). Of these proteins, Bag has specifically been found to inhibit certain factors that are key to the ability of heat shock protein 70kDA (hsp70) to respond to stress (Arndt et al. 2005; Ueda and Boettcher 2009). These findings suggest that stressful situations will lead to down regulation of BAG-2. Another stress induced molecular chaperone is heat shock protein 90kDA (Hsp). This protein is highly conserved and serves a variety of functions including protein folding and degradation (Chen et al. 2005; Li et al. 2008; Zhao et al. 2011). Stressors, including temperature, osmolarity, and pathogens, have all led to increased Hsp expression in marine animals (Zhao et al. 2011). The same pattern is expected to occur in *O. lurida*. Finally, U2 small nuclear RNA auxiliary factor 1-like 4 (U2a) is a splicing factor that has been associated with processing of growth and stress related proteins (Krainer and Maniatis 1985; Maniatis and Reed 1987). The splicing factor works to splice pre–mRNA into functional mRNA allowing the body to potentially adjust the functionality of a gene. Studies have found splicing to be inhibited by stress, possibly to moderate gene expression while the organism deals with the stressor (Wells et al. 1994; Selenko et al. 2001). Given these findings, we expect expression of U2a to be suppressed during stressful events.

Growth typically occurs when energy availability is high within the organism. Muscle glycogen phosphorylase (Pygm) is a protein that breaks down glycogen energy stores in muscle to provide ATP (Carty et al. 1975; Johnson 1992). Research on C. gigas has shown Pygm levels

are up regulated during the growing season, likely to fuel cellular growth (Prudence et al. 2006). We expect the *O. lurida* to display similar physiological control and therefore Pygm levels will be highest when the animal is in a growth stage. Protein kinase, cGMP dependent, has been shown to be associated with a variety of physiological pathways (Lohamm et al. 1997). Of these, two pathways are related to growth and stress. First, Pkrg plays a role in apoptosis induction during high stress situations, most notably displaying increased levels in tumor tissue (Isshiki et al. 2012). Second, Pkrg also has been found to interfere with Ca+ signaling in muscle tissue leading to decreases in muscle contraction (Lincoln et al. 2001). In both instances, increased Pkrg levels are correlated with stress responses and decreased energy expenditure. Finally, insulin-like growth factor 1 receptor (Igfr), a transmembrane receptor associated with IGF-1, has been linked to the anabolic growth of muscle. Certain studies have shown significant decreases in body mass when lacking in the IGF-1 receptor protein and in other cases inability to develop properly has led to death. High levels of Igfr are expected in animals displaying steady growth.

Given the importance of growth rate in restoration efforts and aquaculture possibilities, information on environmental factors affecting growth and stress should be beneficial to the scientific community concerned about *O. lurida* success. Measuring the expression of the six selected genes will provide information towards this goal. Specifically, this study aims to provide information on the effect of 1. Photoperiod 2. Mechanical stress, and 3. Combined photoperiod and mechanical stress, on the stress response and growth physiology of Olympia oyster. We expect both photoperiod and mechanical stress to have significant impacts on *O. lurida* physiology. The shorter photoperiod will induce higher levels of growth and less stress while mechanical stress will produce the opposite response. We also believe there will be a synergistic effect when longer light and mechanical stress are combined due to the stress passing a threshold level.

Methods

Growing and sampling

A total of 44 *O. lurida* were randomly split into two treatment groups. The first group of 22 oysters was subject to 12 hours of light and 12 hours of dark per day. The second group of 22 was subject to 24 hours of light per day. Light was produced using fluorescent light bulbs. All oysters were grown in otherwise identical conditions at 15^{o}C with sufficient air circulation and feed for 14 days. Each treatment group was then randomly split in half again, with one half receiving mechanical stress and the other half not. Mechanical stress consisted of 5 minutes centrifugation at 1000rpm and 15^{o}C with a Sorvall centrifuge (Thermo Fisher Scientific Inc., Wilmington, DE, USA). This experimental design allowed comparison of four treatment types: 1). control (12 hr. light: 12 hr. dark) 2) without mechanical stress 2.) 24 hr. light without mechanical stress 3). control with mechanical stress and 4). 24 hr. light with mechanical stress. Each treatment type contained 11 oysters.

Extraction and Quantification

After treatments, oysters were immediately shucked and tissues samples of gill and

mantle were taken separately. During sampling, tissue was collected in microfuge tubes and quickly placed on dry ice. Tissue samples were then stored at -80°C for two weeks before further manipulation. A small piece of gill tissue (~100mg) was taken from the full tissue sample and RNA was extracted using the standard manufacturer's protocol for TRI Reagent® (TRI Reagent 1995). This involved placing the tissue in 1ml of TRI Reagent®, followed by 0.2ml of chloroform. The mixture was centrifuged at 12,000xg for 15mins at 4°C. Supernatant was collected and 0.5ml of isopropanol was added. The mix was centrifuged at 12,000xg for 8min at 4°C. The RNA pellet that formed was washed using 1ml of 75% ethanol and centrifuged at 7,600xg for 5min at 4°C. Extracted RNA pellets were solubilized in DEPC water and stored at -80°C. In order to confirm success of RNA extraction, concentration and purity of the RNA extract were determined using the NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Then unwanted DNA in the extract was removed using DNase treatment from the TURBO DNA-*free*™ Kit (Ambion Inc., Foster City, CA, USA). A qPCR was run on a subset of DNase treated samples to ensure the treatment was successful.

Reverse transcription was then performed on all Dnase-treated RNA samples using Promega M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). For this procedure, a single reaction volume consisted of 237.32ng of RNA template diluted in 17.75ul PCR H2O. A volume of 0.5uL oligo dT primers (0.25ug) were added and the mix was allowed to anneal in a thermal cycler for 4mins at 70°C. Then 5 uL M-MLV RT Buffer, 1.25 uL 10mM dNTPs, and 0.5 uL M-MLV RT were added, vortexed, and spun down. The cycling parameters for this mix were 60min at 42 °C, followed by 3min at 95 °C. The cDNA product was stored at -20 °C until later use.

A qPCR procedure run on all samples using a CFX96 Touch™ Real-Time PCR Detection System was performed (Bio-Rab Laboratories Inc., Hercules, CA, USA). Samples were prepared using SsoFast™ EvaGreen® supermix (Bio-Rab Laboratories Inc., Hercules, CA, USA). A single reaction volume consisted of 10uL SsoFast™ EvaGreen®, 0.5uL of forward primer (10uM), 0.5ul of reverse primer (10uM), 8ul PCR water, and 1uL of template. The qPCR conditions were 95°C for 30sec, followed by 40 cycles of 95°C for 1sec and 60°C for 5sec. Samples were each run one time. Melt curve was performed by the machine. The results were formatted and processed using Real-time PCR miner which quantified the data into values that statistics could be run on (Zhao and Fernald 2005). Each gene was normalized using a normalizing gene (Actin) and then a statistical analysis was performed. A two-way ANOVA was performed to determine the individual and combined effects of photoperiod and mechanical stress (IBM SPSS). Post hoc tests were performed to determine which levels of independent variables displayed significant differences.

Raw data and plate format from qPCR can be found here: . Data formatted for Real-time PCR miner can be found here: http://eagle.fish.washington.edu/nudibranchs/Data%20analysis.xlsx.

Primer development

A list of Gene primers were designed for genes suspected to be related to growth and reproduction using Primer3Plus. Genes were designed off of an *O. lurida* sequencing template. Genes with sequences matching closely to known genes that function in either stress or growth were chosen. Primers were tested using a GoTaq® Green Master Mix PCR mix (Promega, Madison, WI, USA). Each 25uL reaction volume contained 12.5uL GoTaq® Green, 0.5uL of

forward primer (10uM), 0.5ul of reverse primer (10uM), 10.5ul PCR water, and 1uL of template. The cycling parameters were 95°C for 10min followed by 40 cycles of 95°C for 15sec, 55°C for 15sec, and 72°C for 20sec. PCR product was then run on a 1.3% agarose gel to confirm primers formed a single product during the PCR process. Only primer pairs that displayed this specificity were used further in the study.

Table 1. Sequences of the six sets of gene primers used for qPCR in this study. Expected and actual length of PCR product was approximately equal.

Gene	**Primer sequences**	**Expected/Actual Product length (bp)**
Bag	Forward: TGAAGCCATGGCGGAAAGAT Reverse: TCACTTTGTCCAGAGCTGTCTC	= 101
Hsp	Forward: GTCACGCTCTTTCTCCACCA Reverse: GCACTATGGGTCGTGGAACA	= 149
U2a	Forward: GACGTAGCAGGAGTCGTAGC Reverse: GCCTCGACTCAGTATCTGCC	= 130
Pygm	Forward: TCACCAGTGTTGCCAAGGTT Reverse: GATCTGCTCACTCAGGTCCG	= 131
Igfr	Forward: CTTCCTTCCCGAACAGGTCC Reverse: GGAAGTGGAGCGTGACAAGA	= 101
Prkg	Forward: ACGGGCTGGGATAGAGACTT Reverse: CTCGCATTCTGTTGTAGCGC	= 105

Results

Photoperiod

Two photoperiod treatments were compared for each target gene. A statistical analysis of the data looking exclusively at the photoperiod variable found graphically visible differences in all genes (Figure 1). Of the stress related genes two out of three displayed significant differences between the two treatments group with U2a ($p=0.004$), Hsp ($p=0.054$), and Bag ($p=0.000$). Both genes that were significantly different, U2a and Bag, were found at higher levels in the 24-hour treatment. This differed from Hsp, which was found to be higher in the 12:12 hour treatment. Total levels of U2a and Bag were considerably lower than Hsp levels. The growth related genes also had appreciable differences between the treatment groups although only Pygm ($p=0.018$) was significant. Both Pygm and Igfr ($p=0.106$) were found at higher levels in the 24-hour treatment groups while Prkg ($p=0.123$) displayed the opposite trend. Of the three genes, Pygm was expressed at approximately twice the level of both Igfr and Prkg.

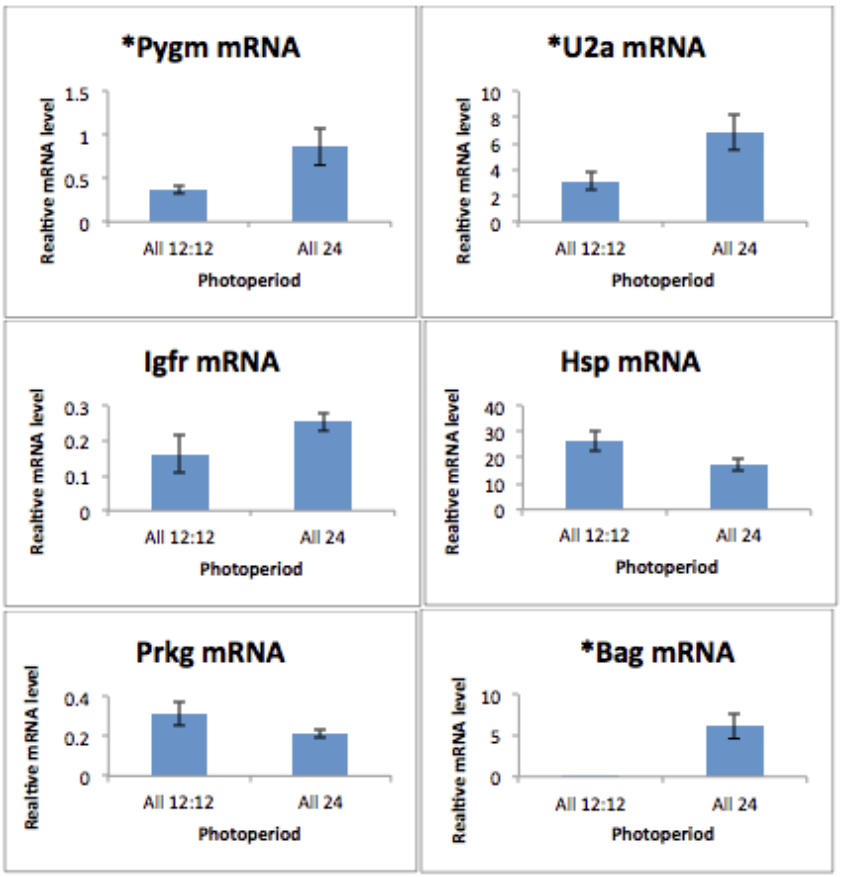

Figure 1. Relative levels of mRNA found for the six genes of interest in this study. Levels from photoperiod treatments of 12hr. light/12hr. dark are compared with 24hr. light treatment. Stress related genes shown on right and growth related genes shown on left. Standard deviation bars are present for each gene. Asterisk denotes statistically significant difference.

Mechanical Stress

Similar to photoperiod, animals were statistically analyzed with mechanical stress as the only factor. Of the six genes, only U2a (p=0.007) and Bag (p=0.000) were statistically significant and both were found at greater quantities in the no mechanical stress treatment group. More Hsp (p= 0.290) was detected in the group that received mechanical stress although the difference was not statistically significant. As with the photoperiod analysis, Hsp displayed the opposite trend of U2a and Bag. The growth related genes also showed these similar trends between themselves. As with photoperiod, Pygm (p=0.077) and Igfr (p=0.167) both had increased expression in the same treatment, mechanical stress. They differed from Prkg (p=0.495) which was found at higher levels in the no stress treatment, although this difference was small. The relationship between stress and growth genes in mechanical stress analysis was opposite of photoperiod. For example, in mechanical stress, Pygm was more abundant in stressed samples while U2a was more abundant in non-stressed samples. This contradicts the pattern in photoperiod in which both Pygm and U2a are at greater levels in the 24-hour

treatment. Therefore, all trends within stress and growth genes are held constant for both photoperiod and mechanical, when compared within the groups. But, when stress and growth genes are compared with each other, photoperiod and mechanical stress display opposing trends.

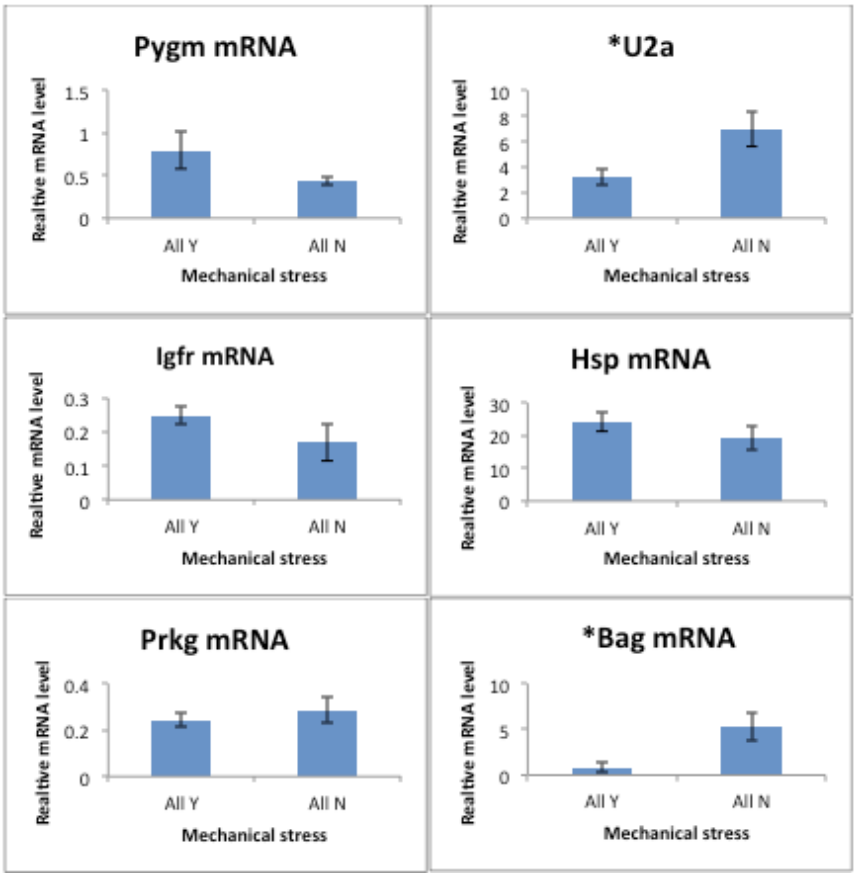

Figure 2. Relative levels of mRNA found for the six genes of interest in this study. Levels from mechanical stress treatments of centrifugal stress are compared with a no stress treatment. Stress related genes shown on right and growth related genes shown on left. Standard deviation bars are present for each gene. Asterisk denotes statistically significant difference.

Combined Trends

An analysis performed across the treatments revealed significant differences between independent variable levels in four of the genes. The post hoc test on these genes showed a significant difference between the photoperiod treatment groups when both were stressed for Pygm (0.001) and Igfr (0.008). Both of these growth related genes displayed highest levels in the 24-hour with stress treatment (Figure 3). The other two genes displaying significant difference were U2a (0.000) and Bag (0.000). The significant difference in both these genes was found between photoperiod treatment groups when both were not stressed. Both of these stress related genes displayed increased expression in the 24-hour no stress treatment (Figure 3). One growth related gene, Prkg (0.863), and one stress related gene, Hsp (0.392) did not show

significant interaction between photoperiod and mechanical stress. These genes do present some visually apparent differences, particularly for Hsp, which had lowest expression in the 24-hour no stress treatment (Figure 3). Notably, of the genes that displayed significant interactions, genes of the same type (growth/stress) behaved similarly. Visually, this is also readily apparent. Genes associated with stress were expressed at higher levels than genes associated with growth.

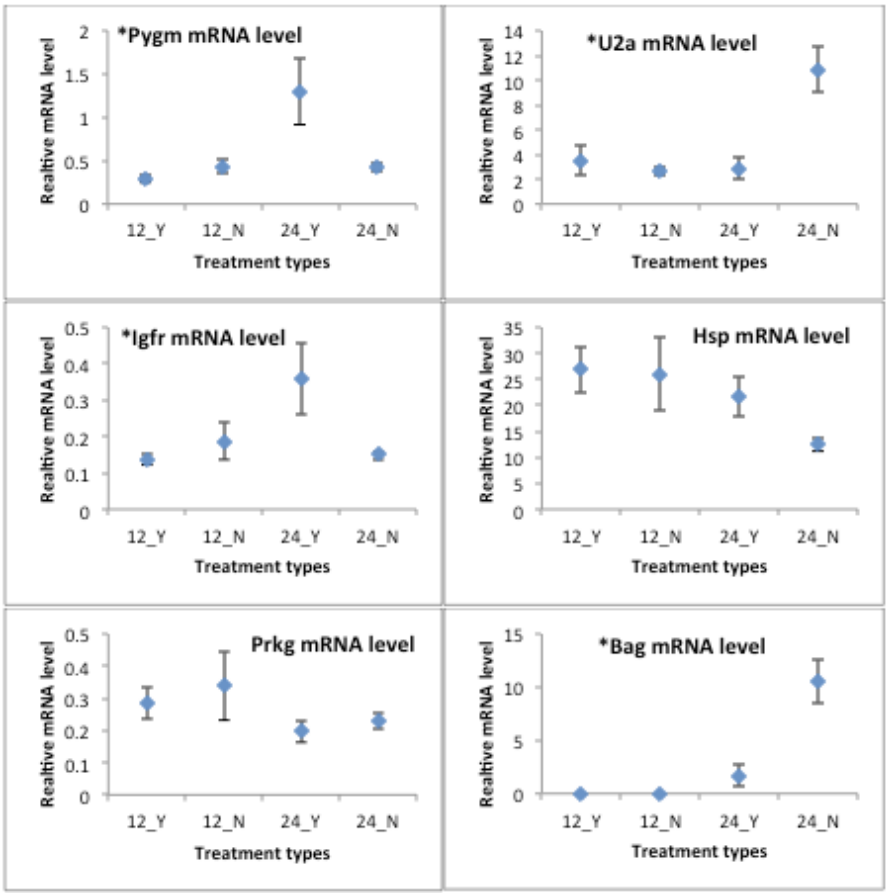

Figure 3. Relative levels of mRNA found for the six genes of interest in this study. Levels from four different treatment types are shown. From left to right – a. 12hr. light/12 hr. dark with mech. stress b. 12hr. light/12 hr. dark without mech. stress c. 24hr. light with mech. stress d. 24hr. light without mech. stress. Stress related genes shown on right and growth related genes shown on left. Standard deviation bars are present for each gene. Asterisk denotes statistically significant difference.

Discussion

Photoperiod

The results clearly point towards the conclusion that differences in photoperiod can induce varying stress responses in *O. lurida*. This can be seen in the significant differences in

expression levels of stress related genes. Both Bag and U2a are inhibitors of stress response and therefore we expected lower levels of these products during stressful periods. Conversely, Hsp directly acts as a stress mediator therefore its levels were predicted to directly correlate with stress. Although only Bag and U2a show statistically significant differences, the greater Hsp levels seen in the 12:12 treatment near statistical significance. These findings suggest that the 24-hour treatment is less stressful on *O. lurida* than the 12:12 treatment, which differed from our original prediction. Interestingly, the growth gene results also seem to form the same conclusion. Genes that are directly correlated with growth, Pygm and Igfr, were both found at greater levels in the 24-hour treatment. While unexpected, the growth pattern follows energy budget theory in that the lower stress treatment also suggested greater growth. Prkg, inversely correlated with growth, shows reduced levels when comparing 12:12 treatment to 24-hour treatment.

Mechanical stress

Results from the mechanical stress analysis differed from photoperiod results with the growth genes. As expected, stress genes, U2a and Bag, were expressed at significantly lower levels in the stressed treatment while Hsp was expressed greater. Again, these findings match with literature-based predictions of their function. The results suggest that inducing mechanical stress does effect gene expression of stress response genes on *O. lurida*, as two out of three genes showed significant differences in expression levels between treatments. Despite consistent conclusions from the stress related genes, growth genes did not prove to provide the same clarity. Higher levels of Pygm and Igfr combined with lower levels of Prkg in the stress treatment all suggested that more growth occurred in the high stress treatment, contradicting previous deductions. While none of the three genes had statistically significant differences, the gap between treatments found in Pygm and Igfr were still visibly large. Furthermore, consistency across all three genes suggests that the results may be more than pure coincidence. If the results are robust, then they suggest mechanical stress, although causing stress to the animal, may also signal growth.

Combined trends

When both independent variables are combined, a more complete picture starts to emerge, explaining some of the curiosities in the mechanical stress analysis. Hsp, which mediates stress, should be expressed in the opposite manner of U2a and Bag, which suppress stress response. This is precisely what is seen across the various treatments. Hsp follows a downward sloping trend with similar levels of expression in both 12:12 hour treatments and the 24-hour with stress treatment. The sharp decline in Hsp levels occurs in the 24-hour without stress treatment. This large difference can explain why Hsp levels were lower in 24-hour treatment and no stress treatment, respectively, from the single variable analyses. The reverse trend was seen for both U2a and Bag, as both are expressed at low levels until a sharp increase in the 24-hour without stress treatment. Similarly to Hsp, the significant difference in single variable analysis for photoperiod and mechanical stress can largely be attributed to the 24-hour

without stress treatment. Due to the consistency across the three genes, it seems likely that the 24-hour without stress treatment is significantly less stressful on the Olympia oyster even though differing from predictions. Notably, it takes both the 24-hour and no stress treatments to elicit this response. Pairing 24-hour with mechanical stress only causes a slightly reduced stress response and pairing no mechanical stress with 12:12 hours causes no reduction is stress response. Considering the sloping trend of the target genes, it seems likely that a 24-hour photoperiod treatment is less stressful to *O. lurida* than the 12:12 hour treatment. But, adding the mechanical stress to the 24-hour treatment makes the reduction in stress much less apparent.

Just as U2a and Bag had similar trends across the treatments, so to, did Pygm and Igfr. While this is not a surprise as both Pygm and Igfr are genes directly correlated with growth, the pattern of expression is harder to explain. Out of the four treatments, only 24-hour with mechanical stress elicited increased levels of expression. The other three treatment groups were fairly similar. The significantly increased levels of Pygm and Igfr suggest that growth rate increased for the 24-hour with stress treatment, which was not expected. This disproportionate increase explains why the mechanical stress analysis found higher Pygm and Igfr levels in samples that had been mechanically stressed. The third gene, Prkg is inversely related to growth and therefore its expression pattern was expected to be opposite of Pygm and Igfr. While Prkg is found at its lowest level in the 24-hour with stress treatment, the level of expression is not as clearly differentiated from the other treatment types as it is for Pygm and Igfr. One possible explanation is that the stresses induced by photoperiod and mechanical stress in this study were not great enough to cause a strong Prkg response. This may be because Prkg is associated with apoptosis, a response withheld for significant stressors. Therefore, large changes in Prkg expression may be reserved for more threatening occasions.

Explanations

Our findings clearly suggest 24-hour light treatment is less stressful to Olympia oyster than the 12:12 light treatment. This result differed from our original predication that 12:12 would be less stressful. The hypothesis was based on the premise that a photoperiod more similar to natural lighting would be preferred by oysters. Research on other marine species shows increased light can both increase or decrease stress and growth rate depending on the species. This includes many marine invertebrates such as fighting conch (*Strombus pugilis*) and southern rock lobster (Jasus edwardsii) who exhibit significant increases in growth from increased light exposure (Dodd 1969; Manzano et al. 1998; Crear et al. 2003). There has been some speculation that this may be due to increased food availability, although this postulation has not been confirmed. Conversely, blue mussel (*M. edulis*) has been found to display the opposite reaction to light (Nielsen and Stromgren 1985). In a process termed, dark enhancement, *M. edulis* grown in low light conditions show significantly larger shell size and heavier dry weight compared to those grown with more light.

A literature search did not reveal any research specific to photoperiod control on oyster growth but research has found that photoperiod can impact maturation of gametes (Fabioux et al. 2005). The study showed longer light treatments increased maturation rate. In oysters, this maturation period naturally occurs during the spring and summer months which is when days

are the longest. We suspect that this correlation may also be applied to growth. The grow out season for oysters general occurs in summer months where in the Pacific Northwest, light is present for up to 16 hours. Therefore, increased light, as in the 24-hour treatment, may stimulate reduced-stress growth. This could be a conditioned response from annual growth cycles in which longer light suggests increased food supply. Despite this, we do not believe actual food supply had any direct impacts on gene expression in this study because oysters were all fed the same mix regardless of treatment type. Two additional factors that could affect *O. lurida* growth are light intensity and wavelength. The impact of these two factors on growth has been documented in a variety of studies, but this study was unable to account for these factors. Further research may attempt to definitively explain why light stimulates growth in *O. lurida* as well as look into other light-related factors that could affect gene expression.

Certainly other explanations for increased stress are also possible. In natural lighting, transition between day and night is gradual with dawn and dusk periods. In experimental settings, a light bulb switched from on to off instantly resulting in abrupt shifts from light to dark. This change from light to dark may have been the reason for signs of increased stress rather than the difference in length of light per day. Additional research accounting for abrupt changes to lighting may provide concrete answers. Another consideration is that the 12:12 treatment signals a stressful situation in the animal. In the natural environment, the 12:12 setting is found during the equinoxes of spring and fall. These times are often periods in which the ecosystem is in a complex transition period. The changes inevitably stress animals in the environment. Therefore, the 12:12 treatment may have signaled the equinox period to the oyster generating a conditioned stress response. In future experimentation, using a broad range of photoperiod treatments will help improve results in this area. The results from this study found the 24-hour with stress treatment to induce a similar stress response to both 12:12 photoperiod treatment groups suggesting the 12:12 was not a sole factor in signaling stress response.

Another interesting implication from our findings was that the 24-hour light with stress treatment, suggested significantly more growth than the same photoperiod treatment without stress. This was unexpected considering energy budget predictions. Furthermore, our highest stress treatment of 12:12 hours with stress produced the lowest levels of growth suggesting high stress did limit growth on oysters, a result consistent with most energy budget models. If the findings truly represent changes in growth pattern, they would indicate some levels of mechanical stress can be beneficial towards growth. This could be similar to the muscle growth model in humans in which exercise leads to muscle hypertrophy. For the oysters, mechanical stress may act as a trigger to induce tissue growth. This theory has not been specifically researched in *O.* lurida and may be a subject for additional research. It would seem mechanical stress can only signal growth under relatively stable conditions where the oyster is not heavily stressed. For example, our highest stress treatment of 12:12 hours with stress produced the lowest levels of growth. In that treatment, mechanical stress did not induce growth, likely because the oysters were already experiencing high levels of stress. While literature suggests otherwise, other stressors may also be able to illicit the same effect. This can only be verified through further experimentation.

Although expression of genes suggested an impact of mechanical stress on growth, no actual growth was seen during the experiment. Since no measurements were taken during the

two week experiment, changes in gene expression could not be verified with visible growth. Therefore, difference in gene levels is only able to suggest growth is occurring instead of showing growth. As a result, further experimentation would be needed to affirm our results. The genes chosen for quantification were carefully selected both for annealing specificity and stress or growth related function. Understanding the role of genes selected for quantification is particularly important when using gene expression because it allows application of gene quantification to targeted physiological processes. Because it is not a well-studied species, the functions of *O. lurida* genes have not been established. Functions for the genes in this study were therefore extrapolated from gene functions of similar, conserved genes from other well-studied species. While functioning of conserved genes is expected to be similar, this is not always true. If *O. lurida* genes do not share conserved functions with genes from other species then the results in this study are not as robust. We must also consider genes having multiple functions in a single species. This means levels of a gene may be indicating physiological changes in another pathway instead of the expected area. If true, gene expression analysis would not reflect physiological changes in growth or stress, but rather a separate, non-target pathway. Although possible, genes expected to act similarly during the study did. The consistency across all genes suggests the targeted function was being measured in the analysis of variation in gene expression.

Conclusion

Although limited in certain aspects, this study found two important implications in *O. lurida* growth physiology and restoration efforts. First, photoperiod may have a significant impact on stress and growth rate of *O. lurida* with longer photoperiods being able to increase growth. Second, mechanical stress may also stimulate growth under conditions in which *O. lurida* is relatively unstressed. Both of these findings are based on gene expression and confirmation by longer studies would be ideal. If valid, results could be implemented into *O. lurida* hatchery settings, or other aquaculture areas to improve growth rate. Any increases in growth efficiency may significantly boost *O. lurida* ability to compete with other oyster species in the region and restore populations to sustainable levels.

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