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The effects of anoxic conditions on thermal tolerance and stress protein levels in four local bivalves

Rachel Allee
Western Washington University

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THE EFFECTS OF ANOXIC CONDITIONS ON
THERMAL TOLERANCE AND STRESS PROTEIN LEVELS
IN FOUR LOCAL BIVALVES

By

Rachel Allee

Accepted in Partial Completion
of the Requirements for the Degree
Masters of Science

Moheb A. Ghali, Dean of the Graduate School

ADVISORY COMMITTEE

Chair, Dr. Deborah Donovan

Dr. Sandra Schulze

Dr. David Shull

MASTER'S THESIS

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Rachel Allee
January 13, 2010

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The Effects of Anoxic Conditions on Thermal Tolerance and Stress Protein Levels in Four Local Bivalves

A Thesis presented to the Faculty
of Western Washington University

In Partial Fulfillment of the requirements
for the degree Master of Science

by
Rachel Allee
January 2010

ABSTRACT

Areas of oxygen depletion in the world's oceans have been on the rise, with the main culprits being anthropogenic causes such as eutrophication and pollution. Coastal marine areas, such as estuaries, are some of the more impacted ecosystems and they have been the focus of recent studies. This study focused on the effects of anoxia and the interactions it has with thermal tolerance in four local intertidal bivalves: *Nuttallia obscurata, Venerupis phillipinarum, Leukoma staminea,* and *Mytilus trossulus*. *Nuttallia obscurata* is a recent invader on which little physiological research has been conducted. Both *V. phillipinarum* and *L. staminea* are commercially important to local fisheries. Finally, *M. trossulus* is a native mussel commonly found in varying parts of the intertidal zone. The bivalves had anoxic conditions induced by using rubber bands to keep them shut. After a period of four days, they were tested for thermal tolerance by placing gill strips from the organism into one of three temperature treatments that varied per species. The gill tissue was evaluated for time to death. Gill tissue was also collected before the thermal tolerance experiment to examine stress protein levels using a Western blot analysis. Increasing temperature caused a decrease in survival time of gill tissue of all four species. However, there were no significant differences in temperature tolerance between the anoxic and control individuals in the bivalves. Levels of constitutive hsp70 were only found in *Mytilus trossulus,* with no difference in hsp70 expression between the control samples and those that experienced a period of anoxia. Thus, there was no evidence of either synergistic or antagonistic effects between the two stressors, anoxia and temperature, in this study. Bivalves are known to

have excellent tolerance of anoxia because of their ability to decrease metabolic demands

and utilize anaerobic pathways to produce needed energy.

ACKNOWLEDGEMENTS

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INTRODUCTION

Areas of anoxic (no oxygen) or hypoxic (oxygen concentrations below 2-4 mg O_2 L^-^{1}) conditions in coastal marine environments have become an object of concern as information about the global extent and local impacts have become available (GESAMP 2001, Rabalais et al. 2002, Paerl 2006). The causes of these anoxic and hypoxic zones are mainly anthropogenic activities such as agricultural runoff, pollution, and climate change (Gray et al. 2002, Jewett et al. 2005). Eutrophication occurs as excess nutrient runoff from agriculture, industry, or municipalities enters water ways and empties into estuaries and other coastal ecosystems. This causes an increase in primary production by algae, oftentimes resulting in an algal bloom. Once the overabundant algae die, they sink to the bottom where decomposition by bacteria depletes the surrounding water of oxygen. Ocean climate cycles such as El Niño/La Niña events affect nutrients and can bring oxygen-depleted bottom water into the shallow coastal waters where low oxygen may already problematic (Grantham et al. 2004, Diaz and Rosenberg 2008).

Obvious consequences of these areas of anoxia or hypoxia are the detrimental effects on the ecosystems within these waters. Areas of severe hypoxia brought about by oxygen-depleted upwelling off of the coast of Oregon caused massive mortality in many marine species such as crustaceans and fish in 2002 (Grantham et al. 2004, Diaz and Rosenberg 2008). One of the largest hypoxic areas in the world is "The Dead Zone," in the northern Gulf of Mexico. "The Dead Zone" is an area of severe hypoxia caused by excessive nutrient runoff from the Mississippi River that has been steadily increasing in size for over 50 years. Its name comes from the inability to catch fish or macroinvertebrates from the bottom

waters due to the low oxygen concentration (Rabalais et al. 2002, Diaz and Rosenberg 2008). In the southern Puget Sound, Hood Canal is a fjord that has been seasonally hypoxic since the 1950's (Parker-Stetter and Horne 2009). In the fall of 2003, large fish kills occurred due to abnormally low dissolved oxygen levels (Newton 2007). As regions of anoxic and hypoxic waters increase in size and number, it is important to assess how trophic interactions and estuarine community composition will be affected.

The coastal intertidal zone is a demanding environment in which animals endure multiple physical stresses including daily exposure to low oxygen conditions. Exposure to periods of anoxia or hypoxia can arise from emersion at low tides, reduced oxygen concentrations in tidepools due to respiration, and wide fluctuations in shallow waters with low water movement (Hagerman 1998, Larade and Storey 2007). In addition to these periodic times of low oxygen, bivalves experience other times of hypoxia or anoxia such as low oxygen concentrations in the substratum where burrowing occurs, or during shell valve closure due to predators in the vicinity (Sobral and Widdows 1997, Greenway and Storey 1999). The amount of anoxia sustained by any species is determined in part by their location within the intertidal zone; those higher up will be exposed longer at low tides (Roberts et al. 1997). In an estuary or coastal area where both anthropogenic and natural anoxia occurs, the ability to tolerate those conditions may create an advantage for some species over others or even facilitate the establishment of an invasive species into an ecosystem (Jewett et al. 2005).

Animals within the intertidal utilize many survival tools when faced with anoxia. Depression of metabolic rate and switching to anaerobic metabolism are the two main ways intertidal invertebrates are able to survive during anoxic conditions (Sobral and Widdows

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1997, Greenway and Storey 1999, David et al. 2005). Bivalves are well known for their high tolerance to anoxia. Decreasing metabolism is the first line of defense in most animals, and in highly tolerant species, metabolism is decreased to less than 10 % of basal metabolic rates (Greenway and Storey 1999, Ortmann and Grieshaber 2003, Lorade and Storey 2007). This decrease in metabolism lowers the amount of energy (ATP) needed to maintain normal function.

Once metabolism is decreased, anaerobic metabolic pathways are utilized to produce energy when there is not enough oxygen to continue glycolysis. Which anaerobic pathways are used depends on the species being examined, and in bivalves multiple pathways are often used. During anaerobic conditions, glucose is converted to pyruvate and then can be combined with an amino acid to produce fermentation end products such as octopine, analopine, strombine, or D-lactate (Livingstone 1991, Hochachka and Somero 2002). In other cases, pyruvate is converted to oxaloacetate and then into either succinate or propionate (Livingstone 1991, Isani et al. 1995, Hochachka and Somero 2002). Having the right tools to survive in habitats that are anoxic on a regular basis is especially important when these conditions are compounded by anthropogenically-caused anoxic events and other stressors experienced by these animals.

Many studies have examined the effect of anoxia, but environmental stresses rarely occur in isolation. For example, as intertidal invertebrates are emersed during a receding tide, a period of anoxia could occur as temperatures are rising. Two main causes of thermal stress faced by intertidal animals are changes in water temperature and changes in air temperature during emersion (Roberts et al. 1997, Feder and Hofmann 1999). Changes in water temperature affect animals residing in tidal pools or in small standing bodies of water

that heat up from outside temperatures. There is also seasonal variation of water temperature, as well as temperature increases due to global warming and pollution (Feder and Hofman 1999, Helmuth and Hofmann 2001).

Temperature fluctuations are considered one of the main factors determining benthic invertebrate distribution within the intertidal zone (Helmuth and Hofmann 2001). In bivalves, species from intertidal zones are more tolerant of temperature changes than those from subtidal zones where temperature is more constant (Vernberg et al. 1963, Helmuth and Hofmann 2001). It has also been found that the mussel *Mytilus trossulus* taken from the intertidal zone showed higher levels of ubiquitin conjugates (indicating protein degradation) and stress proteins than individuals taken from the subtidal zone (Hofmann and Somero 1995). This indicates that not only do intertidal animals see greater temperature fluctuation than those located in the subtidal, but this fluctuation is stressful.

The thermal tolerance of a species will often correlate with both their biogeographical range and depth distribution. Thermal tolerance refers to the range of temperatures at which a species can survive and for how long they can survive at a given temperature. The distributions of intertidal invertebrates are limited by the temperatures at which they can survive. Temperate species are generally able to tolerate a wider range of temperatures (eurythermal) than those species living in environments such as arctic waters where there is less fluctuation in temperature (stenothermal) (Pörtner 2002). Within the habitat range of a temperate species, the populations at the southern edge of the range are often living close to their thermal lethal limit (Jones et al. 2009). As water temperatures increase globally, northern range shifts may be seen for many of these species if the temperatures reach their lethal limit and they are not able to increase their thermal tolerance

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(Pörtner 2002, Jones et al. 2009). Thermal tolerance is also limited by oxygen availability. Extreme temperatures create higher oxygen demand for animals which decreases oxygen levels within the body creating low oxygen conditions within the animal (Pörtner 2002, Kassahn et al. 2009). Oftentimes, an induced or acquired thermal tolerance will be seen in organisms that are gradually exposed to increasing, sub-lethal temperatures (Shamseldin et al. 1997, Kregel 2002). This may allow those populations that are living near their thermal lethal limit to slowly acclimatize to increasing water temperatures.

While multiple stressors could lead to a higher likelihood of physiological distress (synergistic effect), it is also possible that one stressor could increase the tolerance of the organism to other stressors (antagonistic effect). This may be possible due to the induction of stress proteins, which are molecular chaperones that inhibit denaturation of other proteins. Stress proteins can be induced by many environmental factors such as temperature, pollutants, and anoxia (Feder and Hofmann 1999). Thus, if an organism is subjected to a stress that causes the induction of stress proteins, the presence of those proteins may protect the animal from subsequent stressors (Wiegant et al. 1995, Shamseldin et al. 1997). In fact, increased thermal tolerance has most often been seen in organisms that also produced stress proteins (Hahn and Li 1982, Lindquist 1986, Howarth and Skøt 1994).

Stress proteins are very highly conserved across taxa (Feder and Hofmann 1999). Some of the most common stress proteins are heat-shock proteins (hsps). Heat-shock proteins are found in both prokaryotic and eukaryotic organisms, from bacteria to chordates. Within the cell, they are located in the mitochondria, cytosol, and nucleus (Kregel 2002, Fabbri et al. 2008). There are many families of hsps which are named according to molecular weight (KDaltons), with hsp60, hsp70, and hsp90 being the most commonly

studied (Lewis et al. 1999). There are two forms of hsps, constitutive and inducible; the constitutive form performs chaperone activities under normal non-stressed conditions, whereas the inducible form requires a stressor to start the repairing activities (Halpin et al. 2004, Fabbri et al. 2008). Stress proteins are useful when investigating environmental stresses as they indicate a molecular response which can be observed before any external signs of stress are evident (Feder and Hofmann 1999).

Of the many families of hsps, hsp70 is the most highly conserved and most commonly used when studying environmental stresses in organisms (Lewis et al. 1999). Hsp70 functions as a chaperone; when stress is induced in the organism, the proteins help refold denatured proteins so they can resume their function. In addition, they assist in binding and removing damaged proteins to prevent aggregation within the cell (Lewis et al. 1999, Halpin et al. 2004). They encompass multiple genes and are very tightly regulated (Lindquist 1986). Increased levels of hsp70 have been shown after varying stressors such as anoxia/hypoxia, thermal stress, pollutants, and salinity changes in animals across taxa (Lindquist 1986, Kregel 2002). In bivalves, hsp70 has been observed in many species after thermal and other stresses (Fabbri et al. 2008), making it a good choice for this study.

Experimental organisms

Four local species of bivalves were used in this study to investigate interactive effects of anoxia and temperature tolerance: three infaunal species and one epifaunal species. Two of the clams, *Leukoma staminea* (previously *Protothaca staminea*) and *Venerupis phillipinarum,* represent commercially important species to the region, and the third clam species, *Nuttallia obscurata*, is a recent invader. Introduced to western British Columbia in the 1990's via ballast water, *N. obscurata* has quickly spread down through

southern Oregon (Gillespie et al. 2001, Wonham and Carlton 2005) and can reach densities

of up to 800 m^{-2} (Dudas 2005). Concern has been expressed regarding the possible impact

of *N. obscurata* (and even *V. phillipinarum*) on the native commercial species and on each

other. However, previous studies that have examined possible competitive interactions

between these species have not indicated that *N. obscurata* poses a serious threat. Due to *N.*

obscurata's distribution within the high intertidal zone, there is generally little direct

competition with *L. staminea* or *V. phillipinarum*. However, when placed with *V.*

phillipinarum there was decreased growth among both species, and *N. obscurata* was found

to burrow deeper into the substratum than *V. phillipinarum* (Gillespie et al. 2001). An

examination of how these species interact with anoxia and temperature may offer some

insight into possible ways they could impact each other, either directly or indirectly. If one is

able to tolerate anoxia and thermal stress better than the others, it may have a competitive

advantage as these conditions increase in duration and intensity. The fourth bivalve was

Mytilus trossulus, a native epifaunal mussel commonly found in varying parts of the

intertidal zone. *Mytilus trossulus* was included in this study since it represents an epifaunal

bivalve which contrasts to the other experimental bivalves (all infaunal) and has been well

studied in the areas of thermal and anoxia tolerance, as well as levels of stress protein

expression (Roberts et al. 1997, Helmuth and Hofmann 2001, Halpin et al. 2004)

Thermal tolerance in this study was examined by determining how long each

experimental species could survive at a given temperature using a gill-tissue assay. When

examining temperature tolerance in whole bivalves, it is difficult to asses the exact time of

death by relying on behavioral clues such as gaping, siphon response, or mantle retraction.

Physiological studies in bivalves have often been accomplished by using gill tissue as a

proxy for whole organism tolerance (Vernberg et al. 1963, Stevens 1987). Gill tissue was cut into strips and monitored for ciliary activity at regular intervals until there was no activity, providing a precise way to determine when death occurs. In a previous study of the scallop, *Pecten novaezelandiae,* gill tissue was examined for mortality after being subjected to different turbidities and then compared with mortality of the whole organism with similar results (Stevens 1987).

While many physiological studies have been conducted on the mussel *Mytilus sp.,* studies on the three species of clams have been primarily focused on factors such as distribution, predation, and reproduction (Byers 2002, Byers 2005, Dudas et al. 2007). By investigating the physiological tolerances of these four bivalves, I was able to elucidate the relationship between anoxia and thermal tolerance in bivalves to better understand how these two important stressors interact to affect intertidal invertebrates.

Experimental approach

The first goal of this study was to examine the relationship between anoxia and thermal tolerance. The four species had anoxic conditions induced for four days, and the thermal tolerance of the bivalves' gill tissue was tested following the procedure of Vernberg et al. (1963) with different temperature treatments for each species. The second main goal of this study was to examine whether anoxia induces the expression of stress protein, hsp70, in the experimental bivalves. The presence of stress proteins was examined using Western Blot techniques after four days of anoxia.

My experimental questions were:

Question 1: How does anoxia affect temperature tolerance of gill tissue in the four species?

H_0: If anoxia does not affect temperature tolerance, there will be no differences in time to death of gill tissue between those that underwent anoxia and the control group.

Question 2: Are heat shock proteins present in gill tissue of the four species after a period of anoxia?

H_0: If heat shock proteins are not induced in the four species after anoxia, there will be no difference in hsp70 expression between the anoxic and control samples.

METHODS

Study site and experimental organisms

Individuals from each of the four experimental species, *Venerupis phillipinarum, Leukoma staminea*, *Nuttallia obscurata*, and *Mytilus trossulus* were collected from the northern-most part of Chuckanut Bay, WA. Within this portion of the bay, *N. obscurata* is found highest in the intertidal and *L. staminea* is much lower in the intertidal. In between is *V. phillipinarum* which was found mixed with *L. staminea* but also ranging higher near the lower limit of *N. obscurata*. The mussel, *M. trossulus* was found attached to rocks and pilings throughout the area, but individuals used in this study were taken from the low to mid intertidal. This portion of the bay is a mud flat habitat with exposure at moderate to low tides. Water and sediment exchange is limited by a railroad line creating a narrow opening connected to the rest of the bay. Sampling occurred in July, August, October, and December 2008 at low tides.

Inducing anoxia

Individuals from each of the four species were collected and acclimated in aquaria maintained at 15 °C and a salinity of 33 – 34 ppt for five days after collection. To induce anoxia, the valves of the treatment individuals were held shut with a rubber band to prevent water from entering their incurrent siphons. Once treatment animals were forcibly closed, individuals from both control and anoxic treatments were put in the same tank filled with 5 μg filtered seawater to limit the control group from feeding. A preliminary study was done by rubber-banding 28 individuals of each species and checking two of each species per day for death. Death was determined by dissecting out a small portion of gill tissue and observing the terminal gill cilia. The tissue was considered dead if no movement of the cilia was observed. Death began occurring at day five so an experimental treatment of four days was chosen.

To ensure that the rubber-bands were effectively inducing anoxia, mantle-cavity oxygen concentration levels were investigated with an oxygen microelectrode with a guard detector (Unisense OX100; Revsbech 1989). The oxygen probe was calibrated using a two-point calibration system to establish reference points of oxygen depletion and saturation. An airstone was used to create oxygen saturated seawater for the 100% oxygen saturation measurement, while sodium sulfite dissolved in seawater was used for the 0% oxygen baseline. Several individuals (4-6) of each species were set aside from the collection made in December 2008. A Dremel (Racine, WI) was used to drill a small hole in the dorsal aspect of the shell, then the oxygen probe was inserted and oxygen levels were recorded.

One normoxic *M. trossulus* and one *V. phillipinarum* were tested in addition to one 24-hour

anoxic *M. trossulus.*

Measuring thermal tolerance

Ten individuals of each experimental species were collected each day over two to

three consecutive days during low tide in order to stagger the number of individuals to be

processed each day. Each experimental species had individuals split into a treatment group

(N = 5 selected haphazardly for each of three temperatures) and control group (N = 5

selected haphazardly for each of three temperatures). To test the effects of anoxia on

thermal tolerance, gill tissue was evaluated for time to death. The protocol for testing

thermal tolerance followed the procedure of Vernberg et al. (1963) which uses gill tissue as

a proxy for whole animal death. After the period of anoxia, induced by holding the valves

of the bivalves closed, gill tissue was excised from each individual and cut into small strips.

Gill strips from individual specimens were placed into 500 ml Erlenmeyer flasks with 200

ml of seawater such that the tissue of each animal was in its own flask. Once the gill tissue

was placed in a flask, the flask was suspended in baths such that each species was tested at

three different temperatures. Water baths were set up at different temperatures, each

controlled within 0.1 ºC. An airstone circulated the water in each bath to keep temperature

homogeneous. Gill pieces were removed from the flasks at time intervals of 5-10 minutes,

with higher frequency as cilia movement slowed. These pieces were then examined under a

microscope for ciliary activity. A rating system was used to determine time to death: at a

rating of 3 the cilia were all actively moving at a normal rate and at a rating of 0 all cilia had

stopped movement, indicating death (Vernberg et al. 1963). Once a rating of 0 had occurred

in one gill piece, at least two additional gill pieces were examined as verification.

The temperature treatments were determined by a preliminary study of the average

time to death for gill tissue of each species and temperatures were chosen in which death of

the gill tissue occurred between 20 minutes and three hours. Survival times shorter than 20

minutes made it difficult to determine the exact time of death and survival times longer than

three hours were impractical for completing all experiments in a reasonable timeframe.

Leukoma staminea was tested at the lowest temperature range of 35.5 ℃, 36.0 ℃, and 36.5

℃. *Mytilus trossulus* was tested at a low-intermediate range of 36.0 ℃, 36.5 ℃, and 37.0

℃. The other two species were tested at higher temperature ranges with *V. phillipinarum* at

39.5 ℃, 40.0 ℃, and 40.5 ℃ and *N. obscurata* at the highest range of 40.5 ℃, 41.0 ℃, and

41.5 ℃. Thus, these temperature ranges allowed me to use a common window within each

species' optimum temperature-sensitive range to evaluate thermal tolerance.

An additional experiment was done using only *M. trossulus* as the experimental

species. Results from the aforementioned experiment (see Results below) indicated four

days of anoxia may not have been long enough to elicit stress in any of the experimental

species. *Mytilus trossulus* was chosen for the additional experiment because it was the only

species showing hsp70 production (see Results). Collection occurred over two consecutive

days in one low tide cycle during December 2008. A total of 50 individuals were collected

with 24 tested each day. Procedures were the same as above, but anoxic conditions were

maintained for seven days (maximum before death occurred). Also, temperature treatments

were changed to whole degree increments (36.0 ℃, 37.0 ℃, and 38.0 ℃) to elicit more

obvious differences.

Thermal tolerance was measured by documenting time to death of the gill tissue for treatment vs. control groups of each species. Data for mean time to death were log-transformed then compared within species using a 2-way ANOVA, with treatment (anoxia or control) and temperature as main effects, and the interaction of the two. Assumptions for ANOVAs were tested. A correction for Bonferroni inequality was applied to the ANOVA results since the same hypothesis was tested four times. Thus, a treatment was considered statistically significant if $p < 0.0125$.

Measuring stress proteins

After the period of anoxia, portions of gill tissue from individuals of each species were prepared and immediately frozen for later use measuring stress protein levels (N=5 control and N=5 anoxic). Each sample was placed in an Eppendorf tube on ice and was subsequently homogenized in a mixture of 100 μl of a protein grinding buffer (2.5 mL of 10 mM NaOAc at pH 5.2 and 0.386 g DTT) and 400 μl of a reducing buffer (2.4 mL of 1 M Tris at pH 6.8, 4 mL of 100% glycerol, 16 mL of 10% SDS, and a small amount of Bromophenol Blue). The mixture was boiled for 5-10 minutes at 90.0 °C, centrifuged, and the supernatant removed and stored at -20.0 °C until Western blot analysis.

Western blot analysis followed the procedure outlined by Schulze et al. (2005). Protein extractions (10 μl per species) and the protein molecular weight ladder (BlueStep K973-0.5ml) were electrophoresed on a 12% polyacrylamide gel at 100 V for 1 hour. The separated proteins were then transferred onto a nitrocellulose membrane for 1.5 hours at 100 V. The membranes were soaked in a block solution (50 ml PBST and 2 g of 4% powdered milk) for 1 hour then were incubated overnight in a cold room maintained at 4 °C in the

blocking solution with both hsp70 and α-tubulin antibodies. The anti-hsp70 antibody (monoclonal, Thermo MA3-006) was used in dilutions of 1:6000 for *M. trossulus* samples and 1:500 for the other three species and the α-tubulin antibody (Sigma T5168) was used in a dilution of 1:2,000,000 for all species. After incubation, the membrane was blocked for one hour with the secondary antibody (anti-horseradish peroxidase, Thermo 31430) at a 1:10,000 dilution. Detection of hsp70 was done using SuperSignal West Pico chemiluminescent substrate (Pierce 34080) and a cooled CCD camera system (Alpha Innotech FluorChem HD2 Imaging System).

Presence or absence of hsp70 for all species was recorded, and relative amounts of expression were quantified if they were present. Hsp70 expression was quantified using ImageJ software (NIH; http://rsbweb.nih.gov/ij/). Within the band, mean pixel intensity values for each band were measured (see Appendix). The pixel intensity was indicative of the amount of protein expression seen in the individual. Mean pixel intensity (protein expression) of anoxic and normoxic samples were calculated, with standard deviation, and then compared using a Student's t-test. Assumptions of the t-test were evaluated. Within each individual clam, a ratio of hsp70 expression (pixel intensity) to α-tubulin expression was calculated. The ratios were then averaged among normoxic and anoxic individuals. The average ratio for the average normoxic levels and average anoxic levels were compared by setting the control ratio to 1.0 and comparing the anoxic levels to that value.

RESULTS

Mantle cavity oxygen concentrations

Oxygen concentration within the mantle cavities of three individuals that were closed naturally or rubber-banded shut were very close to zero. Calibrated values for the oxygen concentration of air saturated seawater at 14 ºC was 258.3 μmol O_2 L^{-1} and at 18 ºC was 238.7 μmol O_2 L^{-1}, and oxygen concentration of depleted seawater was 0 μmol O_2 L^{-1}. One *M. trossulus* individual had been rubber-banded for 24 hours and had a mantle cavity oxygen concentration of 0 μmol O_2 L^{-1} (seawater at 18 ºC). Two other bivalves (one *M. trossulus* and one *V. phillipinarum*) were not forcibly closed but were not open with siphons extended at the time the measurements were taken and may have been closed for 30 − 60 minutes prior. The *M. trossulus* had an oxygen concentration of 0.490 μmol O_2 L^{-1} (seawater at 14 ºC) and the *V. phillipinarum* had an oxygen concentration of 0.250 μmol O_2 L^{-1} (seawater at 14 ºC).

Thermal tolerance

Thermal tolerance of gill tissue after four days of anoxia was determined by examining time to death for all four species of bivalves. All time to death values were log-transformed for data analysis, but graphed as the original values with 95% confidence intervals. There were no significant interactions between temperature and anoxia for any of the four experimental species ($p > 0.6$ for all species). Results for 2-way ANOVAs regarding thermal tolerance for all four species can be seen in Table 1, with all assumptions

Table 1: ANOVA results for anoxia and temperature effects on thermal tolerance of bivalve gill tissue and the interaction of anoxia and temperature. An asterisk indicates significance with $p < 0.0125$

	DF	F	p
Leukoma staminea			
anoxia	1,24	5.038	0.034
temperature	2,24	7.275	*0.003
interaction	2,24	0.474	0.628
Venerupis phillipinarum			
anoxia	1,24	0.122	0.73
temperature	2,24	27.012	*<0.001
interaction	2,24	0.186	0.832
Nuttallia obscurata			
anoxia	1,24	0.398	0.534
temperature	2,24	28.54	*<0.001
interaction	2,24	0.191	0.828
Mytilus trossulus 4-day			
anoxia	1,24	0.179	0.676
temperature	2,24	5.133	0.014
interaction	2,24	0.216	0.807
Mytilus trossulus 7-day			
anoxia	1,24	1.075	0.31
temperature	2,24	88.51	*<0.001
interaction	2,24	1.042	0.368

having been met. Correction for Bonferroni inequality was applied which adjusted

significance to $p < 0.0125$.

Temperature affected survival time for each species, with survival time decreasing as

temperature increased (Table 1, Figure 1). Temperature was significant for all species ($p <$

0.003) except *M. trossulus* (p= 0.014). However, the effects of anoxia on thermal tolerance

were not significant for any of the four species; there were no differences between the

survival times of bivalves in the normoxic and anoxic treatments (Table 1, Figure 1).

An additional experiment using *Mytilus trossulus* exposed to seven days of anoxia

was done after results indicated differences may be more obvious with a longer period of

anoxia. *Mytilus trossulus* was chosen because it was the only species that had expression of

hsp70. There was no significant interaction between temperature and anoxia treatment ($p =$

0.368; Table 1, Figure 2) and there was still no significant difference in time to death of gill

tissue between the anoxic and control mussels ($p = 0.31$). However, there was a significance

difference between temperature treatments ($p < 0.001$).
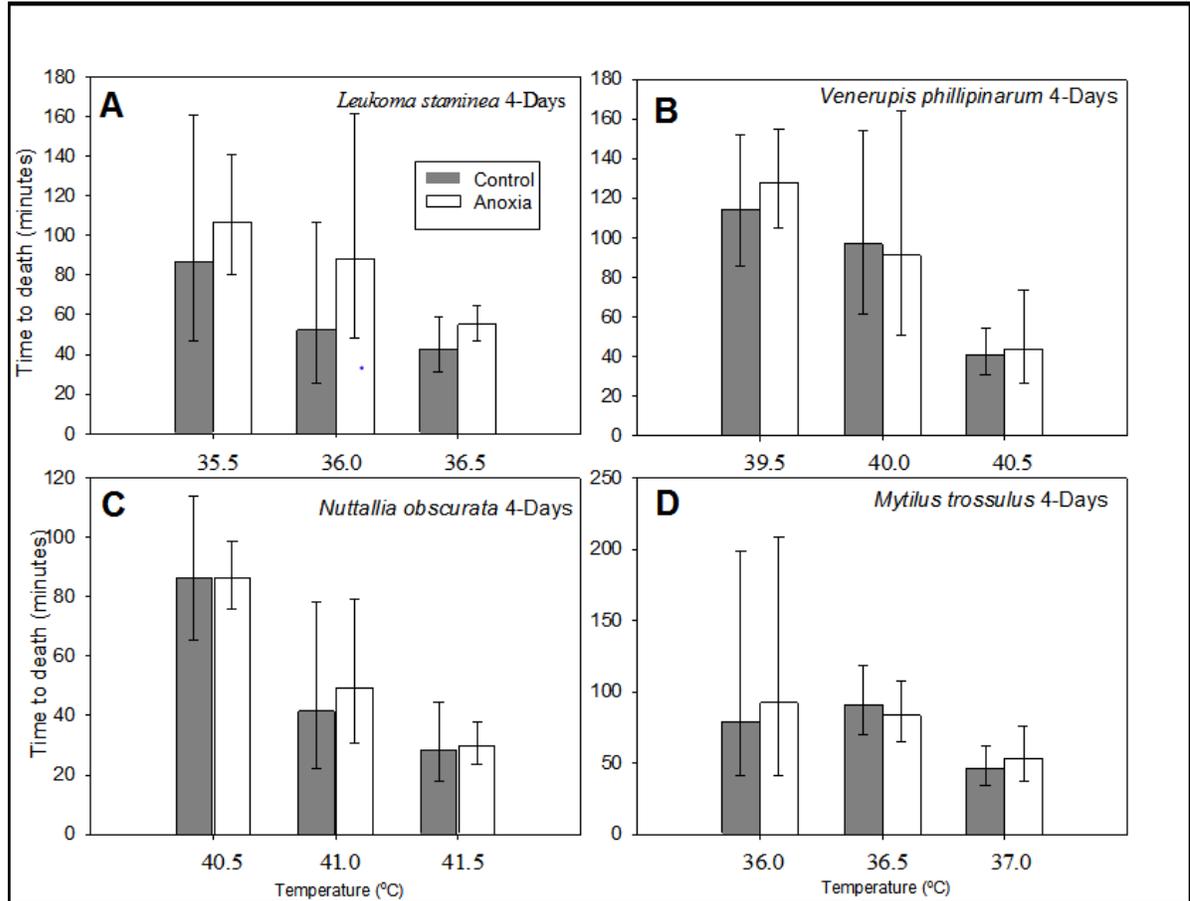
Figure 1: Thermal tolerance after four days of anoxia for *Leukoma staminea* (A), *Venerupis phillipinarum* (B), *Nuttallia obscurata* (C), and *Mytilus trossulus* (D). Temperatures varied by species. Average time to death of the gill tissue was log transformed to normalize variance for statistical analysis, but the untransformed data are presented here. Error bars represent 95% confidence levels.

Figure 2: Thermal tolerance of *Mytilus trossulus* after seven days of anoxia. Average time to death of the gill tissue was log transformed to normalize variance for statistical analysis, but the untransformed data are presented here. Error bars represent 95% confidence levels.

Stress protein levels

Expression of stress protein hsp70 in all four experimental species was evaluated by using portions of dissected gill tissue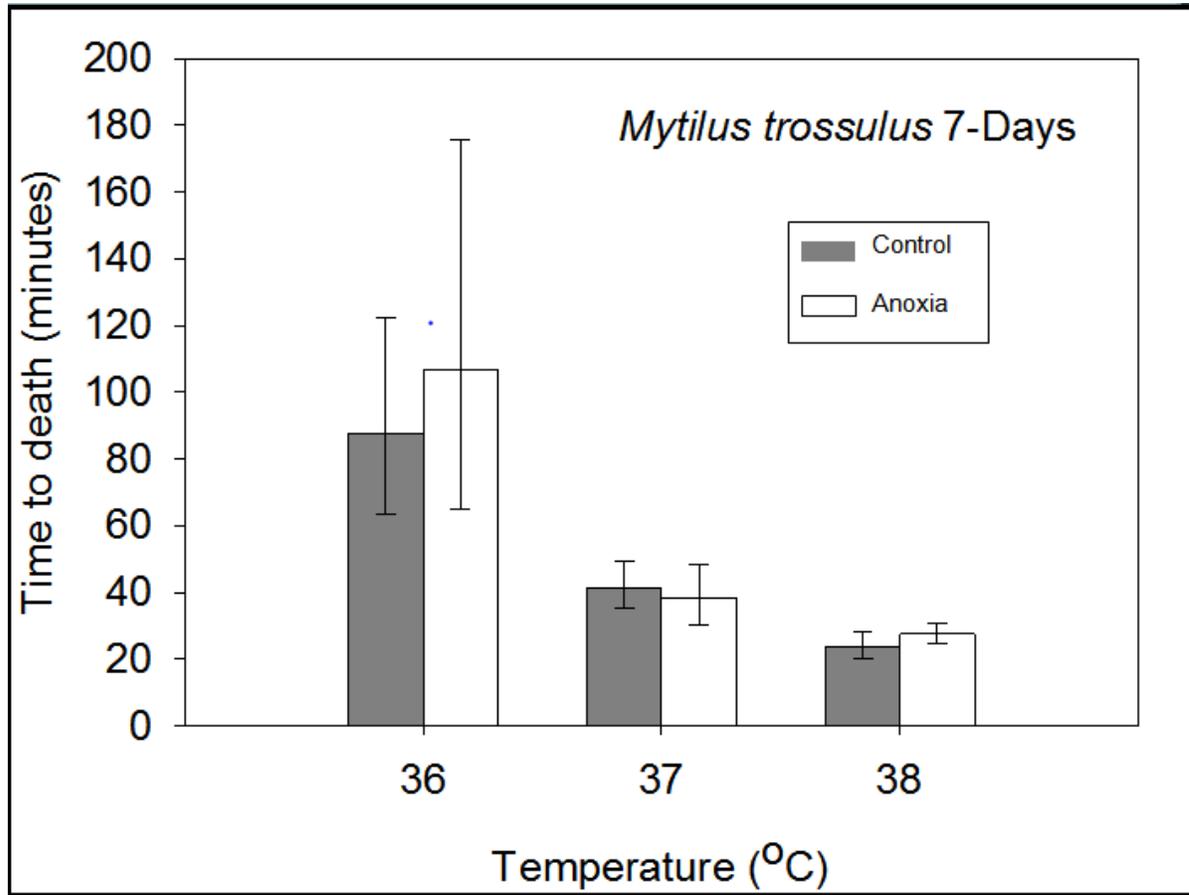 for Western blot analysis. Only background bands were observed in the three species of clams and were not used for analysis. In the mussel *M. trossulus*, no visual difference in expression was seen between the hsp70 expression in 4-day and 7-day samples, so quantification was only done on samples of *M. trossulus* exposed to four days of anoxia due to inadequate number of replicates on a single gel for the 7-day samples. Due to variation within the Western blot protocol, comparisons can only be made on samples from a single gel. Samples from *M. trossulus* showed strong hsp70 expression (Figure 3), but there was no significant difference between levels in control and anoxic mussels ($t_{1,7} = 0.463$, $p = 0.518$; Figure 4). The hsp70:α-tubulin ratio for the control group versus anoxic was nearly a 1:1 ratio (1.00:1.004) indicating that there was no difference in hsp70 expression between the control and anoxic samples.
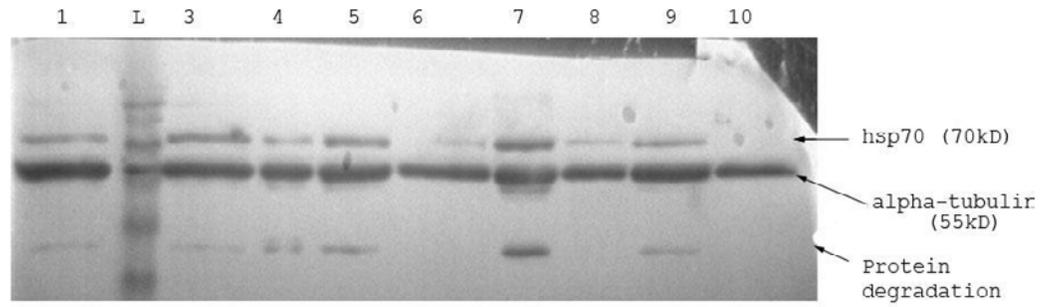
Figure 3: Representative Western Blot showing hsp70 expression in *Mytilus trossulus*. Lanes 1 and 3-6 contained samples from anoxic clams, and 7-10 were samples from control clams. The α-tubulin protein bands were used in quantification of the amount of hsp70 expressed.
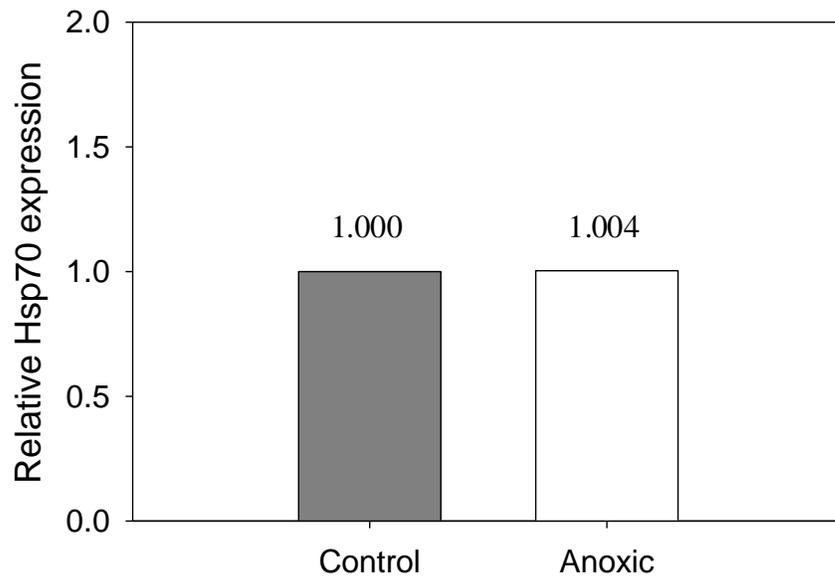
Figure 4: Relative expression of hsp70 in *Mytilus trossulus* in the control clams versus the anoxic clams. The ratio was determined by comparing the amount of hsp70 to α-tubulin within a single sample. The control samples were then set to 1.00 with anoxic samples compared to the control.

DISCUSSION

In this study, I found that several days of anoxia had no effect on subsequent thermal tolerance in the bivalves *Venerupis phillipinarum, Mytilus trossulus*, *Leukoma staminea,* and *Nuttallia obscurata*. This is supported by a lack of induced hsp70 production in *M. trossulus* after the period of anoxia, implying that this species is not stressed after four or seven days of anoxia. All of these species are located in the intertidal zone and see anoxic conditions regularly.

Many studies have examined the effects of a single stress on animals, but in their natural environment animals are exposed to multiple stressors on a daily basis. Studies examining thermal tolerance or anoxia have been done as single factors for many animals, from mammals to lower invertebrates. However, studies looking at the effects of both stresses are less common. Multiple stresses have varying effects depending on many factors including which stresses are used, the order in which they are exposed, the species being examined, and the degree to which the animals are exposed to each stress (Wiegant et al. 1995, Heugens et al. 2006). Two broad categories are used to describe an organism's response to multiple stressors: synergistic, when the combined effects of all stressors are greater than each individually, or antagonistic, when the effects are less when the stresses are combined (Folt et al. 1999, Vinebrook et al. 2004). One major goal of this study was to determine if an initial stress of anoxia would cause an increased tolerance to a thermal stress

in several species of bivalves. The results of my study indicate there is no change in thermal tolerance after a period of anoxia.

No significant increase or decrease in thermal tolerance was measured after a period of anoxia in any of the four species used in my study, which does not support the hypothesis of an initial stress creating an increased tolerance to a secondary stress. Even *M. trossulus* which was subjected to seven days of anoxia, just short of its lethal survival time of nine days, displayed no increase in thermal tolerance. This is not uncommon. In a study on the crustacean *Daphnia magna,* different combinations of stresses resulted in different responses (Heugens et al. 2006). Increased temperature and limited food supply resulted in increased growth rate, whereas increased temperature combined with cadmium exposure yielded a decrease in survival. In my study, the lack of a response after a period of prolonged anoxia may be indicative of the important ability of the bivalves to survive periods of low oxygen availability. They are so well adapted to surviving anoxia that their tolerance to the subsequent thermal stress was not impacted by the prior anoxia. This high tolerance is supported by the lack of induced hsp70 in *M. trossulus* after the period of anoxia.

Constitutive levels of hsp70 were definitively found only in *M. trossulus.* There was no difference in the expression of hsp70 between the control and anoxic clams, thus there was no evidence of the induced form of hsp70. This lack of hsp70 induction after a period of anoxia has also been observed in a number of other animals. In the oyster *Crassostrea gigas,* there was no significant up-regulation of hsp70 in gill tissue until after 14 days of hypoxic conditions followed by a decrease at day 21 (David et al. 2005). In rainbow trout red blood cells, no hsp70 was produced after anoxia, but there was stress protein production

after heat shock (Currie and Tufts 1997). The species used in my study are anoxia tolerant and so may not produce hsp70 after a period of anoxia.

It is also possible that the thermal stress was masking the effects of the anoxia. A modeling study by Folt et al. (1999), which classifies organism responses to multiple stresses, describes scenarios where the effects of two stresses combined are only equal to the strength of the greater stress, so the more mild stress is masked. In my study this would mean that any stress caused by the anoxia was masked by the bigger stress of the high temperatures. If this were the case, I would have expected to see some increase in hsp70 synthesis indicating that the animals were stressed. However, due to the well known nature of bivalves' ability to cope with anoxic stress and the lack of hsp70 induction, it is more likely that they were simply not stressed during anoxic conditions.

The most important way bivalves survive during anoxia is by lowering their metabolic rate to alleviate the need to produce as much ATP. During periods of anoxic stress, marine invertebrates can decrease their metabolic rate to < 10% of their resting rate (Greenway and Storey 1999, Ortmann and Grieshaber 2003, Lorade and Storey 2007) allowing them to survive on the ATP produced by anaerobic pathways for a much longer period of time. In one of the most extreme examples of anoxia tolerance, embryos of the brine shrimp *Artemia franciscana* will still hatch normally after four years of anoxia by entering a state of diapause where their metabolic rate is so low that it is undetectable (Clegg 1997). Studies examining the molecular level mechanisms of metabolic depression are important for understanding how animals are able to cope with anoxic stress and other stressors. A microarray examining gene regulation by the Pacific oyster *Crassostrea gigas* during long and short periods of anoxia showed the main genes regulated during these

periods were involved in cell communication, the immune system, and protein regulation (David et al 2005). Protein synthesis and degradation are very large parts of the energy budget of an animal. Thus, metabolic requirements can be lowered dramatically if protein synthesis and degradation are kept to a minimum. The depression of protein synthesis may explain the lack of hsp70 seen in this study. The need to depress their metabolism to a survival state may have inhibited the bivalves from producing hsp70 to save energy (Hofmann and Somero 1995)

Once metabolic requirements have been lowered, anaerobic pathways are utilized to produce the ATP necessary for the animal to function. There is not enough oxygen to continue aerobic respiration, so anaerobic fermentation pathways can be used. In one set of pathways, glucose (or glycogen) is converted to pyruvate, which is then combined with one of many amino acids to form opines (Hochachka and Mustafa 1972, Hochachka and Somero 2002). Unlike humans, where there is only one main anaerobic fermentation pathway (lactate production), bivalves possess many species-dependent pathways. Some common end products seen in bivalves are D-lactate, octopine, analopine, and strombine. These pathways are fairly inefficient in the time it takes to produce ATP from glucose, but offer a high rate of energy output per glucose molecule (Livingstone 1991). Thus, these pathways are often utilized more during functional hypoxia (swimming and burrowing) and less during environmental anoxia (Livingstone 1991, Carroll and Wells 1995). In a study of several New Zealand bivalves, opines were seen predominantly after functional anoxia, but low levels were also seen after environmental anoxia (Carroll and Wells 1995).

In addition to fermentation pathways, another way marine bivalves produce ATP during anoxia or hypoxia is through pathways that yield end products such as succinate and

propionate. These still utilize the pyruvate created by glycolysis, but instead of following the fermentation pathway, the pyruvate is instead converted into oxaloacetate, and from there through a series of reactions to succinate or propionate. Acetate, formed from acetyl-CoA, is another end product that is commonly found in bivalves during anoxia (Isani et al. 1995). These pathways are much more time efficient but have a lower rate of energy output making them more ideal during environmental anoxia (Livingstone 1991).

Additionally, different pathways can be used during different stages of anoxia. In *Mytilus sp*, large levels of aspartate present during normoxic conditions are converted to succinate during early stages of anoxia, which is then converted to propionate during long term anoxia (Hochachka and Somero 2002). During 14 days of anoxia, *Mytilus galloprovincialis* showed a biphasic metabolic response, with the first phase occurring during the first 24 hours. This first phase was characterized by large amounts of ATP being used and the accumulation of succinate and alanine. The second phase was a stationary phase, which involved a much lower usage of ATP, depletion of glycogen, and production of propionate (Isani et al. 1995). Similarly, in the clam *Corbicula fluminea*, succinate and propionate were produced during hypoxic conditions, but during voluntary shell closure only succinate accumulated (Ortmann and Grieshaber 2003). After two days of voluntary shell closure, levels of succinate were similar to that of clams undergoing just a few hours of hypoxic conditions. The ability to decrease metabolic rate and utilize anaerobic pathways would allow a species to survive long periods of anoxia.

In the bivalves used in my study, utilization of different anaerobic pathways offers some explanation as to why anoxia may not have been a great enough stressor to invoke an increased thermal tolerance. It is possible that during four days of anoxia they were still

utilizing short-term anaerobic pathways and depressed metabolism. However, even after seven days of anoxia for *M. trossulus* there was no increased thermal tolerance. In the invasive clam, *Corbicula fluminea,* voluntary valve-closure was seen for up to four days if conditions were unfavorable (cold temperatures). Even on a daily basis, a circadian rhythm would lead them to be closed for 5-10 hrs at night (Ortmann and Grieshaber 2003). The time frame the bivalves in my study were exposed to anoxia may simply be such a short period of time for these animals that no stress response was needed.

Stress protein production is thought to be a major component in an organisms' response to varying stressors. The lack of hsp70 seen in *Venerupis phillipinarum, Leukoma staminea,* and *Nuttallia obscurata* are possibly a result of a lack of hsp70 synthesis, but there was most likely a low affinity to the primary antibody that was used in this study. Studies examining hsp70 have not been done on all of the species used in this study, so the antibody used was selected based on its use in other bivalves including *Mytilus edulis* and *Crassostrea gigas* (Pempkowiak et al. 2001, Cruz-Rodríguez and Chu 2002, Fabbri et al. 2008). Faint amounts of protein were seen for each species, but there was not enough to verify if it was hsp70 or background proteins. Evaluation with a different antibody would be required to determine whether hsp70 is present in these species after a period of anoxia. Constitutive levels of hsp70 have been found in *V. phillipinarum* and *M. trossulus* using a different antibody (Piano et al. 2004).

The most common stress protein is hsp70, which has been found to be induced after a number of different stressors including thermal stress, toxins and anoxia (Lindquist 1986, Sanders 1993, Feder and Hofmann 1999, Kregel 2002). The lack of hsp70 production after anoxia in *M. trossulus* may be the result of the induction of heat shock proteins other than

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hsp70, which were not measured in this study. In another study examining hsp70 expression in *M. galloprovincialis* and *V. phillipinarum,* no increase in inducible hsp70 levels after thermal stress was observed (Piano et al. 2004). This indicates that at least some of these species may not utilize hsp70 during stresses such as thermal stress or anoxia. In mussel and scallop studies, hsp90 and hsp40 have been induced after hot and cold thermal stress and could possibly play a role in other stresses as well (Lagerspetz et al. 1995, Brun et al. 2008,). In *Artemia* embryos undergoing anoxic conditions for up to four years, large quantities of a small heat shock protein called p26 were found, which acts as a molecular chaperone similarly to hsp70 (Tanguay et al. 2004). It may be beneficial to examine other heat shock proteins within the four species used in this study to determine if there are other proteins being utilized.

Heat shock proteins are not the only stress proteins utilized by cells during anoxia. Many other types of proteins have been used in studies to mark whether an animal is undergoing stress (Sanders 1993, Kregel 2002). In *Mytilus galloprovincialis*, a mitogen activated protein-kinase (MAPK) signaling pathway called p38-MAPK was activated during anoxia and varying salinities (Gaitanaki et al. 2004). This signaling pathway is known to be a regulator during physiological stresses in many animals. Ubiquitin and ubiquitin conjugates are proteins commonly found in stressed invertebrates as they target damaged proteins for degradation (Dutton and Hofmann 2008). Increased production of these proteins has been observed during times of high protein damage such as thermal stress. All of these proteins offer other opportunities to re-evaluate the four species used in this study for anoxia stress-induced protein production. Although they did not appear to induce production of hsp70, they may well be utilizing one or all of these other proteins and

mechanisms. Utilizing Q-PCR methods, which allow quantification of a specific RNA marker, after a period of anoxia could determine which stress proteins are being utilized by the bivalves in my study.

Bivalves are well known for having high tolerance for anoxic conditions. In an oxygen-depleted Swedish fjord, several species of bivalves were among the last to disappear under extreme oxygen deprivation (Nilsson and Rosenberg 2000). The question of how soon these animals become internally anoxic is important for understanding the coping mechanisms used by each species. In my study, we determined that anoxic conditions were present within the mantle cavity of *M. trossulus* and *V. phillipinarum* within an hour. In a similar study, the clam *Arctica islandica* was nearly anoxic after just 40 minutes (Taylor 1976). It has been suggested that anaerobic metabolism begins when oxygen concentration falls to 20-50 mmHg (Ortmann and Grieshaber 2003). In an invasive clam from the Rhine River, aerobic metabolism was able to continue for 4-9 hrs after valve closure (Ortmann and Grieshaber 2003). This was calculated based on the amount of oxygen available in the mantle cavity and body tissues, and the estimated time it would take for the clam to deplete those resources. The ability to utilize anaerobic metabolic pathways once oxygen is depleted within the bivalve allows them to live relatively easily in areas where hypoxia or anoxia is commonly experienced.

The ability to survive long periods of anoxia would hold a competitive advantage over similar species found in the same area. As an invasive species, *N. obscurata,* has adapted to life at the very high intertidal where they would be exposed more often during emersion. To survive here, they must have excellent anoxia tolerance mechanisms that most likely would include utilization of different anaerobic pathways. Survival times during

anoxic conditions for all of these species would aid in determining whether this invasive species will out-compete the others as pollution and increased periods of anoxia become more frequent.

Temperature ranges for each species in my study reflect their locations within the intertidal zone. Located highest in the intertidal, *N. obscurata* would see the most extreme temperature ranges. Temperature readings during July at Chuckanut Bay, where all species were collected, showed temperatures up to 2 ˚C warmer where *N. obscurata* is located compared to the other species' locations lower in the intertidal (Siegrist, unpublished data). *Venerupis phillipinarum* is found from the high-mid intertidal down to the low intertidal and they had the second highest temperature range used in thermal tolerance tests. Having a tolerance to a wide range of temperatures allows them to have more useable habitat within the intertidal. Found in the lower intertidal zone, but on small mounds, was *M. trossulus* which had the second lowest temperature range used for testing. *Leukoma staminea* was found lowest in the intertidal zone and had the lowest temperatures used in the study. This species is not emersed as often and does not experience the high temperatures during the summer that the other species do because of their location within the intertidal. Among two congener species of *Mytilus* located in the same area but one in the subtidal (*M. galloprovincialis)* and the other in the intertidal (*M. trossulus*), tolerance to higher temperatures was seen in *M. galloprinvialis* because it was at the top of its geographical range, but *M. trossulus* had a tolerance to colder temperatures due to being in the southern part of its range (Dutton and Hofmann 2008).

Bivalves can tolerate long periods of anoxia, but several studies suggest that other stresses, including those of anthropogenic origin, can decrease their ability to cope with

natural stressors including anoxia (de Zwaan et al. 1995, Matozzo.et al. 2005, Heugens et al. 2006). Several species of bivalves including *Mytilus edulis* were exposed to cadmium prior to anoxic stress and all but one species of clam showed a significant decrease in survival (de Zwaan et al. 1995). There is a trade-off between the animal needing to increase metabolism to process the toxin and the need to decrease metabolism to cope with the anoxia. In the clam *Chamelea gallina*, anoxia caused their immune systems to become compromised by decreasing haemocyte counts and phagocytosis, making them more prone to infections by bacteria (Matozzo et al. 2005). In *Daphnia* there was a decreased growth rate with exposure to cadmium when combined with high temperatures, but when combined with high food availability no effect of cadmium was seen (Heugens et al. 2006). This reiterates the importance of testing multiple stresses in combination with natural conditions to fully understand what will have the greatest impacts on the animals. Increased eutrophication and pollution within our waterways will cause additional stresses on animals that are already undergoing a number of natural stresses.

As eutrophication and pollution in our estuaries and coastal zones increases, the effects these will have on the local fauna become increasingly important. A conceptual model explaining the importance of multiple stresses on biodiversity and ecosystem functions has been put forth to aid in understanding future effects on these coastal areas (Vinebrook et al. 2004). Species or individuals may be well adapted to one or several stressors, but each new stressor that is added, such as pollution or nutrient runoff, makes it more difficult for a species to survive. Increased periods of anoxia or added pollution may cause a strain on the biodiversity within these estuaries and coastal ecosystems and result in loss of productivity and species or allow new, more tolerant species to invade (Vinebrook et

al. 2004, Jewitt et al. 2005). This allows invasive species such as *N. obscurata* which has high tolerance to anoxia, to thrive and out-compete other species. This could have big impacts on the commercial shellfish fishery, since other clams may be displaced by more tolerant species. Of course, bivalves are not the only animals that live in these estuaries, and many of these other organisms may not be able to cope with large scale anoxia as successfully. Thus it is important to examine how these will be affected by large-scale anoxic events. As eutrophication increases, it is essential that we attempt to understand the short-term as well as long-term impacts it will have on the ecosystems involved, so that we can make smart conservation and resource management decisions.

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APPENDIX
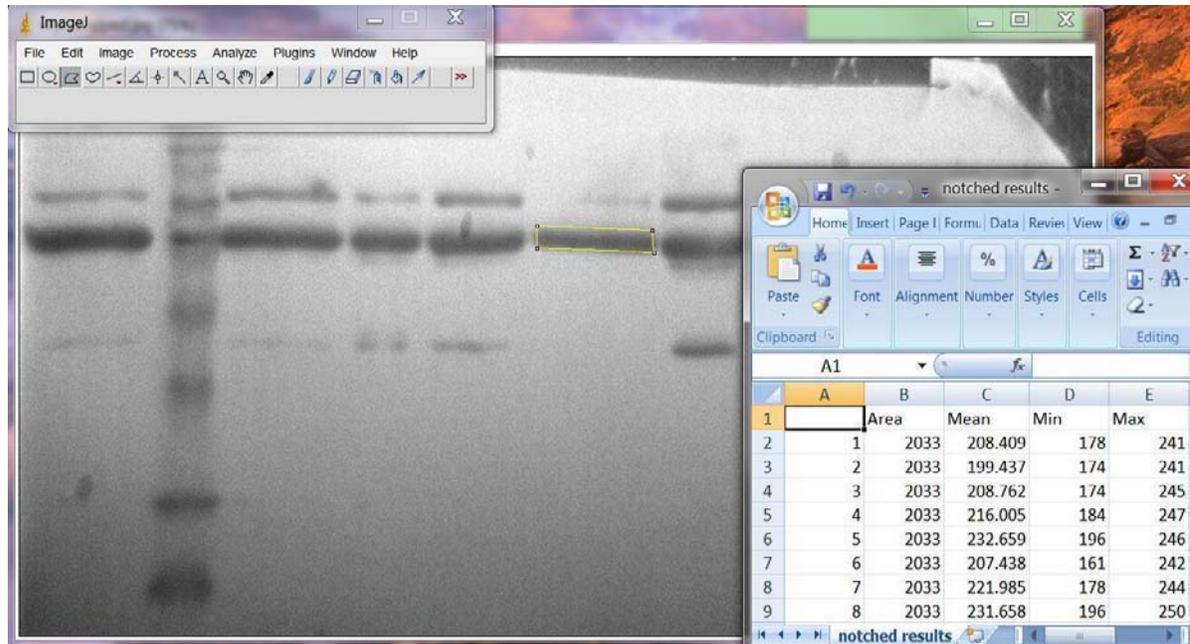
Hsp70 levels were quantified using ImageJ softwaere. Using the software a square the approximate size of a single band on the gel was drawn. The square was placed over every individual band of hsp70 or α-tubulin, keeping the square the same size. Within the square, mean pixel intensity values for each band were measured ("Mean" in the excel table) .