THE PROTEOMIC RESPONSES OF GILL TISSUE IN TIDALLY- AND
SUBTIDALLY-ACCLIMATED MUSSEL CONGENERS (*MYTILUS*

TROSSULUS AND MYTILUS GALLOPROVINCIALIS)

TO ACUTE AERIAL EMERSION

HYPOXIA

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Jaclyn Denise Campbell

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COMMITTEE MEMBERSHIP

TITLE: The Proteomic Responses of Gill
Tissue in Tidally- and Subtidally-
Acclimated Mussel Congeners (*Mytilus*
trossulus and *Mytilus galloprovincialis*) to
Acute Aerial Emersion Hypoxia

AUTHOR: Jaclyn Denise Campbell

DATE SUBMITTED: Febraury 2017

COMMITTEE CHAIR: Lars Tomanek, Ph.D.
Associate Professor of Biological Sciences

COMMITTEE MEMBER: Amy Howes, Ph.D.
Part-time Faculty for Biological Sciences

COMMITTEE MEMBER: Jeffrey Sklar, Ph.D.
Professor of Statistics

ABSTRACT

The Proteomic Responses of Gill Tissue in Tidally- and Subtidally-Acclimated Mussel

Congeners (*Mytilus trossulus* and *Mytilus galloprovincialis*) to

Acute Aerial Emersion Hypoxia

Jaclyn Denise Campbell

Understanding species-specific physiological tolerances to environmental extremes is key in determining the factors that contribute to regulating species distribution. This understanding will aid in determining which species will manage to thrive in a changing global climate. According to the IPCC (2013) it is expected that, in the coming years, many different types of abiotic factors will change as a result of global climate change. The intertidal habitat is a model habitat for studying environmental extremes as it is located at the interface between the marine and terrestrial environments, making it one of the most stressful marine habitats. It is characterized by a 24 hr light: dark cycle and a 12.4 tidal ebb and flow that exposes animals inhabiting this habitat to a wide array of aerial-associated stressors such as changes in temperature, aerial exposure, low oxygen or hypoxic conditions and desiccation stress. Sessile organisms such as marine mussels of the genus *Mytilus*, are an ideal study species for studying physiological tolerance at the environmental extremes of the intertidal habitat. In particular, *M. trossulus* and *M. galloprovincialis* are an excellent study system for examining physiological tolerance at environmental extremes due to the recent change in biogeographic range of both species. *M. galloprovincialis*, a native of the Mediterranean,

has been taking over the coast of California and has been displacing the heat sensitive native *M. trossulus*. The effects of salinity stress and heat stress on the physiologies of these species have been investigated by Braby and Somero (2006a and 2006b), Tomanek and Zuzow (2010) and Tomanek et al. (2012). The results of these studies indicate that the invasive *M. galloprovincialis* is more heat tolerant but is sensitive to hyposalinity while the reverse is true for the native *M. trossulus*. The next logical environmental stress to study is low tide or aerial-emersion as both species can be found both tidally and subtidally. According to Grieshaber et al. (1994) and Müller et al. (2012) *Mytilus edulis* mussels have mechanisms for mitigating aerial-emersion hypoxia; however, very few studies have been performed using the study system of *M. trossulus* and *M. galloprovincialis* in regard to aerial-emersion hypoxia. This study aimed to observe the responses of both *M. trossulus* and *M. galloprovincialis* to aerial-emersion hypoxic stress or low tide. The study also looked to see if the recent habitat history (tidal or subtidal) can play a role in the response of the mussels to hypoxia. The results of the experiment indicate that the invasive *M. galloprovincialis* may be less sensitive to hypoxic stress when compared to the native *M. trossulus*. This difference in sensitivity may be due to the difference in mechanisms of energy metabolism proteins and proteostasis proteins used to mitigate the effects of hypoxic stress. Moreover, tidal acclimation appears to better prepare the mussels for subsequent aerial exposure in both species, possibly based upon the principles of stress-hardening outlined by Kültz (2005).

Keywords: physiological tolerance, *M. trossulus*, *M. galloprovincialis*, aerial-emersion hypoxia, intertidal, stress response

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I. INTRODUCTION

The intertidal habitat is located at the interface between marine and terrestrial habitats (Gracey et al. 2008; Denny and Gaines, 2007). This makes the intertidal habitat one of the most stressful marine habitats, as it has unique physical and biochemical stressors from both the terrestrial and the marine environments (Fields et al., 2014; Petes et al., 2008; Denny and Gaines, 2007). For example, the intertidal habitat is characterized by many abiotic factors such as a fluctuating high and low tide cycle, which exposes its inhabitants to aerial emersion, an environmental stressor similar to terrestrial conditions (Lockwood et al., 2015). Additional abiotic factors present in the intertidal zone that can induce stress include sudden temperature shifts due to aerial emersion, low oxygen due to aerial emersion at low tide, ultraviolet radiation, and desiccation stress during aerial exposure (Altieri, 2006).

Stress is defined as a change in abiotic conditions that reach the limits of the adaptive (evolutionary) niche or beyond the homeostatic response limits set by recent (acclimatization or acclimation) history of an organism (Tomanek, 2015). Environmental stress (i.e.: heat, hypo-salinity, UV exposure) can negatively affect inhabitants of the intertidal habitat by causing cellular damage (Tomanek, 2014; Petes et al., 2008). Many stressors tend to damage macromolecular structures of cells, and can lead to the creation of reactive oxygen species (ROS, free radical oxygen anions), which can also cause damage to the cellular machinery (Tomanek, 2015). Intertidal organisms cope with macromolecular damage by activating a defense reaction known as the cellular stress response that can assess and counter act stress induced damage (Kültz, 2005). An example of this defense reaction is the synthesis of molecular chaperones (heat-shock

proteins) that function in maintaining protein conformation (Tomanek and Somero, 1999) in response to acute heat shock that can cause protein-denaturing conditions (Tomanek, 2015). In addition the stress response may include the down regulation of the organism's metabolism (metabolic depression; Braby and Somero, 2006a; Storey and Storey, 2007) and also switches to alternative anaerobic pathways, as that seen in marine bivalves (Müller et al., 2012; Fields et al. 2014).

One animal in particular that encounters widely varying physical conditions, which exceed the range that most marine organism' can cope with are marine mussels from the intertidal zone. Marine mussels are sessile organisms abundant in the low to mid rocky intertidal region (Braby and Somero 2006). They are a prey source for a variety of predators such as sea stars, whelks and sea otters (Seed and Suchanek, 1992; Braby and Somero, 2006b), a community foundation species (Seed and Suchanek, 1992; Shinen and Morgan, 2009) and a dominant competitor for space (Lockwood et al., 2015), making them a keystone species within the intertidal zone (Altieri 2006). Due to their sessile nature, mussels must endure physical extremes on a daily basis (Lockwood et al., 2015). For example, during periods of low tide the body temperature of a mussel can be 20 °C warmer than during high tide when the body temperature of the mussel matches the surrounding water temperature (Schneider, 2008). Two species of mussel that are of particular interest are the blue mussel congeners *Mytilus trossulus* and *Mytilus galloprovincialis* due to the introduction of *M. galloprovincialis* along the Pacific coast of the USA in the 1940s (Geller, 1999; Sarver and Foltz, 1993; Lockwood and Somero, 2011). This introduction has led to the displacement of the native *M. trossulus* from its southern most biogeographic range (Lockwood and Somero, 2011).

M. galloprovincialis has a higher tolerance to acute heat stress than its congener *M. trossulus* (Tomanek and Zuzow, 2010; Lockwood et al. 2010; Braby and Somero, 2006a; Braby and Somero 2006b). In fact, *M. trossulus* synthesizes heat shock proteins, key regulators of cellular stress, at lower temperatures than does *M. galloprovincialis* (Tomanek and Zuzow, 2010; Kültz, 2005). In addition, *M. galloprovincialis* is able to maintain high levels of antioxidant proteins, another set of proteins that play a large role in combating oxidative stress, whereas *M. trossulus* shows a decrease in antioxidant proteins at the same temperature of 32 °C (Tomanek and Zuzow, 2010; Tomanek, 2015). These studies highlight the physiological differences that can exist between closely related species that occupy slightly different thermal environments.

An abiotic factor that has not been looked at in depth using the study system of *M. trossulus* and *M. galloprovincialis* is hypoxia, specifically, hypoxia that is induced by aerial-emersion such as during periods of low tide in the intertidal habitat (Lockwood et al., 2015). Hypoxia is known as a state of reduced oxygen availability (Grieshaber et al., 1994). Hypoxia occurs when the partial pressures of ambient oxygen or PO₂ has fallen below normoxic values (Grishaber et al., 1994; Somero et al., 2016). Normoxic levels depend on the animal, as well as its level of activity (Grieshaber et al., 1994; Somero et al., 2016). For example, highly active animals tend to have higher rates of oxygen consumption and will have a different corresponding level of critical oxygen concentrations compared to sessile (non-moving) organisms (Somero et al., 2016). Intertidal animals such as marine mussels encounter periods of aerial-emersion induced hypoxia when they experience aerial-emersion during low tide (Grieshaber et al., 1994; Lockwood et al., 2015; Tomanek, 2015; Müller et al., 2012). The response of sessile

organisms to this aerial-immersion induced hypoxia varies but has been characterized by many studies that focus on the sessile marine mussel *M. edulis* (Müller et al., 2012; Tomanek, 2015; Grieshaber et al., 1994).

Understanding species-specific physiological tolerances to environmental extremes (such as elevated temperature, ocean acidification, hypo- and hyper-salinity, low oxygen) is key to determining the factors that contribute to regulating species distribution (Lockwood and Somero 2011). Understanding the pivotal roles played by environmental stressors, such as environmental temperature, in determining distribution patterns will aid in predicting how global climate change will affect where species occur and how well they will perform in the changing environment predicted for the future (Somero, 2010). The purpose of this study was to look at the effects of low oxygen (hypoxia) during acute aerial emersion on two species of blue mussels from the genus *Mytilus* using a proteomics-based approach. By understanding the effects of hypoxia on the proteomes of these two species a better understanding of their physiological mechanisms for mitigating hypoxia will be determined. This understanding of physiological tolerance may provide insight into the effects that global climate change will have on these organisms.

Mytilus mussels as a model organism

The habitat of Mytilus mussels

Marine intertidal mussels filter feed algae while contributing significantly to intertidal community structure as ecological engineers (Nowak, 2013; Gosling, 1992; Braby and Somero, 2006b; Seed and Suchanek, 1992). In the case of mussels, an ecological engineer forms the foundation for a variety of diverse hard-substrate

communities such as in moderate to fully wave-exposed intertidal regions (Seed and Suchanek, 1992). Mussels are one of the most abundant invertebrates within the rocky intertidal habitat (Braby and Somero, 2006). They are considered a prey resource by many species of marine animals such as the California Whelk *Nucella* spp in the high intertidal zone (Braby and Somero, 2006) and by species of sea star such as *Pisaster ochraceus* within the mid to low intertidal zones (Seed and Suchanek, 1992). Mussels, especially those of the genus *Mytilus*, are a dominant competitor for space in the intertidal zone (Lockwood et al., 2015). This is due to their relatively fast growth rate compared to other species of marine invertebrates (Shinen and Morgan, 2009). The species *Mytilus galloprovincialis*, for instance, has a growth rate that is ten times faster than that of other species of mussel such as the ribbed mussel *M. californianus* (Shinen and Morgan, 2009).

In addition to their fast growth rate, they are also considered dominant space occupiers due to their byssal attachment threads and their wedge-shaped shell (Seed and Suchanek, 1992). Their byssal threads and their shell shape have enabled them to successfully use hard strata such as those found along the rocky intertidal habitat to establish dense settlements (Seed and Suchanek, 1992; Sukhotin and Pörtner, 1999). These mussels, however, can also be found living in subtidal as well as intertidal habitats (Seed and Suchanek, 1992). Subtidal habitats that *Mytilus* mussels inhabit are typically man-made structures such as seamounts, dock pilings and offshore oil platforms (Seed and Suchanek, 1992). For example, subtidal *Mytilus* aggregations have been reported to form dense settlements along oil platforms off the coast of California (Simpson, 1977). The abundance of mussels tends to be lower in intertidal habitats as opposed to subtidal

habitats (Seed and Suchanek, 1992). Therefore, the lower limits of mussel distribution is characterized by predation while the upper limits of mussel distribution is characterized by abiotic conditions such as environmental stress (i.e. aerial exposure, increased temperature shifts, change in salinity) (Shick et al., 1988; Seed and Suchanek, 1992).

The evolution of Mytilus and its geographic distribution

The three species of blue mussels within the genus *Mytilus* are as follows: *Mytilus trossulus*, *M. galloprovincialis* and *M. edulis*. These three mussel species are widely distributed throughout both the northern and southern hemispheres inhabiting a wide range of habitats as mentioned above (Figure A; see below) (Hillbush et al., 2000; Braby and Somero, 2006b; Seed and Suchanek, 1992). This is due to their emergence as three species from the original ancestral species *Mytilus trossulus* (Lockwood and Somero, 2011). *M. trossulus* started out in the Northern Pacific region but established populations in the North Atlantic during a large scale trans-Atlantic faunal migration 3.5 million years ago (Lockwood and Somero, 2011). However, the North Atlantic populations became isolated due to glaciation, giving rise to the second species *M. edulis* (Lockwood and Somero, 2011). *M. edulis* began expanding its range throughout the Northern Atlantic area and then eventually into the Mediterranean Sea (Lockwood and Somero, 2011). This population became isolated approximately 2 million years ago giving rise to the third species of blue mussel *M. galloprovincialis* (Lockwood and Somero, 2011). Since these speciation events, the three species of mussels have been evolving under different abiotic conditions such as warm sea water temperatures versus cold sea water temperatures or fluctuating sea water salinity conditions versus constant salinity conditions (see section on *M. trossulus* and *M. galloprovincialis* for a more detailed account) (Lockwood and

Somero, 2011; Tomanek et al., 2012). These differing abiotic conditions have differentially shaped the physiological tolerances of these species via natural selection (Lockwood and Somero, 2011).

Mussels: a study species for physiological tolerance studies

Marine mussels from the genus *Mytilus* can serve as great model organisms for studying the effects of stress and physiological tolerance (Lockwood and Somero, 2011). This is due to the fact that mussels are sessile organisms that must be able to mitigate the effects of any stress that they encounter in their habitat using pronounced behavioral and physiological mechanisms that have developed in response to stress (Lockwood et al., 2015).

An organisms' physiological tolerance is determined by three main factors: genetic factors such as those that have been inherited by an organism, environmental factors such as the abiotic factors that shape the habitat that the organisms has occupied, and developmental factors such as the environmental conditions that have been present during the life cycle of the organism (Prosser and Heath, 1991). This physiological tolerance that defines organisms is most importantly shaped by environmental perturbations that threaten the stability of functional biochemical processes (Hochachka and Somero, 2002). These environmental factors include temperature, hydrostatic pressure, radiation, water availability, oxygen levels, and solute composition/concentration of the medium the organism inhabits (Hochachka and Somero, 2002). The ability of an organism to maintain structural integrity and biochemical functionality in a wide range of habitats is what defines the biogeographic range of an organism (Hochachka and Somero, 2002; Sorte et al., 2010).

There is great debate over which abiotic factors seem to play the largest role in shaping the differing physiological tolerances between the three closely related species of *Mytilus* (Braby and Somero, 2006b). However, field surveys of the hybrid zone between *M. trossulus* and *M. galloprovincialis* by Braby and Somero (2006b) and Schneider and Helmuth (2007), have given insight into which abiotic factors have the strongest influence. The prevailing hypothesis is that temperature and salinity play the most important role in determining *Mytilus* mussel distribution. However, other studies from Altieri (2006), Babarro et al. (2007) and Anestis et al. (2010) are starting to point towards other abiotic factors that could also determine the distribution of species within *Mytilus*. These abiotic factors are those factors associated with low tide or aerial exposure in the intertidal zone such as desiccation stress and low oxygen or hypoxia. This is due to the fact that the three species inhabit intertidal zones (Altieri, 2006 and Lockwood and Somero, 2011).

Understanding the resistance adaptations that organism have developed as a result of their evolution in certain habitats can aid in the understanding of a species distribution globally. In addition to species distribution, physiological tolerances may also be able to shed some light on other mechanisms such as the success of invasive species in non-native areas. In fact, of these three closely related species of *Mytilus*, *M. galloprovincialis* has managed to successfully invade new territories, especially along the California coast where it has been displacing the native *M. trossulus* species (Braby and Somero, 2006; Geller, 1999; Tomanek and Zuzow, 2010; and Lockwood and Somero, 2011). Understanding the mechanism of invasion requires an understanding of the physiological tolerances of both the native *M. trossulus* and the invasive *M. galloprovincialis*. A more

in depth understanding of the invasion of *M. galloprovincialis* could be used as a model system for studying marine invasions (Braby and Somero, 2006b).

***Mytilus galloprovincialis* as an invasive species**

According to Geller (1999), the introduction of *M. galloprovincialis* dates back to the 1940's, when southern California experienced a period of explosive growth from what appeared to be non-native species, according to anecdotal reports. Since this introduction in the 1940's, surveying studies have documented that *M. trossulus* has been disappearing from the southern California coast, while the biogeographic range of *M. galloprovincialis* has been expanding rapidly up the California coast (Geller, 1999). *M. galloprovincialis* has been introduced to the California coast from the Mediterranean region via shipping (Lockwood and Somero, 2011; Geller, 1999). Since its introduction, *M. galloprovincialis* has slowly been extending its geographic range northward up the California coast and has been displacing the native *M. trossulus* species (Lockwood and Somero, 2011). In addition to displacing the native species, *M. galloprovincialis* has also managed to interbreed with *M. trossulus* creating non-viable hybrids that exist within a hybrid zone. This hybrid zone is present along the California coast from Monterey Bay up to Humboldt Bay (Figure 2) (Lockwood and Somero, 2011; Braby and Somero, 2006b).

M. trossulus has evolved within the North Pacific region. Within this region, the mussel encounters conditions such as low nearshore salinity, large diurnal tidal fluctuations, and cold, variable water surface temperatures (Braby and Somero, 2006b). Conversely, *M. galloprovincialis* has evolved within the Mediterranean Sea. The conditions encountered by this species are higher salinity, less tidal fluctuations, warmer

water temperatures and less seasonal variation in both water temperature and salinity (Braby and Somero, 2006b). Thus, both species have adapted to very different environments.

A major ecological concern that is facing marine communities is whether or not communities are able to resist the introduction of an invasive species (Shinen and Morgan, 2009). As species diversity increases within communities, the communities become saturated and the number of available spaces to be colonized within by invasive species reduces in number (Shinen and Morgan, 2009). However, when invasions of non-native species are successful, as has been with *M. galloprovincialis*, the effects can be quite adverse. In fact, marine biological invasions are able to alter nearshore benthic (bottom-dwelling) communities such as intertidal communities (Geller, 1999; Carlton, 1989). Currently, many different marine communities are undergoing homogenization due to success of invasive species like *M. galloprovincialis* (Braby and Somero, 2006b). In this sense, homogenization is referring to a decrease in the diversity in the types of organisms as well as the number of different types of organisms that inhabit these communities (Geller, 1999; Carlton, 1989). In some cases, the alterations of the communities caused by the invasive species are major threats to biodiversity (Shinen and Morgan, 2009). For example, *M. galloprovincialis* has affected other native mussels such as *M. trossulus* but other non-related species such as herbivorous limpets (Shinen and Morgan, 2009). The success of invasive species can be attributed to specific biochemical mechanisms based upon their evolutionary history or even behavioral adaptations. Both mechanisms are outlined below.

Mechanisms of heat tolerance in M. galloprovincialis

According to a study by Tomanek and Zuzow (2010), it is apparent that *M. galloprovincialis* has a higher temperature tolerance than *M. trossulus*. This is due to the fact that *M. galloprovincialis* has evolved within the Mediterranean region, an area characterized by higher sea water temperatures compared to the North Pacific area off the Western coast of North America, where *M. trossulus* has evolved (Tomanek and Zuzow, 2010; Braby and Somero, 2006b; Geller, 1999; and Lockwood and Somero, 2011). In fact, in the study by Tomanek and Zuzow (2010), the effects of acute heat stress were examined. Mussels from two species of the genus *Mytilus* (*M. trossulus* and *M. galloprovincialis*) were exposed to acute heat stress at increasing temperatures. A proteomics approach was then used to determine the differences in physiological tolerance to acute heat stress. One of the major findings of the study indicated that *M. galloprovincialis* had a higher heat tolerance, made apparent by the continued response of the mussel at 32 °C compared to *M. trossulus*, which could only maintain a response at 28 °C (Tomanek and Zuzow, 2010). In fact, *M. trossulus* elicited a heat shock response with the increased expression of Hsp70 and small heat shock proteins at a lower temperature (28 °C) when compared to *M. galloprovincialis* at the higher temperature (32 °C) (Tomanek and Zuzow, 2010). In addition, *M. trossulus*, in general, had higher expression of proteasome subunits than *M. galloprovincialis*. This suggests that protein degradation may also play an important role in setting thermal limits (Tomanek and Zuzow, 2010). Also, the abundance of oxidative stress proteins decreased in *M. trossulus* at 32 °C, indicating an inability to maintain a cellular stress response against acute heat stress (Tomanek and Zuzow, 2010). Conversely, *M. galloprovincialis* was able to maintain high

expression of oxidative stress proteins at 32 °C, indicating a higher tolerance for acute heat stress (Tomanek and Zuzow, 2010). Overall, the study highlights the differences in thermal tolerance between *M. trossulus* and *M. galloprovincialis*.

There are two separate mechanisms that could explain differences in physiological tolerances pertaining to environmental stress from temperature increases (Lockwood and Somero, 2011). The first mechanism is the result of evolved or developmentally-induced differences in gene expression that ultimately led to differences in the concentrations of specific enzymes i.e. those involved in metabolism. In other words, certain selective pressures have created the need for higher concentrations for certain types of enzymes in specific habitats (Lockwood and Somero, 2011). For instance, *M. galloprovincialis* is adapted to warmer waters due to its evolution in the Mediterranean Sea. Therefore, this species typically will have lower metabolic rates than that of *M. trossulus*, a colder-temperature adapted organism evolved mainly along the Northern Pacific Areas on the coast of North America. When in the same temperature water, *M. trossulus* will have a higher metabolic rate than that of *M. galloprovincialis*. In order to maintain this higher rate of metabolism *M. trossulus* has developed higher concentrations of key metabolic enzymes.

Another mechanism to explain the differences in physiological tolerances would be minor differences in amino acid composition of orthologous enzymes that affect the kinetics of the enzyme-catalyzed reactions. In other words, *M. trossulus*, which is adapted to colder temperatures, has key metabolic enzymes that are slightly more efficient than their counterpart *M. galloprovincialis* (Lockwood and Somero, 2011). Thus, this organism would have a higher metabolic rate. Another way this theory can manifest is in

terms of the Michaelis-Menten constant (Lockwood and Somero, 2011). Typically, in the presence of certain stressors such as high temperature stress, enzymes exhibit reduced binding ability towards their substrate. This can occur due to induced shifts in enzyme conformational stability. Thus, in the case of temperature stress, *M. galloprovincialis* is able to maintain stable enzymes that have greater structural stability at higher temperatures unlike *M. trossulus* (Lockwood and Somero, 2011).

In addition to differences in the protein structure, the type of response elicited in an organism can also account for the tolerance of *M. galloprovincialis* to warmer seawater temperatures than *M. trossulus*. Differences in the production of heat shock proteins may be accounting for the differential thermal tolerances seen in the *Mytilus* congeners (Tomanek and Zuzow, 2010). The difference may be in the expression of particular isoforms of heat shock protein. Another mechanism highlighted by Tomanek and Zuzow (2010) is the difference in the expression of a certain type of protein that can elicit a different strategy altogether. It could be that by relying on a particular protein that can stabilize a particular type of cellular machinery such as the cytoskeleton, in the case of *M. galloprovincialis*, is more effective than trying to maintain all cellular machinery in times of stress. These differences in the type of cellular stress response utilized by the *Mytilus* congeners could be accounting for the physiological differences between genetically similar species thus giving them different biogeographical ranges. However, it is important to note that it is not one type of abiotic factor that sets distributional patterns but in fact a set of multiple types of stressors (Somero et al., 2016). Therefore, it is imperative that the physiological capacities be determined for each type of stress in these genetically similar organisms.

Mechanisms of hyposalinity tolerance in M. trossulus

In a study by Tomanek et al. (2012), the proteomic responses of *M. trossulus* and *M. galloprovincialis* to hyposaline stress were characterized. Mussels were acclimated to constant salinity conditions for four weeks. After acclimation, mussels were exposed to two different hyposalinity conditions followed by a recovery period. One of the major findings of the study suggests that *M. trossulus* was able to respond to greater hyposaline exposure than *M. galloprovincialis*. However, the proteomic response of both congeners showed common themes. There was an increase in ER molecular chaperones indicating an increase in protein unfolding in response to hyposaline stress. Changes in the proteins involved in vesicular transport and cytoskeletal modifications also occurred, indicating adjustments for changes in cell volume. In addition to cytoskeletal modification changes in energy metabolism proteins and ROS scavenging proteins were also utilized in order to reduce the production of ROS. However, the differences in the relative protein abundance of energy metabolism proteins between the two species indicated the presence of a species-specific response (Tomanek et al., 2012). There was an increase in energy metabolism at mild hyposaline stress during recovery in *M. galloprovincialis* indicating a difference in energy metabolism adjustment in response to hyposaline stress (Tomanek et al., 2012). This study indicates that while *M. galloprovincialis* may be better adapted to warm temperatures, it is not equipped to tolerate decreases in salinity concentrations like its congener *M. trossulus*. This study, along with the acute heat stress study (Tomanek and Zuzow, 2010) indicate that the evolution of these two species in different habitats (Pacific North West versus Mediterranean) have generated pronounced differences in these two genetically similar congeners in physiological tolerance.

Behavioral mechanisms for invasion

Despite *M. trossulus* ' better tolerance towards hyposalinity and *M. galloprovincialis* ' better tolerance towards heat stress, only *M. galloprovincialis* has managed to establish itself as an invasive species (Lockwood and Somero, 2011). In addition to biochemical mechanisms, the success of *M. galloprovincialis* can be attributed partly to certain behavioral-based competitive strategies such as direct strategies that utilize physical interference (i.e. crowding or pushing). In a study by Shinen and Morgan (2009), behavioral mechanisms such as direct (interference) competition and indirect competition (exploitation) were explored between *M. galloprovincialis* and two other native *Mytilus* species (*M. trossulus* and *M. californianus*) in both field conditions and laboratory conditions. This was done by looking at the relative growth and survival of each species under space-limited conditions such as growth in a polyculture (more than one species of mussel is grown together). The growth and survival rates were then compared to the control monoculture (only one type of species is grown) conditions. It was observed that *M. galloprovincialis* is able to reduce the growth rate and survival of *M. trossulus* when a two-species polyculture of *M. galloprovincialis* and *M. trossulus* was utilized. Another finding from the study by Shinen and Morgan (2009), was that the mortality of *M. californianus*, a ribbed mussel, increased as a result of what seemed like smothering from *M. galloprovincialis* climbing on top of *M. californianus* when grown in a two-species polyculture of *M. galloprovincialis* and *M. californianus*.

Overall, the invasive nature of *M. galloprovincialis* is due to a number of factors such as the tolerance to warmer sea water temperatures as was described by Tomanek and

Zuow (2010), Lockwood and Somero (2011) as well as Braby and Somero (2006b). It could also be due to the observations seen by Shinen and Morgan (2009), that *M. galloprovincialis* uses behavioral mechanisms such as physical interference to climb over and smother other mussel species such as *M. californias* in wave-protected areas of Southern California (Geller, 1999). In addition, *M. galloprovincialis* is also able to grow ten-times faster than *M. trossulus*. This allows *M. galloprovincialis* to reduce the amount of useable space for other sessile organisms in intertidal communities such as *M. trossulus*; thus, when in space-limited competitive hierarchy, *M. galloprovincialis* is a dominant competitor (Shinen and Morgan, 2009). However, in the face of changing conditions induced by global climate change, it is important to determine if these invasive species have the physiological characteristics necessary to not only displace native species but also to mitigate the effects of global climate change.

In order to determine what makes an invasive species, such as the blue mussel *Mytilus galloprovincialis*, successful at the physiological level, studies that focus on comparing congeneric species should be utilized. This is due to the fact that species of the same biotype living in different ecological conditions display different physiological characteristics (Sukhotin and Pörtner, 1999). Thus, using the invasion of *M. galloprovincialis* in the biogeographic range of *M. trossulus* as a study system can provide insights into how interspecific physiological differences can facilitate the relative success of invasive species over native species (Lockwood and Somero, 2011). Certain differences between *Mytilus* congeners are topical and can disappear by placing the mussels in equal life conditions (Sukhotin and Pörtner, 1999). However, other physiological characteristics that are maintained for prolonged periods indicate the

presence of genetic differences (Sukhotin and Pörtner, 1999). Thus, it is important to track the physiological responses the organisms have to individual stressors in order to predict the effects that global climate change may have on species composition by looking at the molecular phenotype.

The rocky intertidal: a model environment for stress response

Both *Mytilus trossulus* and *M. galloprovincialis* are known to inhabit areas along the rocky intertidal region (Schneider, 2008; Lockwood et al., 2015). The rocky intertidal habitat is one of the most stressful habitats in nature (Guderly et al., 1994; Werner et al., 1983). This is due to the fact that the intertidal is characterized by many different types of abiotic factors such as tidal cycles, diel cycles, solar heating and wave action (Lockwood et al., 2015). The habitat exists at the interface between marine and terrestrial environments, giving it characteristics of both regions. For example, the intertidal region experiences a 24-hour light and dark daily rhythm from the terrestrial environment, while also experiencing a 12.4-hour ebb and flow from the tidal cycle of the marine environment (Lockwood et al., 2015).

The tidal cycle and its influence on physiology

The tidal ebb and flow exposes sessile organisms such as *M. trossulus* and *M. galloprovincialis* to aerial conditions during low tide. These aerial conditions can expose organisms to a variety of stressors such as low oxygen, and temperature shifts which can lead to an organism experiencing stress such as an increase in internal temperature and a decrease in aerobic metabolism (Altieri, 2006; Schneider, 2008; Babarro et al., 2007; Fields et al., 2014). A study by Helmuth (2002), looked at how body temperature changes

during low tide using *M. californianus*. This was done by using temperature loggers that thermally matched mussels in mussel beds at eight different sites of varying latitudes. One of the major findings from this study indicated that *M. californianus* mussels, during low tide, can have an internal body temperature that is 20 °C warmer than during high tide where the mussel experiences the same internal temperature of the surrounding water. In addition, a study by Babarro et al. (2007), looked at effects of both anaerobiosis and temperature increase on individuals of species *M. galloprovincialis*. The results of the experiment show that *M. galloprovincialis*, in periods of anaerobiosis, has aerobic rates of 6-17% of aquatic aerobic values when exposed to aerial conditions. Also, a study by Fields et al. (2014), which looked at the effects of aerial-emersion hypoxia on the salt marsh mussel *Geukensia demissa*, exposed *G. demissa* to different lengths of hypoxia followed by periods of recovery. This study also confirmed that, in response to aerial exposure, a decrease in aerobic metabolism was observed. In response to both the diel cycles and the tidal cycles, organisms have developed circatidal biological rhythms that match the rise and the fall of the tide (Lockwood et al., 2015). These rhythms allow organisms to anticipate and respond accordingly to predictable daily changes (Lockwood et al., 2015). In a transcriptomics study by Connor and Gracey (2011), the influence of tidal rhythms and light/dark cycles was determined on the physiology of *M. californianus* by monitoring rhythms of gene expression in simulated and natural tidal conditions. This was done by sampling mussels in conditions of twelve hour light: twelve hour dark cycle regime alongside six hour episodes of seawater immersion and aerial-emersion (which simulates the tidal cycle) every two hours. The study identified 236 transcripts that oscillated with a tidal period of 12 hours and a larger group of 2,365 transcripts that

oscillated on a circadian period of roughly 24 hours. Thus, intertidal organisms such as blue mussels have adapted specific physiological mechanisms that can be used to mitigate the effects of a stressful environment such as the intertidal.

The intensity and the relative importance of these different stressors can vary due to the organism's location along the intertidal zone (Werner et al. 1983). The intertidal region can be divided into three regions: the high intertidal region, mid intertidal region and the low intertidal region (Helmuth et al., 2006; Gracey et al., 2008). The ecology of the three intertidal zones is heavily influenced by the changing diel and tidal cycles (Lockwood et al., 2015). For example, regions within the high intertidal zone experience longer exposure to low tide and larger temperature shifts which exposes mussels to more extreme abiotic stresses when compared to the mid to lower intertidal regions (Fields et al., 2014; Schneider, 2008). Most mussel beds on the west coast of North America are located within primarily the mid intertidal range (Lockwood et al., 2015). Within this region, mussels experience two periods of low tides and two periods of high tides (Lockwood et al., 2015). Some mussel beds can be found at the higher intertidal zones where they experience high tide only once a day (Gracey et al. 2008). Thus, the location of these mussels along the intertidal habitat can heavily impact their physiological strategies (Gracey et al., 2008). For example, mussels that are collected from the high intertidal zone have expression patterns that are characterized by a period of growth and proliferation and a period of recovery to restore cellular energy (Gracey et al., 2008). This contrasts the expression patterns seen in mussels of the mid intertidal region that experience less drastic abiotic stress (Gracey et al., 2008; Lockwood et al., 2015). Thus,

the differences in intensity of abiotic stressors create an ideal study site for examining physiological tolerance.

The intertidal is a habitat ideal for studying physiological limits, as many types of stressors are extreme in the rocky intertidal (Somero, 2008). The magnitude and the rapidity of changes in the physical environment make organisms from these habitat, especially sessile organisms such as *Mytilus* mussels great model organisms for physiological and biochemical adaptations (Fields et. al 2014). This extreme environment can act as a predictor of how organisms will be able to withstand the changing environment to come. They act as warning signs for the impacts of global climate change (Helmuth et al., 2006).

Global climate change and its influence on the environment

In many regions of the world global climate change has led to alterations in abiotic factors such as temperature, pH and oxygen levels (Somero et al., 2016). These abiotic factors can have wide-ranging effects on the physiologies of many species of marine organisms (Somero et al., 2016). Climate change is a change in the state of the climate that can be identified by changes in the mean and/or the variability of its properties, and that persists for an extended period, typically decades or longer (IPCC, 2013). This is in regards to climate which is defined as the average weather over a period of time ranging from months to thousands or millions of years (IPCC, 2013). The properties of climate that are predicted to experience change that are of the great interest to this study are zonal mean precipitation events and averaged surface ocean temperatures. According to the IPCC (2013), it is predicted that precipitation events are expected to increase in occurrence in the high and some of the mid latitudes, which can

lead to changes in salinity of ocean water in those areas. Also, globally averaged surface and vertically averaged ocean temperatures are expected to increase in the near term (IPCC, 2013). This increase in temperatures and precipitation events are likely to affect the distribution of species (Somero et al., 2016). In fact, these increases in surface ocean temperatures have led to an increase in eutrophication events by algal species (Somero et al., 2016). These eutrophication events can lead to a rapid decrease in oxygen, leading to oxygen minimum zones (Somero et al., 2016). These oxygen minimum zones, occurring mainly near the equator, have caused species to shift their distribution more northward (Somero et al., 2016).

Environmental stress studies: a way to predict the effects of GCC

With global climate change causing alterations in abiotic factors, it is important to have an understanding of how organisms mitigate the effects of stress. This will help to predict where species will occur in the future as well as predict how well these organisms will thrive in the changing ecosystems caused by global climate change (Somero, 2010). Therefore, in order to determine how successful *M. galloprovincialis* will be as an invasive species and to determine the continued success of *M. trossuulus* it is important to have a fundamental understanding of how these two species respond to different types of stressors. Understanding how these organisms respond to individual stressors will provide a basis for multiple stressor studies (Somero et al., 2016). According to a review by Somero et al. (2016), it is unlikely that the changing global climate will cause only one type of stressor such as changes in ocean water temperatures, changes in levels of dissolved O₂ in sea water or ocean acidification to increase in intensity. In fact, it is expected that these three classes of stressors will all change in intensity due to global

climate change (Somero et al., 2016; IPCC, 2013). For example, with global climate change it is expected that episodic upwelling events of cold deep water will be increasing in frequency (Somero et al., 2016). This will cause organisms to experience rapid decreases in temperature, which can occur in conjunction with falling pH and concentrations of dissolved oxygen (DO) (Somero et al., 2016). Therefore, exposure to multiple abiotic stressors can lead to effects that are the sum of effects of individual stressors (additive), effects from stressors that could have counter-acting or offsetting effects (antagonistic) or stressors amplifying the influence of other stressors (synergistic) (Somero et al., 2016). Thus, it is important to understand how these stressors affect the physiologies of organisms by way of multiple stressor studies (Somero et al., 2016). However, before multiple stressor studies can be done single stressor studies must be conducted in order to determine what the effects (as listed above) are of a single stressor. This will help pinpoint which types of stressors will impact organisms the most with global climate change.

Global climate change is expected to increase the temperatures surface sea waters, which may negatively impact organisms that have a low tolerance to increasing temperatures such as *M. trossulus* (IPCC, 2013; Tomanek and Zuzow, 2010). In addition to changing temperatures, global climate change will also lead to a higher incidence of precipitation events, increasing the amount of runoff that flows into the ocean and thus causing drops in salinity or creating hyposaline conditions (Tomanek et al., 2012; IPCC, 2013). Thus, organisms that are susceptible to low salinity conditions such as *M. galloprovincialis* will be negatively affected by this change. To date, many studies such as those by Tomanek and Zuzow (2010), Schenider (2008), Lockwood et al. (2010), and

Tomanek et al. (2012) have looked at the effects of increasing temperature and decreasing salinity concentrations on the physiologies of marine mussels including *M. galloprovincialis* and *M. trossulus*. One type of abiotic factor that has been over-looked in this two species study system when considering global climate change is the concentration of dissolved oxygen in the ocean water.

The increasing temperatures of global climate change will create warmer water that holds less oxygen (Somero et al., 2016; Keeling et al., 2010). In addition, this increase in water temperature can lead to a decrease in the circulation of sea water, a process that is necessary for keeping the oceans ventilated (Somero et al., 2016). This will lead to an increase in the amount of hypoxic water present as well as an increase in the size of the oxygen minimum zones (OMZ) that are currently present (Somero et al., 2016). This increase in the occurrence of hypoxia within the ocean have already led to large-scale mortalities in sessile animals (Somero et al., 2016). With this in mind, it is important to understand how hypoxia or low oxygen levels, caused from global climate change will affect sessile marine organisms such as *M. trossulus* and *M. galloprovincialis*.

Hypoxia and its effects on the physiology of marine mussels

Hypoxia is defined as a state of reduced oxygen availability when the partial pressure of ambient oxygen falls below normally experienced levels of oxygen by organisms or “normoxic” levels (Grieshaber et al., 1994). The normal oxygen levels that organisms experience vary between species, and depend on the type of organism or species, as well other types of physiological parameters such as activity level of the organism and resting metabolic rate of the organism (Somero et al., 2016; Grieshaber et

al., 1994). Hypoxia is experienced by marine organisms as a result of two scenarios: the functional levels of the organism increase in order to achieve certain behavioral functions (functional hypoxia) or as a result of changing environmental conditions of the surrounding habitat (Environmental hypoxia) (Somero et al., 2016; Grieshaber et al., 1994; Müller et al., 2012).

Environmental versus physiological hypoxia

Functional hypoxia depends on the organism's activity level or the organism's resting metabolic rate (Müller et al., 2012; Somero et al., 2016). Functional hypoxia is experienced by organisms that experience behavioral changes. These behavioral changes can occur as a result of fleeing predators or trying to catch prey (Müller et al., 2012; Somero et al., 2016). Both types of scenarios require an increase in energy demand in order to either successfully avoid a predator or successfully catch a prey. This increase in energy demand triggers a response that increases anaerobic metabolic processes (Müller et al., 2012; Somero et al., 2016). In order to acquire large amount of energy rapidly organisms will switch from primarily aerobic metabolism to rapid anaerobic metabolism of ATP in order to satisfy the increased energy demand (Müller et al., 2012; Somero et al., 2016).

Environmental hypoxia occurs when the habitat itself that the organism occupies has a change in oxygen concentrations (Müller et al., 2012; Somero et al., 2016; Grieshaber et al., 1994). Changes in oxygen concentration can occur as a result of changes in abiotic factors (Somero et al., 2016). An example of changes in abiotic factors that lead to changes in oxygen concentrations occur when ventilation of dissolved oxygen in sea water does not occur properly due to fluctuating water temperatures as will be

experienced by marine organisms with the changing global climate (Somero et al., 2016; Grieshaber et al., 1994). Another type of scenario that can result in environmental hypoxia occurs in the intertidal region (Tomanek, 2015). Environmental hypoxia is experienced by sessile organisms such as marine mussels within the intertidal habitat, as it is brought on during periods of low tide, when marine mussels experience aerial emersion (Tomanek, 2015; Grieshaber et al., 1994). In order to avoid desiccations stress, mussels will close their valves and seal off their shells, creating low oxygen conditions within their shells (Lockwood et al., 2015; Anestis et al., 2010). Thus, during these periods of aerial-emersion there is less than ideal levels of oxygen available to these sessile marine mussels, as they are unable to move into areas that are not aurally-emersed (Tomanek, 2015; Lockwood et al., 2015).

Due to the fact that marine mussels are sessile organisms, they are more likely to encounter environmental hypoxia instead of functional hypoxia (Somero et al., 2016; Tomanek et al., 2015; Lockwood et al., 2015). Specifically, marine mussels encounter environmental hypoxia due to aerial-emersion from low tide created by an alternating tidal cycle, which is characteristic of the intertidal zone (Lockwood et al., 2015; Müller et al., 2012; Anestis et al., 2010). However, aerial emersion-induced hypoxia is not the only type of environmental hypoxia that marine mussels encounter. Marine mussels encounter hypoxic water when the concentrations of dissolved oxygen within sea water drop (Somero et al., 2016). These drops in dissolved oxygen concentration can occur as a result of severe eutrophication events. Eutrophication events are caused by an increase in nutrients utilized by organisms such as algae that lead to dense growth, otherwise known as algal blooms. These eutrophication events or algal blooms cause dramatic decreases in

oxygen concentrations (Rabalais et al., 2009; Hallegraeff, 2010; Somero et al., 2016). These eutrophication events can occur due to excessive runoff from land due to increased precipitation events or from increasing sea water temperatures, both consequences of global climate change (Somero et al., 2016; Rabalais et al., 2009; Hallegraeff, 2010; IPCC, 2013). These algal blooms can lead to the formation of hypoxic water (Somero et al., 2016; Rabalais et al., 2009; Hallegraeff, 2010). Though the current focus is on aerial emersion, marine mussels' resistance to aerial emersion can also reveal how well a marine mussel can survive hypoxic water created by these eutrophication events that will increase in severity and frequency due to global climate change (Altieri, 2006; Somero et al., 2016). In fact, a study by Altieri (2006), looked at the tolerance of *M. edulis* mussels to either aerial exposure or submerged hypoxia after reciprocal transplants of mussels between upper intertidal conditions and subtidal conditions. The results revealed that mussels that had been exposed to intertidal conditions or acute periods of aerial-emersion had a lower rates of mortality during extended periods of aerial-emersion exposure and hypoxic-water exposure. Therefore, determination of population level variation in tolerance to environmental hypoxia or aerial-emersion is of practical importance (Altieri, 2006).

Strategies utilized by mussels to tolerate aerial-immersion hypoxia

The strategies utilized by marine mussels during these periods of environmental hypoxia center around maintaining certain levels of ATP or available energy for use in maintenance of the organism (Müller et al., 2012). In order to achieve these energy levels necessary for cellular maintenance, marine mussels such as members of the genus *Mytilus* utilize a series of physiological adaptations. These adaptations are useful for

maintaining not only essential levels of energy but also mitigate the rising oxidative stress levels that can result from low oxygen conditions.

When marine mussels first encounter aerial emersion, the first strategy that is utilized is the closing of their shell in order to avoid desiccation of gill tissue (Müller et al., 2012; Lockwood et al., 2015; Anestis et al., 2010; Grieshaber et al., 1994). Gill tissue is a vital organ to mussels as it is the site of gas exchange (Bayne et al., 1976). In addition to being the site of gas exchange, gill tissue is also involved in facilitating water transport and separation of particles that are drawn in with ambient water that is being filtered into the mussel for feeding and gas exchange (Nowak, 2013; Riisgård et al., 2011; Bayne, 1976). However, this closing of the shell is what creates the hypoxic conditions that are experienced by the mussel, as shell closure reduces the rate of oxygen consumption to 4-17% of its immersed rate (Müller et al., 2012; Lockwood et al., 2015; Anestis et al., 2010). Once the mussel has closed its shell, the mussel utilizes two strategies. The first strategy is depression of metabolic rate during valve closure in air (Demers and Guderly, 1994; Fields et al., 2014; Grieshaber et al., 1994). This is done in order to compensate for the decreased rate of oxygen consumption, as this metabolic depression can decrease the metabolic rate up to 5% of its normal rate (Demers and Guderly, 1994; Fields et al., 2014; Babarro et al., 2007). Blue mussels, specifically *M. edulis*, decrease metabolic rate by decreasing the activity of key glycolytic enzymes, such as phosphofructokinase, by decreasing the activity of the enzyme that activates phosphofructokinase, fructose-2,6-biphosphate (Storey, 1987). In addition, *M. edulis* will also phosphorylate phosphofructokinase and pyruvate kinase, another key glycolytic enzyme, in order to produce less efficient forms of these enzymes and decrease the rate of glycolysis (Demers

and Guderly, 1994; Storey, 1987). The second strategy utilized by marine mussels involves the switch to anaerobic metabolism, which leads to the accumulation of opines, such as succinate, propionate and other certain volatile fatty acids (Demers and Guderly, 1994; Müller et al., 2012; Fields et al., 2014).

Anaerobic metabolism in marine mussels

During aerial exposure, a switch to anaerobic metabolism occurs after metabolism has been depressed. During this switch, there is an increase in proteins involved in anaerobic pathways towards succinate and propionate rather than lactate (Tomanek, 2015; Fields et al., 2014; Müller et al., 2012; Grieshaber et al., 1994). This anaerobic mechanism has evolved specifically in mussels that undergo periods of hypoxia because it has the potential to produce more ATP when compared to fermentative pathways that produce lactate (Müller et al., 2012; Fields et al., 2014). For instance, succinate and propionate-based fermentative pathways produce six ATP molecules per glucose versus the two molecules of ATP produced by lactate-based fermentation (Fields et al., 2014; Müller et al., 2012). Not only does this type of metabolism increase the amount of ATP produced but it also delays acidosis (Fields et al., 2014; Müller et al., 2012). This is done by producing three moles of ATP per one mole of H^+ versus lactate fermentation that produces one mole of ATP per one mole of H^+ generated (Fields et al., 2014; Müller et al., 2012).

The formation of alanine during anaerobic metabolism

Alanine is formed from glycogen or pyruvate along with the degradation of L-alanine, another free amino acid available in invertebrates (Müller et al., 2012; Grieshaber et al., 1994). Alanine is formed from the transamination of aspartate by glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and malate dehydrogenase (Grieshaber et al., 1994). Low oxygen levels, high levels of pyruvate and high levels of NADH act as a trigger for the transamination of aspartate into alanine (Grieshaber et al., 1994). According to a review by Grieshaber et al. (1994), which characterized the physiological and metabolic responses of a number of invertebrate species to hypoxia, this type of transamination is characteristic of anoxia-tolerant marine invertebrates that have higher pools of free amino acids for osmotic regulation. Glycogen is one of the major energy sources of marine mussels during extended periods of hypoxia (Grieshaber et al., 1994). It can be stored in higher amounts in the tissues of marine invertebrates as it does not tend to increase the osmotic pressure within the cell (Grieshaber et al., 1994). In fact, glycogen content in marine invertebrates accounts for 10%-35% of the dry weight of the organism (Grieshaber et al., 1994). Glycogen is fermented via the Embden-Meyerhof-Parnas pathway located within the cytosol (Grieshaber et al., 1994). The Embden-Meyerhof-Parnas is one of the most common types of glycolytic pathways seen within the cytosol (Grieshaber et al., 1994). The fermentation of glycogen provides two or 0.5 mol ATP more than the transphosphorylation of a phosphagen (discussed later) or the fermentation of aspartate to succinate (Grieshaber et al., 1994). Succinate, in contrast to alanine, is produced as a part of the Krebs cycle from the reductive branch of malate dismutation (Müller et al., 2012).

The formation of succinate during anaerobic metabolism

The reductive branch of malate dismutation is what is responsible for the formation of succinate (Müller et al., 2012; Grieshaber et al., 1994). Malate dismutation is a fermentative process for the degradation of carbohydrates (Müller et al., 2012). The succinate is formed from acetate and propionate in a split pathway in anaerobically functioning mitochondria (Müller et al., 2012; Grieshaber et al., 1994).

Phosphoenolpyruvate (PEP) from glycolysis is converted into oxaloacetate via an ATP-linked PEP carboxykinase (Müller et al., 2012). This is then subsequently reduced by cytosolic malate dehydrogenase which reoxidizes the glycolytic NADH (Müller et al., 2012). The malate formed from this process is then transported into the mitochondria for malate dismutation. In this process a portion of the malate is oxidized into acetate, while another portion is reduced to succinate. This succinate can then be further metabolized into propionate. Acetate and propionate are accumulated in a ratio of 1:2 making this process a redox balancing process (Müller et al., 2012). Thus, the process of transaminating aspartate and then further converting the end products to succinate is coupled to both glycogen fermentation and the production of alanine (Müller et al., 2012).

Opine formation during anaerobic metabolism

Another form of anaerobic metabolism by *M. edulis* is the formation of opines (Müller et al., 2012; Grieshaber et al., 1994). It is a cytosolic fermentation pathway that condenses pyruvate with a free-floating amino acid by a specific dehydrogenase that reduces a Schiff base with glycolytic NADH, which results in the formation of an iminoacid or an opine (Müller et al., 2012). For example, the condensation of pyruvate

and arginine by octopine dehydrogenase results in the formation of octopine as the opine end product, whereas the condensation of other types of amino acids such as alanine or glycine forms alanopine or strombine (Müller et al., 2012). A Schiff base is essentially a sub-class of imines and acts as a substrate for the formation of the iminoacid or opine in opine fermentation (Müller et al., 2012; Grieshaber et al., 1994). These anaerobic opine end products, as stated above, are less acidic than lactate. In addition, only one amino acid is condensed to form these opine end products, thus making this process osmotically neutral (Müller et al., 2012). These amino acids that are used in this process come from the pool of amino acids that are used for osmotic balance (Müller et al., 2012). In addition to being osmotically neutral, opine fermentation maintains the redox balance of the cell (Müller et al., 2012). The opines from fermentation are accumulated within the cell until oxygen is present again (Müller et al., 2012). Once oxygen is present again, the opines are reoxidized (Müller et al., 2012).

Other non-fermentative strategies utilized during hypoxia

In addition to these fermentative pathways, other types of anaerobic methods are used in order to fulfill the cellular maintenance ATP demand. One such strategy is the use of phosphagens. Phosphagens are phosphorylated guanidinium compounds that serve as rapidly available stores of phosphate bond energy that can be used as needed for ATP generation without the presence of oxygen (Grieshaber et al., 1994). The form of phosphagens used by many invertebrates, such as marine mussels, is phospho-L-arginine. Phosphagens are a fast form of ATP energy (Grieshaber et al., 1994). They are typically used during the beginning stages of hypoxia, before the switch to anaerobic metabolism or fermentative pathways has been made (Grieshaber et al., 1994). This is due to the fact

that just a single step reaction is needed to transphosphorylate the phosphagen into ATP (Grieshaber et al., 1994).

In conclusion, the acute hypoxic response of blue mussels can be characterized into two phases: defense and rescue. The first phase or line of defense is characterized as the coordinated suppression of ATP consumption and ATP producing pathways to reach and maintain a new steady state level of ATP (Anestis et al., 2010). It is important that these mussels have the ability to switch to anaerobic mechanisms especially those that are living in intertidal regions (Anestis et al., 2010; Lockwood et al., 2015; Fields et al., 2014). However, increased anaerobic capacity is a short-term solution for the exposure to hypoxic conditions. In long-term emersion, upregulation of fermentative pathways disappears, and a shift towards the cellular stress response occurs in order to protect existing tissues (Fields et al., 2014). Therefore, the second phase of the hypoxic response is known as the rescue phase and is characterized by rescue mechanisms that initiate the expression of several oxidative stress proteins (Anestis et al., 2010). The timing of the onset of the second phase varies from hours to up to a day and depends upon the species type and aerial-exposure time (Fields et al., 2014; Grieshaber et al., 1994; Müller et al. 2012).

The cellular stress response and hypoxia

During extended periods of hypoxic exposure, marine mussels will start to experience stress which signals a shift towards the second phase of the hypoxic response also known as the rescue phase (Anestis et al., 2010; Fields et al., 2014). However, before the cellular stress response in regards to aerial emersion or hypoxia can be examined, we must first define what environmental stress is.

Environmental stress

Environmental stress is encountered in an organism when any physical or chemical factor that perturbs the physiological and biochemical systems of an organism past a certain threshold leads to suboptimal values of their normal traits (Tomanek, 2015; Somero et al., 2016). In addition to hypoxia from aerial emersion, other types of abiotic stressors such as temperature, osmotic perturbations and pH perturbations, can lead to stress by creating suboptimal conditions in an organism (Tomanek, 2015; Somero et al., 2016). However, it is important to note that a change in the intensity of any one of these abiotic stressors will not necessarily lead to an organism experiencing stress (Somero et al., 2016). Stress is only encountered by an organism when the displacement of traits by these stressors is outside of the organism's optimal value of ranges (Somero et al., 2016; Tomanek, 2015). In regards to the hypoxic response in mussels, a rescue response is coordinated that involves an increase in the abundance of certain proteins (Fields et al., 2014; Tomanek, 2015). However, this type of cellular stress response may not be just common to hypoxia. In fact, there exists a cellular stress response that is non-specific and is activated in the presence of any type of environmental stress.

Environmental stressors and the cellular stress response

There exists a common theme to most forms of environmental stress (Kültz, 2005). That theme is damage is typically inflicted to macromolecular structures of the cell during the acute phase of any environmental stress (Kültz, 2005; Tomanek, 2015). This macromolecular damage activates a response that involves a common set of cellular processes known as the cellular stress response (Tomanek, 2015; Kültz, 2005). Thus, the cellular stress response or CSR is essentially a defense reaction that detects

macromolecular damage such as the deformation or damage of DNA, the unfolding or denaturation of proteins, etc. (Tomanek, 2015; Kültz, 2005). The CSR assesses and counteracts stress-induced damage, which in turn temporarily increases tolerance towards the environmental stress causing the damage and removes terminally damaged cells via apoptosis (Tomanek, 2015; Kültz, 2005). The capacity of the CSR is both species dependent and cell-type dependent (Tomanek, 2015). The CSR is indeed characteristic to all cells, and the targets of the CSR include cellular functions such as cell cycle control, protein chaperoning and repair, DNA and chromatin stabilization and repair, removal of damaged proteins and certain aspects of metabolism (Kültz, 2005).

The CSR is aided by expression of certain types of chaperone proteins within the cell (Tomanek, 2015; Kültz, 2005). High expression of these chaperone proteins at all times confers some level of stress resistance (Kültz, 2005). Aspects of the CSR or protein members of the CSR arsenal include redox-sensitive proteins, proteins involved in sensing, repairing and minimizing damage (i.e. chaperones), oxidoreductases involved in energy metabolism as well as cellular redox regulation, and cell cycle control (Kültz, 2005). In order for these proteins of the CSR to be properly expressed, the CSR must also have some transcriptional factors that coordinate to help produce these proteins (Kültz, 2005). Transcriptional responses to stress are coordinated by inducible and redox-sensitive transcription factors and transduction pathways (Anestis et al., 2010). An example of this would be the signaling pathways of JNKs and p38 kinases which seemed to be involved in the phosphorylation of heat shock factors (HSF) in marine bivalves, and promote the induction of the heat shock proteins, a type of chaperone protein, in response to several stress conditions (Anestis et al., 2010; Tomanek, 2015; Kültz, 2005). As most

types of environmental stressors induce macromolecular damage, it seems as though the cellular stress response is only a general response to all types of environmental stress (Kültz, 2005). However, the CSR can coordinate into a specific response depending on the environmental stressor type.

Stressor-specific stress responses

According to Tomanek (2011) and Tomanek (2012), there are types of stressors that warrant a specified stress response. One such study done by Tomanek et al. (2011), looked at the effects of low pH on the eastern oyster *Crassostrea virginica*. When oysters from the species *C. virginica* were acclimated to low pH, the oyster elicited a broad complement of antioxidant proteins in response to a low pH of 7.5 (Tomanek, 2011). This complement of proteins included the increased expression of copper-zinc superoxide dismutase (SOD), the cytoplasmic form of peroxiredoxin (Prx2), the endoplasmic reticulum (ER) isoform of peroxiredoxin (Prx4), and the mitochondrial and peroxisomal isoform of peroxiredoxin (Prx5) (Tomanek, 2015; Tomanek, 2011). In addition, *C. virginica* increased expression of nucleoredoxin (homolog of thioredoxin or Trx) in response to the low pH of 7.5 (Tomanek, 2015; Tomanek, 2011). This suggests that the expression of the SOD-Prx-Trx complex of proteins occurs in response to low pH (Tomanek, 2015). This could be in response to increasing levels of reactive oxygen species (ROS) formation (Tomanek, 2015). Reactive oxygen species or ROS are oxygen species with an unpaired electron that react with and damage biological macromolecules such as protein, lipids and DNA (Tomanek, 2011). They form as a consequence of oxygens structure (Tomanek, 2015). The two outer electrons on oxygens outer shell have a parallel spin (Tomanek, 2015). Thus, in order for oxygen to become reduced the

reaction must proceed through single electron transfer reactions (Tomanek, 2015). Many forms of ROS include superoxide anions, hydrogen peroxide and a hydroxyl radical (Tomanek, 2015). ROS are usually the source of macromolecular damage during periods of environmental stress in a majority of organisms (Tomanek, 2015; Kültz, 2005). In another study by Tomanek et al. (2012) that looked at hyposaline stress in the gill tissues of *Mytilus*, a different stress response from that of low pH was observed. In response to hyposaline stress, a decrease in the abundance of ER chaperones was observed (Tomanek, 2015; Tomanek et al., 2012). Thus, we see that two different types of environmental stressors elicited a seemingly different response which appears to support the hypothesis that there is a stress-specific response (Kültz, 2005; Tomanek, 2015).

Specificity in the cellular stress response can arise due to the types of lesions or damage that are caused to proteins, DNA and membranes that could potentially vary depending upon the type of stressor experienced (Kültz, 2005). Specificity can also arise from stressor specific interactions, posttranslational modifications and compartmentalization of stress proteins resulting from different relative levels of induction with a common set of stress proteins (Kültz, 2005). The CSR appears to elicit some level of stressor-specific response. Though different types of stressors can elicit different responses, there is still one unifying theme that links stressors together which is that stressors, in any form, lead to the creation of oxidative stress (Kültz, 2005).

Oxidative stress: a means of creating stress resistance

Oxidative stress is defined as a state where the rate of reactive oxygen species (ROS) formation is excessive and overwhelms the antioxidant capacity of organisms (Hermes-Lima and Zenteno-Savin, 2002). ROS-type molecules can act as a signal for the

activation of defense mechanisms (Hermes-Lima and Zenteno-Savin, 2002). They can act as second messengers in transduction pathways (Zenteno-Savin, 2002; Kültz, 2005). The production of ROS can occur during periods of stress or even during periods of recovery, such as those that occur after hypoxic/anoxic events where reperfusion of tissues upregulates the metabolism of an organism rapidly and the over-generation of ROS can occur (Hermes-Lima and Zenteno-Savin, 2002; Tomanek, 2015). Oxidative stress is typically experienced due to a change in cellular redox potential, which can be disrupted in the presence of a specific stressor (Kültz, 2005). The molecular events that lead to an increase in ROS depend on the type of stress disrupting the redox potential (Kültz, 2005). During times of oxidative stress, cellular oxidases such as NADPH oxidase are activated which increase ROS as well (Kültz, 2005). Together, ROS and cellular redox potential changes act as secondary messengers in cells exposed to stress (Kültz, 2005). With environmental stress, however, a type of resistance can be formed with frequent exposures to stress.

Environmental stress tolerance varies based upon two properties: the species of the organism and the cell-type the stress is occurring in as well as its differentiation state (Kültz, 2005). The stress tolerance stemming from the genome is formed from gene-environment interactions during development or the organisms' life history (Kültz, 2005). As in the case of the blue mussels, *M. galloprovincialis*'s tolerance to heat stems from the fact that the organism has evolved in the Mediterranean Sea, while *M. trossulus*'s tolerance for low salinity comes from the fact that the organism has evolved in the North Pacific off the coast of North America (Tomanek and Zuzow, 2010; Tomanek et al., 2012; Tomanek, 2015). However, there is another type of conditioning to stress that can

occur without the evolution of the organism and is based upon preconditioning of the organism to the stress at low doses (Kültz, 2005). This phenomenon is known as stress-hardening (Kültz, 2005). This type of preconditioning can result in an increased stress tolerance due to the fact that the organism has already been introduced to the stress, and has already mounted a response to the stress (Kültz, 2005). This stress-hardening has been seen in many studies involving species of marine mussels and their hypoxia tolerance. In the study by Altieri (2006), marine mussels from the species *Mytilus edulis* that had been acclimatized to intertidal conditions had a higher tolerance towards both aerial-emersion hypoxia and aqueous hypoxia when compared to subtidal or immersed *M. edulis* mussels. This study indicated that an inducible tolerance to low-oxygen environments was gained from experiencing low, nonlethal doses of the aerial-emersion hypoxia (Altieri, 2006). This provides strong evidence for stress hardening.

In stress-hardening, after the initial stress has been experienced by the organism, stress proteins remain active/elevated for a time (Kültz, 2005). The length of the time depends on the species, cell type, history of prior stress exposure, gene environment interactions during development and stress severity (Kültz, 2005). Activated or elevated levels of the stress proteins during this time confer resistance in many different type of stress due to their involvement in many general aspects of cellular protection (Kültz, 2005). However, this increased tolerance or stress-hardening appears to have an energetic cost associated with maintenance of the physiological mechanisms that aid in the survival during aerial exposure (Altieri, 2006). This was due to the fact that intertidal *M. edulis* mussels that had been subsequently transplanted back to subtidal conditions lost this increased aerial-exposure or low-oxygen tolerance (Altieri, 2006). The question

that now still remains is how low-oxygen conditions can lead to an increase in reactive oxygen intermediates, when metabolism is typically decreased in marine mussels and oxygen consumption also decreases.

The effects of hypoxia on the cellular stress response

When oxygen levels are low during hypoxia, this leads to a repression of the metabolic state. This lowered metabolic state leads to a lowered ATP demand (Tomanek, 2015). This can cause an increase in NADH/NAD⁺ within the mitochondria, which inhibits electron transfer down the electron transport chain (Tomanek, 2015). This inhibited electron transfer leads to a back-up of electrons from NADH via FMN to O₂ (Tomanek, 2015). In addition, low ATP affects the activity of glycolytic enzymes and pyruvate dehydrogenase (Tomanek, 2015). Taken together, hypoxia leads to a high pool of reduced coenzyme Q(CoQH₂/CoQ), a high proton gradient and a low rate of ATP synthesis (Tomanek, 2015). This all leads to the flow of electrons back from CoQH₂ to complex I of the electron transport chain (Tomanek, 2015). This back flow of electrons is what leads to the creation of many different types of ROS that can cause macromolecular damage in these marine mussels (Tomanek, 2015).

In order to characterize the cellular stress response and these concepts of stress-hardening, a good place to start is by looking at the molecular phenotype of cells. One such experimental method that can be utilized when observing the molecular phenotype at the cellular level is proteomics.

Proteomics as a tool for studying the cellular stress response

Proteins are the biochemical machinery of organisms (Tomanek, 2014). The types of proteins utilized by organisms in response to a changing environment represent the type of functional processes that organisms need to thrive in any type of condition, particularly those conditions that create environmental stress (Tomenk, 2014; Tomanek, 2011). The proteome then represents the entire protein pool of organisms, and is therefore the protein complement of an organism's genome (Campos et al., 2012; Woods et al., 2014). Due to the fact that the proteome is essentially the end product of gene expression it represents the final molecular phenotype, and can provide powerful insights into the adaptations that organisms have to changing abiotic factors (Tomanek 2014; Tomanek, 2011). The proteome is a dynamic and complex network that responds quickly to changing abiotic and biotic factors, sometimes independently of the genome (Tomanek, 2014). The proteome can change independently of the genome by altering already produced proteins, instead of making new proteins with transcriptional activation (Tomanek, 2014; Tomanek, 2011). These alterations include the use of posttranslational modifications, and protein-protein interactions (Tomenk, 2014; Tomanek, 2011). Posttranslational modifications or PTMs are chemical modifications to proteins that occur after protein synthesis or translation and can alter their function (Tomanek, 2011). Some examples of posttranslational modifications include phosphorylation, acetylation, and ubiquitination (Tomanek et al., 2011).

The power and limitations of proteomics

Proteomics is an analytical tool that can be used to study the proteome of organisms in a specific tissue at any given time (Tomanek, 2014; Tomanek, 2012;

Campos et al., 2012). Proteomics is a powerful tool for studying the proteome of organisms as it can be used to study the processes of protein synthesis, posttranslational modifications, and protein degradation, especially in response to a changing environment (Tomanek, 2011; Tomanek, 2014; Campos et al., 2012). In addition, proteomic analyses have the potential to report alterations in structural proteins, and key proteins of the oxidative stress defense mechanisms, cell signaling, protein stabilization, energy metabolism and metabolism of lipids, and amino acids (Campos et al., 2012). In fact, proteins that are present within a cell have the potential to be present in many different forms within a cell (up to 20 different forms according to Suckau et al., 2003) making proteomics an important analytical tool for identifying each of these different forms (Suckau et al., 2003). Thus, the stated goal of proteomics then is to characterize the entirety of all protein forms, including posttranslational modifications and protein-protein interactions (Tomanek, 2011).

Many technical limitations in proteomic analyses restrict access to the entire proteome (Tomanek, 2011). These limitations range from sample preparation only isolating specific types of proteins to lack of communication with other individuals who have the technical expertise to facilitate the completion of a proteomics project (Tomanek, 2014). These limitations prompt the question of why proteomics is the preferred method for studying the molecular physiology of organisms as opposed to other techniques such as transcriptomics. Transcriptomics is another method of gene expression profiling that involves the study of transcript levels (Lockwood et al., 2015; Evans, 2015). Transcriptomics focuses on just one level of regulation in the protein synthesis pathway- the regulation of transcription to form mRNA transcripts (Lockwood

et al., 2015; Evans, 2015). These mRNA transcripts are obtained by using microarray or sequencing of all mRNA to characterize gene expression (Lockwood et al., 2015; Evans, 2015). Though these techniques have helped to provide a comparative framework to elucidate evolutionary outcomes of natural selection in terms of transcriptional regulation, a major limitation exists when using transcriptomics to study the physiological response of organisms. This major limitation is that, according to Feder and Walsh (2005), variation in transcript levels in general explain less than half of the variation in protein abundances. In addition, transcriptomics does not take into account modifications that can occur independently of the genome (as mentioned above) (Tomanek, 2014; Tomanek, 2011). In fact, according to Suckau et al., 2003, the number of expressed proteins that are present within a cell tend to exceed the number of genes within the genome. Thus, proteomics is the clear choice for studying an organism's response to a changing environment as proteins, not mRNA transcripts, are the final molecular phenotype of cells and have a direct effect on organismal physiology and organismal fitness (Feder and Walsh, 2005; Tomanek, 2011).

Though proteomics should be the focused method for studying organismal response to changing environmental conditions it should not be the only approach considered (Aggarwal and Lee, 2003). Proteomics can provide a basic understanding of what is occurring at the cellular level in response to changing environmental conditions, and can provide the key molecular components of living systems (Aggarwal and Lee, 2003). However, information coming from only one level such as the level of the proteome is not sufficient for fully explaining the behavior of biological systems in response to a changing environment (Aggarwal and Lee, 2003). This is due to the fact

that in some studies, such as studies concerning human liver cells and yeast cells, there is some disconnect between mRNA expression levels and protein expression levels (Aggarwal and Lee, 2003). Thus, there is a need for an integrated approach that utilizes many types of measurements such as measurements at the transcriptomics level, the proteomics level and even the metabolomics level in order to fully understand biological systems (Aggarwal and Lee, 2003). In order to accomplish this integrative technique, studies that utilize each of these techniques individually need to be done and their results combined to gain a greater understanding of what is occurring at the physiological level of organisms in response to a changing environment (Aggarwal and Lee, 2003).

The different types of proteomic workflows

Proteomic studies typically differ in the types of workflows utilized. There are two types of major workflows for proteomic analysis (Tomanek, 2011). The first major type of proteomic workflow involves the separation of proteins by two-dimensional gel electrophoresis (2D GE), followed by subsequent identification by either matrix-assisted laser desorption ionization (MALDI) mass spectrometry or by electrospray ionization (ESI) mass spectrometry following enzymatic proteolytic cleavage typically using trypsin digestion (Tomanek, 2011). The other type of workflow typically utilized in proteomic analysis first involves the digestion of proteins, which are then separated by liquid chromatography before identification with ESI mass spectrometry (Tomanek, 2011). The type of proteomic workflow determined for use in any given proteomic analysis depends on two parameters (Tomanek, 2011; Woods et al., 2014). The first parameter is the type of sample that is being used as the source of proteins (Tomanek, 2011). Thus, sample collection is crucial for contributing to how powerful the results of any proteomic

analysis will be (Tomanek, 2011). The more targeted the sample collection is the more powerful the analysis will be (Tomanek, 2011). The second parameter that determines which proteomic workflow that is utilized is how the proteins will be quantified (Tomanek, 2011). Proteins can be quantified in two ways. The first method of quantification is a gel-based technique that is dependent upon protein spot detection within a 2D GE-based gel (Tomanek, 2011). If proteins are quantified using this technique then the first proteomic workflow mentioned earlier is utilized (Tomanek, 2011). The other type of protein quantification is gel-free and uses either stable isotope labeling or label-free spectral counting (Tomanek, 2011). If proteins are quantified using this technique then the second type of proteomic workflow (described above) is utilized (Tomanek, 2011). Despite these differences in the methods utilized, most proteomic studies follow the same three step approach: sample preparation, protein separation and analysis with mass spectrometry, and data analysis using bioinformatics (Tomanek, 2011; Woods et al., 2014).

Sample preparation

Sample preparation, the first step in the proteomic workflow, is essentially the separation of proteins from other interfering macromolecular compounds such as nucleic acids and polysaccharides within a tissue (Tomanek, 2011; Woods et al., 2014). In other words, the aim of sample preparation is to convert the proteins within a tissue sample into a suitable physiochemical state for subsequent protein separation later on in the proteomic workflow (Shaw and Riederer, 2003; Woods et al., 2014). This means that the proteins need to be solubilized, disaggregated, denatured and reduced in order to completely disrupt intra- and intermolecular interactions and to ensure that each spot that

is isolated truly represents only a single polypeptide (Shaw and Riederer, 2003; Görg et al., 2004). These molecular interactions need to be disrupted all while maintaining the protein's native charge (which is a result of their amino acid composition) and the protein's molecular weight (Shaw and Riederer, 2003; Woods et al., 2014; Görg et al., 2004). It is important that the inherent charge properties and the molecular weight of the proteins be maintained as proteins will be separated based upon their isoelectric point or pI and molecular weight later on in the proteomic workflow (Görg et al., 2004; Tomanek, 2011; Shaw and Riederer, 2003). In addition, these charge properties are important for detection of posttranslational modifications or PTMs (Görg et al., 2004; Shaw and Riederer, 2003; Tomanek, 2011). It is also crucial that sample preparation resolve as many proteins as possible in order to facilitate analysis or protein identification later down the line (Shaw and Riederer, 2003).

Sample preparation involves the disruption of cells or homogenization of tissue that is acting as the source of proteins (Shaw and Riederer, 2003; Görg et al., 2004). This homogenization is achieved by using a urea-based buffer (Shaw and Reiderer, 2003; Tomanek, 2011). In addition to a urea-based buffer sample preparation also uses other elements such as thiourea, detergents and reducing agents (Shaw and Riederer, 2003). Urea is a neutral caotrope that denatures proteins by disrupting noncovalent bonds and ionic bonds between amino acid residues (Shaw and Riederer, 2003). Urea can also disrupt hydrogen bonds which leads to protein unfolding and denaturation (Görg et al., 2004). The chaotropic quality of urea is ideal for denaturing proteins for better separation from other cellular elements, while the neutral quality of urea helps to maintain the isoelectric point or pI of the protein for subsequent separation from other proteins (Shaw

and Reiderer, 2003). Thiourea is used for breaking up hydrophobic interactions, and can even improve the stabilization of hydrophobic membrane proteins, which can increase the number of proteins that are isolated for subsequent identification (Shaw and Riederer, 2003; Görg et al., 2004). Detergents such as sodium dodecyl sulfate or SDS are used to aid in denaturing proteins for subsequent protein separation (Shaw and Riederer, 2003). Also, detergents are used to prevent hydrophobic interactions occurring between exposed hydrophobic protein domains, which can lead to a loss of proteins due to aggregation and precipitation (Görg et al., 2004). In addition, reducing agents are used for the reduction of disulfide bonds between cysteine amino acids, which help to maintain quaternary structure of proteins (Shaw and Riederer, 2003). Thus, reducing agents are necessary for the cleavage of intra- and intermolecular disulfide bonds to complete protein unfolding (Görg et al., 2004).

Another subsequent sample preparation step that should be utilized, especially in proteomic analyses that involve the use of marine organism, is the use of a protein precipitation step (Görg et al., 2004; Tomanek, 2011). This step is necessary for marine organisms in order to eliminate any salts that could interfere with gel-based and chromatographic separation methods of proteins later in the proteomic workflow (Tomanek, 2011; Görg et al., 2004). By applying a TCA acetone-based precipitation step, not only will interfering salts be removed from solution but proteases that may be present in solution will also be deactivated (Görg et al., 2004). Inactivating proteases prevents protein degradation that could result in artificial protein spots and loss of certain high molecular weight proteins (Görg et al., 2004). In addition, a TCA acetone-based precipitation can also aid in the removal of polysaccharides and nucleic acids that could

increase the viscosity of the solution that will be applied in two-dimensional gel electrophoresis for the separation of proteins (Görg et al., 2004). In addition, polysaccharides and nucleic acids can block the pores on the gel matrix which further prevents protein separation (Görg et al., 2004).

Separation of proteins: the first dimension

Once the sample has been prepared and the proteins have been isolated from the tissue the proteins must be separated from each other. Due to the fact that proteins can have posttranslational modifications that can modify their structure, it is important to track these changes by use of two-dimensional gel electrophoresis (Michel et al., 2003; Woods et al., 2014; Tomanek, 2011; Görg et al., 2004). Two-dimensional gel electrophoresis separates proteins based upon 2 properties: the point at which the charges of the amino acids that make up the protein are neutral or the isoelectric point and by the proteins molecular weight (Michel et al., 2003; Tomanek, 2011; Görg et al., 2004). Two-dimensional gel electrophoresis can resolve more than 5,000 proteins simultaneously, and can even detect less than one nanogram of protein per spot (Görg et al., 2004). This method is preferred for protein separation as opposed to the liquid chromatography-based separation technique noted above (Görg et al., 2004; Tomanek, 2011). This is due to the fact that liquid chromatography-based techniques perform analysis on peptides, rather than whole protein, which causes a loss of information about molecular weight and pI (Görg et al., 2004). In addition, 2D GE reveal levels of relative protein expression in tangible amounts for further structural analysis using MALDI MS or ESI MS (Görg et al., 2004).

The first dimension of gel electrophoresis separates proteins based upon their isoelectric point or pI (Michel et al., 2003; Tomanek, 2011). This separation is accomplished by utilizing an immobilized pH gradient or IPG gel strips (Tomanek, 2011). It is important to separate proteins by pI as it is a direct way of identifying posttranslational modifications (Michel et al., 2003). Understanding when PTMs are occurring and on the types of proteins they occur is important as PTMs are responsible for the activation of different functions for a particular protein (Michel et al., 2003). These PTMs are the quickest way to modify existing proteins, especially in response to changing environmental conditions (Michel et al., 2003). One of the most common methods of immobilized pH gradient separation is the process of off-gel separation (Michel et al., 2003). This process allows for the direct recovery of proteins in solution without adding any buffer (Michel et al., 2003). The sample is placed in a chamber with the IPG strip. The IPG gel strip acts as a buffer for the thin layer of sample in the liquid chamber (Michel et al., 2003). Then, an electrical field is applied in a perpendicular fashion to the liquid chamber that contains the IPG gel strip and the sample (Michel et al., 2003). This electric field penetrates into the channel and extracts charged species into the gel (Michel et al., 2003). Thus, the proteins are charged according to their pI and are found along the IPG strip based upon the immobilized pH gradient that is imposed on the IPG gel strip (Michel et al., 2003). This technique is utilized for 2D GE as it gives a high separation rate and allows for easy recovery of the purified compounds (Michel et al., 2003). Once the proteins have been separated by their pI, they are then separated based upon their molecular weight.

Separation of proteins: the second dimension

The second dimension of 2D GE utilizes the technique of vertical sodium dodecyl sulfate or SDS gel electrophoresis (Tomanek, 2011). This process separates proteins based upon their molecular weight, and the gel that the proteins are separated onto act as the medium in which proteins will be extracted for future protein identification (Tomanek, 2011). The vertical system of gel-electrophoresis is ideal for large-scale proteomic analysis which gives higher through-put and maximal reproducibility for a large number of proteins being separated at once (Görg et al., 2004). However, before proteins can be separated in the second dimension they must be equilibrated while they are on the IPG gel strips (Görg et al., 2004). This is due to the fact that the proteins bind strongly to the IPG gel strip matrix (Görg et al., 2004). This equilibration step improves transfer of proteins from the first dimension to the second dimension (Görg et al., 2004). Once the second dimension has been obtained, proteins need to be visualized (Görg et al., 2004). This can be accomplished by using an anionic dye such as Coomassie blue stain (Görg et al., 2004). Coomassie blue stain is typically utilized in proteomic analyses as it has a high compatibility with subsequent protein analysis and characterization methods such as MS (Görg et al., 2004). In addition, proteins tend to have a higher affinity for the dye than the gel matrix, which creates minimal background staining leaving clearly labeled proteins (Patton, 2002).

Principles of Mass spectrometry

Once proteins have been separated, identification using mass spectrometry is then utilized. A mass spectrometer that is used in proteomic analyses has three components: an ionization source, a mass analyzer and a detector (Woods et al., 2014). The first

component, the ionization source, has two major types: a matrix-assisted laser desorption ionization (MALDI), and electrospray ionization (ESI) (Woods et al., 2014). These two ionization techniques differ in the type of spectra they produce and the method of analysis (Tomanek, 2011). Initially, the protein sample that has been extracted from a protein gel is ionized (Woods et al., 2014). The ions produced by MALDI methods are separated in a mass analyzer based on their mass-to-charge ratio (measured in Daltons) or m/z (Woods et al., 2014; Tomanek, 2011). This gives the protein a unique label known as the peptide mass fingerprint or PMF (Tomanek, 2011). The ionization of the protein is dependent on the electrical potential at the ion source and on the pH at which they are analyzed (Woods et al., 2014). Typically, proteins are protonated at a low pH where the ionization is positive and are deprotonated at a high pH where the ionization is negative.

Another component of the mass spectrometer, the detector, detects these ions as they are created (Woods et al., 2014). The detector gives the end product which is a mass spectrum, which is measured in a plot of ion abundances versus the m/z ratio (Woods et al., 2014). From here, the final component of the mass spectrometer, the mass analyzer, is utilized. There are 3 main types of mass analyzers but the most commonly used type of mass analyzer in proteomic analyses is the time of flight or TOF analyzer (Woods et al., 2014). This is due to the fact that TOF mass analyzers have relatively low costs associated with them and have high resolution measurements along with high mass accuracy measurements (Woods et al., 2014). Once ionization of proteins occurs, the ions that are created are accelerated through a known electric field and then travel from the ion source to the detector (Woods et al., 2014). The TOF mass analyzer measures the time it takes for the ions with different masses to travel from the ion source to the

detector (Woods et al., 2014). Typically, mass spectrometers will have two or sometimes even three mass analyzers to increase accuracy using the combined force of the mass analyzers (Woods et al., 2014).

Tandem Mass Spectrometry

The type of mass spectrometry utilized for this particular proteomics study that looks at the effects of hypoxia on two species of marine mussels involves the use of MALDI-TOF/TOF tandem mass spectrometry methods (Tomanek, 2011). This type of mass spectrometry is used for the determination of the mass of a peptide or protein and the identification of a protein using peptide mass fingerprinting (Woods et al., 2014). To obtain the PMF of the proteins, the procedure starts by co-crystallizing the peptide mixture under acidic conditions with a UV-absorbing matrix that has been spotted on an Anchorchip technology plate (Woods et al., 2014). Next, a laser beam ionizes the matrix, causing the peptides to desorb and start to fly under the electrical field (Woods et al., 2014). The matrix molecule transfers a proton to the peptides which then become ionized and fly through the mass analyzer tube, which in this case is a TOF analyzer (Woods et al., 2014). Once the ionized peptides have hit the detector they are detected according to their mass-to-charge ratio (m/z), generating the protein mass fingerprint or PMF. The detector generates a spectrum from this mass to charge ratio or PMF, where one peak corresponds to a single peptide, while many peaks correspond to many peptides (Woods et al., 2014). This provides the MS peptide data that can be matched to a theoretical digest extracted from all proteins that are included in a particular database (Woods et al., 2014; Tomanek, 2011). In order to confirm the match that is generated by the PMF, a number of peptides are selected and further fragmented into ions, which creates a peptide

fragment fingerprint or PFF that contains information about the amino acid sequence of the protein of interest (Tomanek, 2011). The combined data of both the PMF and the PFF is known as tandem mass spectrometry (MS/MS) (Tomanek, 2011). In MALDI-TOF/TOF mass spectrometry, peptides originate from a single protein that was obtained from in-gel digestion of proteins separated by 2D GE (Tomanek, 2011). These peptide fragments can be linked and combined with PMF to undergo a database search that contains both MS data (data from just the PMF) and MS/MS data (data that takes into account both the PMF and PFF) (Tomanek, 2011).

Once MS and tandem MS data have been obtained, protein identification can be obtained by using a particular database (Tomanek, 2011; Woods et al., 2014). Identifying peptides through the interpretation of MS spectra is challenging due to the limited information contained within available databases (Tomanek, 2011; Choudhary et al., 2001). This is especially true for non-model organisms whose genomes have not been fully sequenced (Tomanek, 2011). In addition very few protein sequences for marine organisms such as marine mussels are able to be found within protein databases (Tomanek, 2011). In addition, there only exists partial genomic information for a number of marine organisms, with an even greater number of EST databases available for marine organisms (Tomanek, 2011).

Identification of proteins from MS/MS data

There are two methods for protein identification and characterization using the three databases mentioned above. One method, peptide mass fingerprinting, compares a set of measured peptide molecular-mass values from a proteolytic digest against values calculated by *in silico* digestion of sequences from a protein database (Choudhary et al.,

2001). However, this method is limited and cannot be used for short stretches of sequence, such as those available from transcriptomic data or EST databases, or for long continuous sequences such as genomic data (Choudhary et al., 2001). For transcript-type data or long genomic type data, the preferred approach for protein identification identifies discrete peptides using tandem MS/MS data, which requires the use of the PFF to obtain a short stretch of amino acid sequence (Choudhary et al., 2001). This characterization is accomplished by using a particular database for searching using the MS and tandem MS data obtained from the mass spectrometer (Tomanek, 2011). There are three types of databases that are utilized: protein databases, genomic databases and translated expression sequence tag databases or EST (Tomanek, 2011). EST databases are extremely useful for protein identification as they can be used to translate transcripts into proteins (Tomanek, 2011). EST databases are available for many types of organisms, such as the genus *Mytilus* (Tomanek, 2011). This is thanks to the work of many transcriptomics studies such as those accomplished by Connor and Gracey, 2011, and Lockwood et al., 2010.

II. MANUSCRIPT

Abstract

The intertidal zone is a habitat that is located between terrestrial and marine environments and is characterized by both a diurnal and tidal cycle exposing organisms to alternating periods of high tide and low tide. These alternating tides can expose organisms to a wide variety of environmental stressors. One such stressor is prolonged hypoxic conditions during aerial emersion (low tide) which is typically experienced by sessile organisms such as intertidal mussels. Understanding how these organisms can respond to stressors can reveal insights into the nature of the cellular stress response of marine invertebrates. This information is invaluable for future studies that look into the effects of the ever changing climate of the planet. Furthermore, studying closely related congeners can also reveal the ‘winners’ and ‘losers’ that will result due to the changes in the global climate that are occurring. The closely related *Mytilus* congeners *M. trossulus* and *M. galloprovincialis* can provide insights into the cellular stress response as well as reveal mechanisms of invasive species. *M. galloprovincialis*, a heat-tolerant and low-salinity sensitive congener, originated in the Mediterranean but has been slowly taking over the Pacific Western coast of the United States since its introduction in the early 20th century. Its invasion is also displacing the native heat-sensitive, low-salinity tolerant *M. trossulus*. We acclimated both species to subtidal (constant emersion) and intertidal (6 h low: 6 h high tide) conditions for three weeks before exposing animals to zero, six, twelve, and 24 h of aerial-induced hypoxia while also running a control under acclimation conditions. It is presumed that differences may depend on the recent tidal history of the animals. Changes in protein abundance in gill tissue collected during the

experiment were analyzed with 2D GE and MALDI TOF/TOF tandem mass spectrometry. It is presumed that heat-tolerance correlates closely with hypoxia tolerance, suggesting that *M. galloprovincialis* may be the more hypoxia-tolerant of the congeners, making it a successful invasive species. Principal component analyses of the proteomic changes during hypoxia suggest that mussels greatly differ in their response towards hypoxic stress. The native species appears to rely on a switch to anaerobic mechanisms to maintain basal levels of energy for cellular maintenance while also heavily relying on the ubiquitin-proteasome proteolytic pathway for protein homeostasis. The invasive species, however, appears to heavily utilize signaling molecules to coordinate a response that involves the use of TCA cycle intermediates to maintain basal levels of energy for cellular maintenance while increasing protein translation, potentially for use in the unfolded protein response.

Keywords: proteomics, hypoxia, *Mytilus trossulus*, *Mytilus galloprovincialis*, invasive species

Introduction

The intertidal zone is a habitat that is located at the interface between the terrestrial and the marine environments (Gracey et al., 2008). The environment is characterized by a 24 hour light: dark cycle as well as a 12.4 hour tidal ebb and flow which can expose organisms to alternating periods of high tide and low tide based upon their position within the intertidal zone (Lockwood et al., 2015; Connor and Gracey, 2010). Sessile intertidal organisms that inhabit the intertidal zone can be exposed to multiple stressors during both high tide and low tide. When these sessile organisms are immersed during high tide, they can experience temperature stress due to changes in ocean temperature, hypoxic stress due to changes in dissolved oxygen content of the water, salinity stress and pH stress (Denny and Gaines, 2007; Harley et al., 2006). During low tide, sessile organisms are aerially-emersed which exposes them to a different suite of stressors which include decreasing oxygen partial pressures inside the cavity due to shell closure, desiccation stress, and cold and heat stress induced by the surrounding air (Newell, 1979; Widdows et al., 1979; Schneider, 2008). Global climate change is predicted to change several of these environmental variables due to warming seawater and air temperatures, expanding and shoaling oxygen minimum zones (OMZ), greater precipitation extremes leading to the dilution of coastal waters and ocean acidification (Gilly et al., 2013; IPCC, 2013; Kroeker et al., 2013). One way to study the effects of these changes on intertidal organisms is to compare species which have already shown shifts in distribution patterns according to predictions based on their differences in tolerance ranges.

One such species pair are the two blue mussel congeners *Mytilus trossulus* and *M. galloprovincialis* along the Pacific coast of North America. The former is the comparatively more heat sensitive but hyposaline-tolerant native congener (Braby and Somero, 2006b; Schneider, 2008). The latter originated in the Mediterranean Sea and is more heat tolerant but more sensitive to hyposaline stress. *M. galloprovincialis* successfully invaded southern California at the beginning of the last century (Geller, 1999) and has since replaced the native *M. trossulus* congener up to a shifting line along the coast of northern California (Hilbish et al., 2010), with a hybrid zone existing further north and in San Francisco Bay (Braby and Somero, 2006a; Schneider and Helmuth, 2007). Both species can be found living in subtidal and intertidal habitats (Lockwood et al., 2015).

Mussels of the genus *Mytilus* respond to aerial emersion by closing their shells in order to avoid desiccation of gill tissue leading to a decrease in the rate of oxygen consumption to 4-17% of their immersed rate (Müller et al., 2012; Lockwood et al., 2015; Anestis, 2010; Fields et al., 2014). Studies such as those by Grieshaber et al. (1994), and Müller et al. (2012) have identified that marine mussels such as *Mytilus edulis* are able to mitigate the effects of environmental hypoxic stress during periods of aerial-emersion. This is accomplished through the use of a two-step strategy that starts with metabolic depression which eventually shifts towards maintaining certain levels of ATP or available energy to provide the energy necessary to shift towards rescue mechanisms in order to maintain basic functions of the organism (Müller et al., 2012; Grieshaber et al., 1994; Fields et al., 2014). One strategy used to induce metabolic depression is the phosphorylation of key glycolytic enzymes such as phosphofructokinase, which

decreases the efficiency of the enzymes and helps to compensate for the decreased rate of oxygen consumption (Demers and Guderly, 1994; Fields et al., 2014; Babarro et al., 2007; Storey, 1987). In addition to the switching off of certain biochemical pathways, marine mussels can also switch to anaerobic pathways that involve the accumulation of succinate and propionate and are more efficient at producing ATP than lactate or opine formation while also avoiding acidosis (Müller et al., 2012; Fields et al., 2014). This switch to anaerobic pathways is used to maintain certain levels of ATP in order to power the subsequent rescue mechanisms that marine mussels switch towards during periods of hypoxic stress (Müller et al., 2012; Grieshaber et al., 1994; Fields et al., 2014). These rescue mechanisms are used to repair damaged proteins such as the respiratory machinery of the mitochondria or actin monomers that make up the actin cytoskeleton during periods of stress (Kültz, 2005; Tomanek and Zuzow, 2010; Fields et al., 2014; Grieshaber et al., 1994). This rescue response occurs at the cellular level and can include the action of chaperone proteins (Kültz, 2005; Tomanek, 2015; Grieshaber et al., 1994; Fields et al., 2014). Though these hypoxic response mechanisms have been identified in the posterior adductor muscle of *M. edulis* during prolonged hypoxia, a direct comparison of the response of the two blue mussel congeners *M. trossulus* and *M. galloprovincialis* to aerial-emersion hypoxic stress has not been conducted yet.

The current study aims at elucidating the differences in the proteomic response to aerial-emersion hypoxia in the two *Mytilus* congeners. In addition, due to the fact that these species can be found in both subtidal and intertidal regions, we investigated how acclimation to these two different habitat conditions affects the response to aerial-emersion hypoxic stress. It may be that prior exposure to aerial-emersion hypoxia in

small doses such as alternating periods of low tide may better prepare the mussels for subsequent long-term exposure. The response to aerial-emersion hypoxia under different acclimation conditions will be elucidated by using a mass spectrometry based proteomics approach on gill tissue. This is based upon the fact that protein abundance changes represent modifications of the molecular phenotype of the cell as well as functional changes (Feder and Walser, 2005). Moreover, mass-spectrometry based proteomics that utilize expressed sequence tag (EST) libraries will allow hypotheses about the hypoxic response in non-model organisms to be elucidated (Tomanek et al., 2011; Tomanek, 2011; Tomanek, 2012).

This proteomics study aims to answer two main questions. The first addresses whether or not the cellular mechanisms utilized by *M. trossulus* to mitigate the effects of aerial-emersion hypoxic stress differ from the cellular mechanisms utilized by *M. galloprovincialis*? We hypothesize that there will be some similarities and differences between the cellular mechanisms utilized by *M. trossulus* and *M. galloprovincialis* to mitigate the effects of hypoxic stress. These cellular mechanisms will be identified by dividing the identified proteins into three main functional categories: energy metabolism proteins, protein homeostasis or proteostasis proteins and cytoskeletal proteins. Therefore, the cellular mechanisms will be identified separately for each functional category. Then these functional categories will be compared between species to identify the similarities and the differences between the mechanisms utilized by each species. We are predicting that the species will have similar cellular mechanisms in terms of energy metabolism proteins. This is based upon the work of Müller et al. (2012), Greishaber et al. (1994), and Fields et al. (2014), which have identified cellular mechanisms at the

energy metabolism level in marine mussels. We are also predicting that the differences will be between the proteostasis functional category and the cytoskeletal category. This is based upon the work done by Tomanek and Zuzow (2010) and Tomanek et al. (2012), which identified many differences in the cellular mechanisms at the proteostasis level and the cytoskeletal level in response to heat stress and salinity stress.

The second question aims to answer whether or not the organisms habitat affects the response utilized to mitigate the effects of hypoxic stress. We hypothesize that the environment, either subtidal or tidal, will affect the response of the organisms to hypoxic stress. We are also predicting that mussels acclimated to a tidal regime will show differences in their proteomes during both control and experimental conditions when compared to subtidally acclimated mussels.

Mussels from both species were exposed to either a subtidal or tidal acclimation regime for three weeks. After this three week acclimation period, mussels either continued their acclimation condition to act as a control group, or were exposed to constant low tide conditions for up to 24 hours. During this exposure period, gill tissue was collected at the following time points: zero hours of hypoxic exposure, six hours, twelve hours, and finally 24 hours of hypoxic exposure. Our results in the study indicate that the heat sensitive, hyposaline tolerant native *M. trossulus* may be more sensitive to hypoxic conditions compared to the heat tolerant, hyposaline sensitive *M. galloprovincialis*. In addition, the acclimation conditions or prior exposure may also play a large role in developing the mussels' response to hypoxic conditions in both species as tidally acclimated mussels appeared to utilize more efficient strategies for mitigating the effects of aerial-emersion hypoxic stress.

Results

Mussels were exposed to aerial-emersion hypoxia for zero hours, six hours, twelve hours and 24 hours. Proteins from gill tissue were separated and analyzed using two-dimensional gel electrophoresis (2D GE). Protein spots were detected using the Delta 2D program software for each species separately. This is due to the fact that even closely related species have evolutionary variation in primary sequence and PTMS of orthologous homologs, giving proteins with different changes to molecular mass and pI (Tomanek, 2010). Using the Delta 2D software, two fusion images were created that consisted of gels from all treatment groups (*M. trossulus*: n=78; *M. galloprovincialis*: n=81) (Figure 1A; Figure 1B). A total of 323 spots were detected for *Mytilus trossulus* and a total of 270 spots were detected for *M. galloprovincialis* (Figure 1A; Figure 1B). Of the 323 spots detected, 181 spots were successfully identified using tandem mass spectrometry giving an identification rate (ID rate) of 56.03% for *Mytilus trossulus* (supplementary material table S1). Of the 270 protein spots detected for *M. galloprovincialis* 200 protein spots were successfully identified using tandem mass spectrometry giving an ID rate of 74.1% (supplementary material table S2).

A three-way nonparametric permutation ANOVA ($P < 0.02$) was utilized to determine if acclimation type, hypoxic exposure or the length of hypoxic exposure had a significant effect on protein abundance. A p-value of 0.02 was used rather than the typical 0.05 in order to control for false positive ID rate. In addition to this three-way ANOVA, Tukey's post hoc analysis was also considered for individual proteins with high variation within the principal component analysis (see below). For the factor level of Oxygen*Time*Acclimation, 60 proteins were significant for *M. trossulus* while 36

proteins were significant for *M. galloprovincialis*. For the two-way interaction effect concerning oxygen and time as the factors 49 proteins (*M. trossulus*) and 68 proteins (*M. galloprovincialis*) were deemed significant. For the two-way interaction effect between oxygen and acclimation 22 proteins (*M. trossulus*) and 20 proteins (*M. galloprovincialis*) were deemed significant. For the two-way interaction effect between time and acclimation 61 proteins (*M. trossulus*) and 81 proteins (*M. galloprovincialis*) were deemed significant. For the acclimation main effect 69 proteins (*M. trossulus*) and 73 proteins (*M. galloprovincialis*) were deemed significant. For the time course main effect 86 proteins (*M. trossulus*) and 75 proteins (*M. galloprovincialis*) were deemed significant. And finally, for the oxygen main effect 31 proteins (*M. trossulus*) and 38 proteins (*M. galloprovincialis*) were deemed significant. In total, *M. trossulus* had 147 significantly changing proteins while *M. galloprovincialis* had a total of 166 significantly changing proteins.

Principal Component Analysis

Principal component analysis (PCA) was used to analyze the contribution of significantly changing proteins (three-way ANOVA; $P > 0.02$) to separating treatments and time points along newly generated orthogonal axes called principal components (PC) that account for the variation in protein abundances (Abdi and Williams, 2010). We constructed separate PCAs for mussels acclimated to either intertidal or subtidal conditions experiencing normoxic and (aerial-emersion induced) hypoxic conditions to minimize the number of treatment groups and to compare the effects of the two acclimation regimes on how mussels respond to either normoxia or hypoxia. PCAs including tidally and subtidally acclimated groups with both oxygen (normoxic and

hypoxic) treatments were also constructed. However, we were unable to discern major differences how oxygen treatments separated mussels, but noticed that acclimation conditions showed a discernable effect on how mussels responded to oxygen availability when we conducted different PCAs for the oxygen treatments.

In addition, we conducted separate PCAs for the following three functional categories: cytoskeleton, energy metabolism and oxidative stress, and protein homeostasis (proteostasis), which will be discussed separately.

Discussion

Energy Metabolism Proteins

M. trossulus- normoxia

Tidally acclimated *Mytilus trossulus* experiencing normoxia only showed limited separation among the four time points [06:00 (0h), 12:00 (6h), 18:00 (12h) and subsequent 06:00 + (24h of exposure)] along PC1 (x-axis; Figure 2A) and no separation along PC2 (y-axis; Figure 2A). However, subtidally acclimated mussels showed a greater separation along PC1, which accounts for 15.55% of the variation, between the two early (06:00 and 12:00) and the late (18:00) time points, which are located in negative and positive sections of PC1, respectively (Figure 2A). In addition, the 06:00+ (24h of exposure) time point was separated from all other treatments along PC2 (Figure 2A).

Proteins with positive loading values for PC1 generally showed greater abundance at 18:00 in subtidally acclimated mussels experiencing normoxia and included the tricarboxylic acid (TCA) cycle enzymes aconitase (spot#26, 24), succinate dehydrogenase (subunit B; SDHB) (47) and NADP-dependent isocitrate dehydrogenase 2

(NADP-ICDH2) (124), as well as the electron transport chain (ETC) enzyme ubiquinol-cytochrome C reductase (137), and the oxidative phosphorylation enzyme ATP synthase (β) (108) (Table 1A; Tukey's *post hoc* protein abundance profiles, Figure 4A). This trend is also reflected in a subset of proteins belonging to clusters II (SDHB, ubiquinol-cytochrome C reductase and ATP synthase) and III (two aconitases and NADP-ICDH) in the hierarchical clustering (heat map) of metabolic proteins (Figure 3). Based upon the PC loadings and the changes in abundance, these enzymes indicate that there is an increase in aerobic metabolism occurring at 18:00 in the subtidal but not the intertidal animals under normoxic conditions. In addition, according to the results of the three-way ANOVA (Figure 3), all six proteins either showed a time of day (ToD) main effect or an oxygen and ToD interaction effect.

Along the negative section of PC1, where the subtidal 06:00 and 12:00 and most of the intertidally acclimated groups are located, the proteins with high (negative) loading values include several aldehyde oxidizing enzymes such as two isoforms of α -aminoadipic semialdehyde dehydrogenase (AASDH; 113, 116) and one of methylmalonate-semialdehyde dehydrogenase (MMSDH; 93) (Table 1A). The former is generally associated with lysine catabolism and the latter with branched-chained amino acid, specifically valine, degradation (Michal, 2012 #1727; Salway, 2004). Additional enzymes include two isoforms of NADP-ICDH (145, 185), 4-hydroxyphenolpyruvate dioxygenase- a protein involved in the degradation of the amino acids tyrosine and phenylalanine-, glutamine synthetase, the antioxidant enzyme cystathionine γ -lyase (176), ATP synthase β subunit, and UDP-N-acetylhexosamine pyrophosphorylase, a hexosamine pathway protein (56) (Table 1A). The abundances of these particular proteins

are lower in subtidally acclimated mussels at 18:00 in comparison to all other treatments and time points, according to the Tukey's *post hoc* abundance profiles, (Figure 4), and the hierarchical clustering (cluster III; Figure 3).

Methylmalonate dehydrogenase and α -aminoadipic semialdehyde dehydrogenase are also involved in the detoxification of reactive aldehydes that form as by-products of lipid peroxidation when reactive oxygen species (ROS) react with polyunsaturated fatty acids (Kültz, 2005; Tomanek, 2014; Halliwell, 2015; Brocker et al., 2010; Kotchoni et al., 2006). ALDHs in general are able to detoxify aldehydes by oxidizing their carbonyl group into the corresponding carboxylic acid (Kotchoni et al., 2006). For example, AASDH detoxifies and converts the aminoadipate semialdehyde, which is known to damage proteins through cross-linking reactions, into α -aminoadipic acid (Brocker et al., 2010). In addition, the oxidation of the carbonyl of aldehydes is coupled to the reduction of NADP^+ to NADPH in the reaction mechanism of some ALDHs (Kotchoni et al., 2006). Thus, not only do ALDH enzymes detoxify aldehydes but specific isoforms can also add to the pool of NADPH to be used by glutathione (GSH) to detoxify ROS.

NADP-ICDH2 (NADP-dependent) is an enzyme that converts the TCA cycle metabolite isocitrate to α -ketoglutarate (Chandel, 2015). This reaction also leads to the formation of NADPH. Finally, cystathionine γ -lyase is involved in the conversion of methionine to homocysteine and subsequently to cysteine, one of the three amino acids of GSH, and is up-regulated during stressful conditions (Dilly et al., 2012; Tomanek, 2015).

UDP-N-acetylhexoasmine pyrophosphorylase is an enzyme which leads to the O-linked attachment of β -N-acetylglucosamine to proteins (Zachara et al., 2004). This

posttranslational modification (PTM) supports the activity of molecular chaperones during periods of oxidative stress by altering their regulation or localization, increasing their synthesis, and the association of chaperone proteins to target proteins that have experienced damage due to ROS (Zachara et al., 2004).

Taken together, the proteins with high variation along PC1 and their corresponding protein abundance values (Figure 2A; Table 1A; Figure 4) suggest that subtidally acclimated mussels only decrease their antioxidant defenses at 18:00 during normoxia while the abundance of enzymes of aerobic metabolism increased. This trend suggests a trade-off that requires a decrease in energy metabolism proteins when the danger of damaging ROS is already high may be occurring in the 06:00 and 12:00 subtidal time points respectively.

Positive loadings for PC2 include the cytosolic and mitochondrial MDH, mNADP-ICDH, mALDH, S-formylglutathione hydrolase and glutathione S-transferase (GST) (Table 1A), which showed lower abundances at 06:00+ (Figure 4). Four of these proteins are found in cluster III and two in cluster IV (Figure 3). At the same time, negative loadings involve a number of enzymes representing several metabolic pathways, some involving the conversion of methionine into homocysteine for eventual GSH production (S-methyl-5'-thioadenosine phosphorylase) and tyrosine metabolism (4-hydroxyphenylpyruvate dioxygenase) (Dilly et al., 2012; Tomanek, 2015; Chandel, 2015). Six of these proteins cluster together, showing higher abundances at 06:00+ under normoxia (Fig. 3, cluster I). Thus, it is possible that the separation of treatments along PC2 is partly achieved through changes in the abundance of four biochemical pathways or reactions: linking production of reducing equivalents to reactions that occur in the

mitochondria (i.e. TCA cycle enzyme mNADP-ICDH), the transfer of reducing equivalents produced via the malate-aspartate shuttle (cMDH and mMDH), the synthesis of cysteine from methionine for incorporations into GSH, an antioxidant that requires reducing equivalents and its transfer to proteins by GST. The fact that the enzymes of the two former reactions are mostly lower at 06:00+ while the conversion of methionine into homocysteine is increased may suggest a trade-off between the availability of GSH and the need for producing and transferring reducing equivalents and GSH to proteins.

In summary, subtidally but not tidally acclimated mussels show a pronounced time-of-day effect on the changes in the abundance of proteins involved in energy metabolism and antioxidant responses under normoxic conditions. However, as the 06:00+ is not overlapping with the group of mussels from the beginning of the experiment at 06:00, it is not clear if this represents a possible trend that indicates a shift in energy metabolism based on an endogenous rhythm, as has been proposed for the transcriptome of *Mytilus* by Connor and Gracey (2011), who showed that the expression of the majority of transcripts was entrained by the circadian rhythm, with only a low percentage of transcripts being affected by the circatidal rhythm.

M. trossulus- hypoxia

In *M. trossulus* the PCA for mussels experiencing hypoxic conditions (Figure 2B) showed a separation between tidally acclimated mussels (negative section) from the subtidally acclimated mussels (positive section) along PC1, which accounts for 15.11% of the variation within the PCA. Proteins with the highest positive loading values are part of the TCA cycle (mMDH, aconitase, and NADP-ICDH [162]), the cysteine and glutathione biosynthesis pathway (cystathionine γ -lyase; S-methyl-5'-thioadenosine

phosphorylase) and involved in ROS scavenging (Cu/Zn superoxide dismutase [Cu/Zn SOD]) (Table 1B). It is possible that S-methyl-5'-thioadenosine phosphorylase is involved in the conversion of methionine into homocysteine according to the work done by Dilly et al. (2012). This homocysteine can be further converted into cysteine, one of three amino acids that make up glutathione (Dilly et al., 2012; Tomanek, 2015). Based upon the findings of Dilly et al. (2012) in metazoans a proposed version of this biosynthetic pathway in bivalve mussels can be found in Figure 20 of the appendix section. Seven of the ten proteins cluster together (Figure 3; cluster I), with additional ones appearing in clusters III and IV, including cystathionine γ -lyase, arginase and Cu/Zn SOD. Proteins from cluster I showed higher abundances at 12:00 under hypoxia in subtidally acclimated mussels, including the three TCA cycle enzymes (Protein abundance profiles, Figure 4). These three may indicate a higher activity of the malate-aspartate shuttle (mMDH), as predicted according to the response of *Mytilus* to hypoxia (Müller et al., 2012), a shut-down of TCA cycle activity through the hypoxia-sensitive response of aconitase (see below) and an increase in the production of NADPH through the activity of NADP-ICDH2, for ROS scavenging. Furthermore, subtidally acclimated mussels may require increased synthesis of glutathione during prolonged hypoxia given the greater abundance of cystathionine γ -lyase at 18:00 and 06:00+ (Fig. 3; cluster III; Figure 4F). Interestingly, constitutive levels of Cu/Zn SOD were higher in subtidally in comparison to tidally acclimated *M. trossulus* (Fig. 3; cluster IV; Figure 4G).

Proteins with the highest negative loading values for PC1 include three isoforms of succinate dehydrogenase (subunit B; SDHB) (47, 44, 46), ATP synthase (β -subunit) (141), NADP-ICDH2 (124), MMSDH (93), AASDH (116) and ALDH (66) (Table 1B).

These proteins showed high levels in all of the tidally acclimated mussel groups in response to hypoxia (06:00, 12:00, 18:00 and 06:00+) (Figure 3; Figure 4). Although tidally acclimated *M. trossulus* experiencing normoxia also showed higher levels of MMSDH, AASDH and ALDH, only hypoxia lead to an increase in SDHB isoforms and ATP synthase (Figure 4). However, levels of ubiquinol-cytochrome *c* reductase complex (137) decreased in tidally acclimated *M. trossulus* in response to both normoxia and hypoxia (Figure 4).

PC2 showed the greatest separation between the 06:00 and the 12:00 and 18:00 time points of the subtidally acclimated *M. trossulus* in response to hypoxia (Figure 2B). The 06:00+ mussels were located in-between these times along PC2 (Figure 2B). Thus, PC2 can be interpreted as indicating differences between the normoxia-experiencing subtidal acclimation control at 06:00 and the hypoxia-exposed groups at varying exposure times. However, only 9.04% of the variation in the data was explained by PC2. Nevertheless, several interesting patterns emerge from the loading values (Table 1B): one is that propionyl-CoA carboxylase, part of *Mytilus*' anaerobic metabolic pathway during prolonged hypoxia (Müller et al., 2012), ATP synthase β , and citrate synthase were higher in mussels experiencing hypoxia relative to the subtidal acclimation control experiencing only normoxia (Figure 4). The second pattern is higher protein abundance levels (and negative loadings) of three enzymes involved in the oxidation of branched chain amino acids, specifically valine (enoyl CoA hydratase, 3-hydroxyisobutyrate dehydrogenase and MMSDH), in the subtidal acclimation control (Figure 4C). Interestingly, propionyl CoA carboxylase is also part of this pathway (Salway, 2004). PC2 could therefore represent a switch from valine oxidation during normoxia (and more

so during acclimation to subtidal conditions) to the anaerobic pathway leading to the accumulation of succinate first and then subsequently to propionate during prolonged hypoxia. A possible reason for the higher levels of citrate synthase in the subtidally (and intertidally) acclimated *M. trossulus* experiencing hypoxia may be its role in producing the intermediate metabolite (citrate) for the NADP-ICDH reaction. Furthermore, although ATP synthase does play a role during prolonged hypoxia in *Mytilus* in producing ATP from the build-up of a proton gradient generated by the reducing equivalents of two reactions (malic enzyme and pyruvate dehydrogenase), it is unclear why a greater capacity for oxidative phosphorylation may be needed given the reduced production of reducing equivalents during hypoxia. It is possible that these two isoforms represent PTMs that are functionally different during hypoxia. Another known role of ATP synthase β during periods of hypoxia is that of an ATP consumer in order to maintain the mitochondrial membrane potential (Solaini et al., 2010).

M. trossulus– summary of energy metabolism

Subtidally acclimated *M. trossulus* showed a stronger separation by time-of-day than tidally acclimated mussels, more so during normoxia, but also in response to emersion-induced hypoxia (Figure 2A; Figure 2B). However, mussels from both acclimation regimes showed a separation between the early (06:00 and 12:00) and the later (18:00) time point along PC1 during normoxia, suggesting an endogenous rhythm as a cause for these shifts in both, even if the separation was greater in subtidally acclimated mussels (Figure 2A). Although in reverse orientation, the same two time points also clustered in tidally but not in subtidally acclimated mussels during hypoxia, suggesting that emersion-induced hypoxia disrupted the clustering and separation more in subtidally

acclimated *M. trossulus*. The loading values and their subsequent patterns of protein abundance suggested that this separation of time-of-day may be in part due to a trade-off between higher levels of metabolism during dusk (18:00) while antioxidant enzymes are in low abundance, supposedly because of lower levels of oxidative stress, during normoxia (Table 1A; Figure 3; Figure 4). This is in part supported by a previous study on the endogenous rhythm of the *Mytilus* transcriptome (Connor and Gracey, 2011) and a proteomic study on the endogenous rhythms of *Mytilus californianus* (Elowe, 2016).

The abundance changes of specific enzymes provide further insight into the possible changes preparing differently acclimated mussels for hypoxia. For example, changes in the abundance of aconitase isoforms (24, 26) show that the decrease during hypoxia is much greater in subtidally than tidally acclimated *M. trossulus* (Figure 4A). The reverse is the case for a third aconitase isoform (29) (Figure 4A). The cytosolic MDH (209) shows a similar pattern as the two aconitase isoforms, with a decrease in abundance after 24h of hypoxia (Figure 4F). One isoform of 4-hydroxyphenylpyruvate dioxygenase (156) and 3-hydroxyisobutyrate dehydrogenase (214), which are involved in tyrosine and valine oxidation, respectively, also showed a decrease after 24h of hypoxia in subtidally acclimated mussels (Figure 4). One additional enzyme of valine oxidation, enoyl-CoA hydratase, also showed a decrease after 12h of hypoxia, while levels of MMSDH did not change (Figure 4C). Based on these differences, we hypothesize that subtidally acclimated *M. trossulus* showed a strong down-regulation of TCA cycle activity and of the supply of TCA cycle with amino acids (tyrosine and valine) and reducing equivalents (cMDH) in response to prolonged hypoxia. In fact, based upon the work of Müller et al. (2012), Wanders et al. (2012) and Hsia et al. (1971), a pathway has

been proposed that links the oxidation of valine to supplying the TCA cycle with the intermediate succinyl-CoA (Figure 21). Thus, a decrease in the abundance of enzymes such as enoyl-CoA hydratase, 3-hydroxyisobutyrate dehydrogenase, propionyl-CoA carboxylase (another enzyme involved in the oxidation of valine) and other TCA cycle proteins supports the down regulation of the TCA cycle during prolonged periods of hypoxia. This response was not observed in tidally-acclimated mussels (Figure 4).

Among the enzymes that increased more in subtidally than in tidally acclimated mussels in response to hypoxia were α -amino adipic semialdehyde dehydrogenase, which is involved in the production of the osmolyte betaine and is known to have antioxidant activities by turning aldehydes into carboxylic groups. Another isoform of 4-hydroxyphenylpyruvate dioxygenase (165), and both NADP-ICDH and cystathionine γ -lyase, two antioxidant enzymes, were higher in mussels from both acclimation regimes after at least 12h of exposure to hypoxia, indicating an increasing demand for reducing equivalents and glutathione for ROS scavenging (Figure 4).

M. galloprovincialis– normoxia

The PCA for *M. galloprovincialis* experiencing normoxia (Figure 5A) showed a separation of time points for the subtidally and only for one time in the tidally acclimated group along PC1. The subtidal 06:00 and subsequent 06:00+ (and tidal 06:00+) are separated from the subtidal 12:00 and 18:00 groups along PC1, which explains 11.46% of the variation in the data (Figure 5A).

The positive loadings for PC1 include proteins that generally showed higher levels at 06:00 and 06:00+ and lower levels at 18:00 in both acclimation regimes (Table

2A; Figure 6). They are part of cluster II, and eight of the ten are either significant for a time-of-day or an oxygen + time-of-day interaction effect (Figure 6). The proteins represent an antioxidant enzyme (Cu/Zn SOD), two TCA cycle enzymes (mMDH and aconitase), four are part of the valine oxidation pathway [3-hydroxyisobutyryl-CoA hydrolase (143), 3-hydroxyisobutyrate dehydrogenase (147 and 166), Enoyl-CoA hydratase (224)], one a lectin, involved in the binding of divalent metal ions such as calcium (EP protein) and one involved in regulating the production of nitric oxide (dimethylarginine dimethylaminohydrolase or DDAH1) (Table 2A).

Enzymes with negative loading values generally showed the opposite pattern of those with positive loadings for PC1, with lower levels at 06:00 and 06:00+, mainly in subtidally acclimated *M. galloprovincialis*. Seven of the ten proteins cluster together (Figure 6; cluster IV). Four of them are TCA cycle (SDHB, two citrate synthase isoforms, and mMDH), and one an ETC (ubiquinol cytochrome *c* reductase) enzyme (Table 2A). Three are associated with nitrogen metabolism (arginase, ornithine aminotransferase and glutamate dehydrogenase) and two are putatively associated with the biosynthesis of glutathione (O-methyltransferase and S-adenosylhomocysteine hydrolase). These differences suggest that morning hours start with high levels of ROS scavenging and valine (branched-chained amino acids) oxidation that produces the TCA cycle intermediate succinyl-CoA. Based upon the overall lower levels of TCA cycle proteins in the early morning hours (06:00 and 06:00+), it appears that TCA cycle and ETS activities are lower when compared to the mid and late day times. In addition, there is a higher activity of GSH synthesis that accompanies the higher levels of metabolic enzymes during mid and late day times.

PC2 separates the early time-of-day time points (06:00 and the 12:00) from both acclimation groups from the 18:00 and subsequent 06:00+ groups and accounts for 11.10% of the variation (Figure 5A). Seven of the ten proteins with positive loadings are found in cluster II, with lower levels at 18:00 and 06:00+, while seven of the ten with negative loadings are found in cluster III, with higher levels at 18:00 and 06:00+ (Figure 6). The enzymes represent a number of possible pathways, including the malate-aspartate-shuttle (mMDH), the urea (arginase) and TCA (aconitase and dihydrolipoyl dehydrogenase) cycle, calcium signaling (calcyphosin, EP protein and EF hand domain-containing protein), glutamate (glutamine synthetase and glutamate dehydrogenase 1) and tryptophan (3-hydroxyanthranilate 3,4-dioxygenase) metabolism, and the hexosamine pathway, which can lead to the glycosylation of lipids and proteins ((UDP-N-acetylhexosamine pyrophosphorylase). Additional enzymes represent ROS scavenging (peroxiredoxin 5) using reducing equivalents (NADP-ICDH) and the glutathione biosynthesis pathway (S-methyl-5'-thioadenosine phosphorylase and cystathione γ lyase) and, finally, elimination of carbonyls to alcohols (carbonyl reductase 3), possibly downstream of the formation of aldehydes as a byproduct of lipid and protein oxidation by ROS. As several of these pathways are represented by enzymes with either positive or negative loadings, it is not possible for us to provide a consistent interpretation for these changes between the early and late time points other than that these processes show a dependence with exposure duration to normoxia.

M. galloprovincialis– hypoxia

The PCA for *M. galloprovincialis* exposed to emersion-induced hypoxia showed a separation of the majority of subtidally and tidally acclimated mussels (PC1) and early

to late time points (PC2), with some exceptions (Figure 5B). PC1 (12.21%) included several antioxidant proteins (CGL, ETHE1, GST, and DyP-type peroxidase), TCA cycle enzymes (SDHB and mMDH) and proteins involved in signaling of nitric oxide (DDAH1) and calcium (EF hand) with positive loadings (Table 2B). Seven of these enzymes cluster together and showed an increase in abundance during hypoxia in subtidally acclimated mussels (Figure 6, cluster III). Proteins with negative loadings increased in abundance during hypoxia in subtidally acclimated mussels and include proteins involved in regulating thyroxine (thyroglobulin), glycolysis (triose phosphate isomerase), and the TCA cycle (aconitase) or they decreased during hypoxia in subtidally acclimated mussels and include proteins involved in TCA cycle (citrate synthase), ETS (ubiquinol cytochrome c reductase) and oxidative phosphorylation (ATP synthase) (Figure 7). To summarize, prolonged hypoxia led to increased levels of several antioxidant enzymes, specific glycolytic and TCA cycle enzyme, and a decrease in specific ETS enzymes and oxidative phosphorylation enzymes in subtidally acclimated *M. galloprovincialis*. In tidally acclimated *M. galloprovincialis* mussels prolonged hypoxia led to an increase in the use of signaling molecules such as H₂S and NO, both of which can be attached to proteins and generate posttranslational modifications.

PC2 (10.66%) showed a separation of the early and late time points with some exceptions, similar to the PCA for mussels experiencing normoxia (Figure 5B). Among proteins with positive loadings TCA cycle (citrate synthase and mMDH), ETS (cytochrome c reductase) and urea cycle (arginase) enzymes decreased with prolonged hypoxia in tidally and subtidally acclimated mussels, indicating a depression of major metabolic pathways, cMDH and a lectin showed an increase at 18:00 in both acclimation

groups and procollagen-proline dioxygenase, mGDH1 and amine oxidase showed an increase, mainly in subtidally acclimated mussels, at 06:00+ (Figure 7). Proteins with negative loadings either increased at 18:00 (S-methyl-5'-thioadenosine phosphorylase, O-methyltransferase and glutamine synthetase) or 06:00+ (TCA cycle enzymes and valine oxidation) or were higher at both (two TPI isoforms) (Figure 7; Table 2B).

M. galloprovincialis- summary of energy metabolism

Subtidally acclimated *M. galloprovincialis* showed greater separation along PC1 during both normoxia and hypoxia than tidally acclimated mussels (Figure 5A; Figure 5B). In both cases, the 06:00 and 06:00+ clustered separately from the 12:00 and 18:00 time points in subtidally acclimated mussels (Figure 5A; Figure 5B). In contrast, tidally-acclimated mussels generally overlapped along PC1 for both treatments (Figure 5A; Figure 5B). This is similar to *M. trossulus*. With some exceptions, PC2 showed a separation of the early (06:00 and 12:00) from the late time points (18:00) for mussels from both acclimations and oxygen treatments.

Similar to *M. trossulus*, the abundance changes of several proteins provide insights into the responses of differently acclimated *M. galloprovincialis* to normoxia and hypoxia. Although one aconitase isoform (16) showed lower levels in response to hypoxia in mussels from both acclimations, none of the pairwise comparisons and ANOVAs showed significance with oxygen treatments (Figure 6; Figure 7). However, there is an increase in aconitase levels in subtidal mussels experiencing hypoxia from early to late time points that indicates a return to normoxia levels within 18h. Together with another aconitase isoform (172), which did not change either, *M. galloprovincialis*, does not seem to decrease aconitase levels in response to hypoxia as much as *M.*

trossulus. One of two citrate isoforms showed a decrease at 12:00 after 6 h of exposure to hypoxia in subtidal mussels only (Figure 7). A decrease in levels of mDLHDH (part of α -ketoglutarate dehydrogenase) and one of three mMDH isoforms (159) showed similar but non-significant patterns (Figure 7). One (31) of two SDHB isoforms showed lowest levels at 12:00 in both normoxia and hypoxia in subtidal but not tidal mussels. The same isoform increased significantly after 6h of hypoxia in tidal mussels. The second SDHB (28) increased in subtidal but decreased in tidal mussels at 06:00+ during hypoxia when compared to normoxia (Figure 7). Both, cytochrome c reductase and ATP synthase decreased with hypoxia at 12:00 and 18:00, respectively, in tidal and subtidal (ATP synthase only) mussels (Figure 7C). Together, there was a suggestive trend towards a decrease in TCA cycle enzymes in mussels from both acclimations and a significant decrease in ETS and oxidative phosphorylation enzymes in tidal mussels, with two SDHB isoforms (28, 31) following an opposite pattern in tidal (31 only) and subtidal mussels. This is consistent with SDHB playing an important role in the anaerobic pathways of *Mytilus* (Müller et al., 2012).

Three enzymes involved in the oxidation of the branched-chained amino acid valine (two enoyl-CoA hydratases [224, 214], 3-hydroxyisobutyryl-CoA hydrolase [143] and two 3-hydroxyisobutyrate dehydrogenases [147, 166]), which can lead to the production of propionyl-CoA and uses several steps that are going in the reverse direction of the anaerobic pathways of *Mytilus* that lead to the production of propionyl-CoA from succinyl-CoA, showed a consistent trend towards higher levels with hypoxia. Two of them showed significant oxygen by time interactions [224, 143], but no significant pairwise comparisons. However, the hydrolase (143) showed a significant decrease with

hypoxia after 24 h of exposure. This could indicate that valine oxidation continues during hypoxia in order to produce succinyl-CoA, which can be converted into succinate, an amino acid that is accumulated during prolonged periods of hypoxia (Müller et al., 2012).

Two enzymes involved in glutamate metabolism (GDH1 and glutamine synthetase) show a trend towards higher levels during hypoxia, with significant two- and three-way interactions. A third one (ornithine aminotransferase) showed a decrease (oxygen main effect) during hypoxia. This suggests that glutamate is mainly funneled into the TCA cycle as α -ketoglutarate. This funneling of α -ketoglutarate can lead to the production of succinyl-CoA, a precursor to succinate which accumulates during prolonged hypoxia (Chandel, 2015; Müller et al., 2012).

Three enzymes that represent the glutathione synthesis pathway were either higher with hypoxia (MAT, 214), showed an oxygen-by-time interaction (MAT [215], S-adenosylhomocysteine hydrolase [249]) and either an acclimation main (CGL, 26) or an acclimation-by-time interaction (CGL 257) effect, with generally higher levels in tidal mussels, but almost no significances in pairwise comparisons. This suggests that *M. galloprovincialis* acclimated to tidal conditions increased glutathione synthesis enzymes and that hypoxia overall increased levels of additional enzymes from this pathway. Similarly, glutathione S-transferase A was generally higher in tidal mussels (acclimation main effect), possibly protecting proteins from oxidation of their thiol groups through glutathionylation. This increased production of glutathione may in part be due to the activity of one specific enzyme involved in the glutathione production pathway: cystathionine γ -lyase. Cystathionine γ -lyase is an enzyme that can produce hydrogen sulfide (H₂S) (Banerjee, 2011; Kabil and Banerjee, 2014; Ju et al., 2013). Cystathionine

γ -lyase appears to have high levels in both the tidal and subtidal groups (Figure 7E). The activity of cystathionine γ -lyase can also be enhanced by high calcium concentrations (Kabil and Banerjee, 2014). This could explain the higher levels of calcyphosin, a calcium binding protein, EP protein precursor, another calcium binding protein and EF hand domain-containing protein in the tidal groups (Figure 7G). H₂S can have many functions, especially during periods of low oxygen. Normally, H₂S inhibits the activity of Complex IV of the electron transport chain (Ju et al., 2013). H₂S is degraded by protein ETHE1 in order to avoid inhibition of the electron transport chain. However, when oxygen levels are low, ETHE1 activity can decrease as enzymatic activity is oxygen-dependent (Kabil and Banerjee, 2014). This is supported by the decrease in ETHE1 protein in the subtidal and tidal hypoxia groups; however, the decrease is less drastic in tidal groups (Figure 7G). In addition to inhibition of the ETS, H₂S can act as a potent secondary messenger that can actually have antioxidant capacities (Ju et al., 2013). In fact, H₂S can play a role in regulating cellular function via redox regulation (Ju et al., 2013). H₂S can increase the activity of many antioxidants such as Cu/Zn SOD and can dissociate into a powerful one-electron chemical reductant that can participate in hydrogen atom transfer in order to detoxify free radicals (Ju et al., 2013). Moreover, H₂S can limit the amount of ROS produced by decreasing the activity of the ETS, a large source of ROS and by synergistically inducing the production of GSH (Ju et al., 2013). H₂S increases cellular glutamate uptake and induces cysteine transport activity, both of which are involved in the formation of GSH (See Figure 20) (Ju et al., 2013). Moreover, H₂S can also induce a PTM known as S-sulphydration, which is added to cysteine residues in order to act as an important redox signaling mechanism (Ju et al., 2013).

Overall, it appears that the tidally acclimated mussels are increasing levels of H₂S as a response towards oxidative stress.

In addition to signaling through the use of H₂S it appears that tidally acclimated *M. galloprovincialis* mussels also utilize the signaling molecule nitric oxide (NO). NO can regulate the activity of Cu/Zn SOD, thioredoxin/thioredoxin reductase and glutathione peroxidase through S-nitrosylation (Ju et al., 2013). Levels of N(G), N(G)-dimethylarginine dimethylaminohydrolase appear to be higher in the tidally acclimated mussels (Figure 7G). This enzyme is involved in degrading inhibitors that prevent the activity of nitric oxide synthase, the enzyme that produces NO from arginine and NADPH (Chandel, 2015; Stuehr, 2004). Moreover levels of arginase, an enzyme that uses arginine to form urea, are low in the tidally acclimated hypoxic groups, allowing more arginine to go towards nitric oxide formation (Figure 7).

Several enzymes are involved in antioxidant activities, specifically scavenging of ROS by the peroxiredoxin-thioredoxin system (Tomanek, 2015). Cu/Zn-SOD increased at 18:00 in subtidal and decreased at 06:00+ in tidal *M. galloprovincialis* in response to hypoxia. It showed much higher levels at 18:00 and 06:00+ in subtidal in comparison to tidal mussels, indicating a higher demand for ROS scavenging in the former. The mitochondrial peroxiredoxin-5 increased at 18:00 in subtidal and tidal mussels, a strong indication that *M. galloprovincialis* experiences oxidative stress after 12 h of hypoxia, regardless of acclimation. However, tidally-acclimated mussels increased NADP-ICDH (248), which can provide reducing equivalents for ROS scavenging.

Comparing the congeners

Both congeners were similarly affected by the tidal and subtidal acclimation regime. Both normoxic and hypoxic conditions led to a higher degree of separation between the subtidally than the tidally acclimated mussels in both congeners. This suggests that subtidally acclimated *Mytilus* are entrained more by the circadian rhythm and that the entrainment is reduced through the addition of a circatidal rhythm. Recent work has shown that *Mytilus* undergoes both circadian and circatidal rhythms at the level of the transcriptome and proteome (Connor and Gracey, 2011; Elowe, 2016). When compared, the circadian rhythm exerts a much greater effect than the circatidal rhythm at the level of the transcriptome but less so at the level of the proteome. However, the magnitude of changes in both transcripts and proteins have yet to be evaluated in response to a subtidal acclimation regime that lacks a circadian rhythm. Based on our results, it seems that a circatidal in combination with a circadian rhythm generates much smaller changes in protein abundance than a circadian rhythm alone. Greater changes in protein abundance may be energetically costly and increase the susceptibility of mussels to environmental stress. Thus, subtidal and tidal mussels may have different capabilities to cope with stressors, such as hypoxic conditions typical for near shore oxygen minimum zones.

Among the subtidal mussels, there was a consistent separation between the clustering of the early morning (dawn) and the late (dusk) time points under normoxic conditions, which did not expose mussels to emersion-induced hypoxia. This further supports the conjecture that mussels are undergoing endogenous changes in the proteome. However, while the 06:00 and 06:00+ groups overlapped in subtidal *M. galloprovincialis*

experiencing normoxia, this was not the case for *M. trossulus*, for reasons we cannot explain. The dawn hours were marked with increasing levels of proteins involved in GSH production and enzymes involved in ROS scavenging. The mid to late time-of-day hours appeared to be marked by a decrease in these same processes with a concomitant increase in TCA cycle, ETS and oxidative phosphorylation proteins. This pattern indicates a potential trade-off between high levels of ROS production, possibly from processes not directly associated with mitochondrial energy metabolism, requiring antioxidant responses, and high levels of metabolic activity in the mitochondrion. To provide substrate for the TCA cycle, both species showed an increase in valine oxidation. Valine oxidation leads to the production of succinyl-CoA, which can be fed into the TCA cycle (Figure 21).

Both species experienced metabolic depression, as indicated through a decrease in several TCA cycle, ETS and oxidative phosphorylation enzymes, in response to emersion-induced hypoxia, a strategy typical for most intertidal bivalves (Müller et al., 2012). However, the mediation of metabolic depression differed between congeners. While *M. trossulus* showed a decrease in the abundance of two aconitase isoforms, *M. galloprovincialis* showed a decrease in citrate synthase in order to decrease the activity of the TCA cycle. Aconitase is a TCA cycle enzyme that is sensitive to high ROS levels, and can act as an oxygen-sensing enzyme (Andreyev et al., 2005). *M. galloprovincialis* may also reduce metabolism during hypoxia through the inhibition of Complex IV through low levels of ETHE1, an enzyme that degrades H₂S, a potent inhibitor of complex IV.

The congeners also differed in how acclimation affected their response to hypoxia. Subtidal *M. trossulus* decreased TCA cycle activity through the oxygen-sensing enzyme aconitase, with a concomitant decrease in amino acid metabolism feeding into the cycle. In addition, subtidal *M. trossulus* showed an increase in cystathionine γ -lyase, possibly to synthesize GSH, an increase in NADP-ICDH2 to increase the production of NADPH and an increase in the aldehyde-scavenging enzyme α -aminoacidic semialdehyde dehydrogenase. Subtidal *M. galloprovincialis* mussels also showed a decrease in ETS and oxidative phosphorylation enzymes. However, the decrease in the abundance of TCA cycle enzymes was much lower in subtidal *M. galloprovincialis*. A unique response was seen in the increase in the glycolytic enzymes triosephosphate isomerase. Similarly, the increase in abundance of several nitrogen metabolism enzymes (glutamate dehydrogenase) in the subtidal *M. galloprovincialis* suggests that these mussels are funneling glutamate into specific parts of the TCA cycle. Thus, it appears that the main strategy of subtidal *M. galloprovincialis* mussels is a switch towards anaerobic metabolism via glycolysis, while maintaining high levels of GSH production and high levels of antioxidant enzymes such as Cu/Zn-SOD and peroxidoredoxin-5.

In contrast to the subtidal mussels, tidally acclimated mussels differ in how they respond to both normoxia and hypoxia. In the tidal *M. trossulus* we see a decrease in the major metabolic pathways such as the TCA cycle and oxidative phosphorylation. However, a major difference from the subtidal was the increase in SDHB and ATP synthase. These two enzymes are involved in anaerobic metabolism, suggesting a switch towards anaerobic metabolism leading to the accumulation of succinate and propionate. This is further supported by enzymes of the malate-aspartate shuttle (cMDH and

mMDH), which also increased in response to hypoxia (Müller et al., 2012). Just as in the subtidal *M. trossulus* group we also see an increase in GSH production and the production of the reducing equivalent NADPH, thereby increasing the defenses against greater production of ROS. In contrast, although both tidal and subtidal *M. galloprovincialis* showed reduced abundances of several metabolic enzymes, tidal mussels showed an increase in levels of several proteins involved in calcium signaling, nitric oxide and H₂S production. In fact, tidal *M. galloprovincialis* showed an increase in EF hand domain-containing protein, calcyphosin and EP protein, all proteins involved in binding to calcium. The binding of calcium could help control levels of H₂S production, as it can increase the activity of cystathionine γ -lyase, an enzyme that produces H₂S (Kabil and Banerjee, 2014). This H₂S secondary messenger can act to increase the production of GSH and maintain redox balance during periods of oxidative stress (Ju et al., 2013; Kabil and Banerjee, 2014). In addition, there appears to be higher activity of nitric oxide signaling due to the increase in (N)G, N(G)-dimethylarginine dimethylaminohydrolase, yet another protein modulator that can increase the antioxidant response during periods of hypoxia (Stuehr, 2004). An increase in calcium, hydrogen sulfide and nitric oxide signaling could be activating the antioxidant response to hypoxia that would increase tolerance to emersion-induced changes in redox balance in comparison to subtidally acclimated *M. galloprovincialis* counterparts and *M. trossulus*.

Proteostasis proteins

M. trossulus- normoxia

The PCA for proteins involved in proteostasis in *M. trossulus* under normoxia showed a separation of the subtidal 06:00 and 12:00 groups (positive section) from the

18:00 group (negative section) along PC1, accounting for 18.96% of the variation in protein abundance (Figure 8A). The 06:00+ group was positioned in-between. The tidal mussels showed the opposite distribution of time points, with the tidal 18:00 overlapping with the subtidal 06:00 and 12:00 groups. With the exception of 06:00, time points of the tidal groups showed a greater spread than subtidal mussels. Furthermore, the overall degree of separation of sampling times between tidal and subtidal mussels was similar, in contrast to what was observed for metabolic enzymes.

Six of the ten proteins with the highest positive loadings for PC1 were either proteasome subunits or a lysosomal cathepsin Z, all of which clustering together (Figure 9; cluster IV). In addition, other enzymes with high positive loading values included the mitochondrial prohibitin, which acts as a mitochondrial chaperone, an antioxidant and a regulator of the Erk-MAPK signaling pathway (Chowdhury et al., 2014; Zhou et al., 2013); the small heat shock protein 24.1 (sHsp24.1), which is involved in holding aggregation-prone proteins (Haslbeck and Vierling, 2015) and the Hsp70 co-chaperone DnaJ (Hsp40), which recognizes and delivers denaturing proteins to Hsp70 and stimulates the hydrolysis of Hsp70-bound ATP (Kim et al., 2013). Another protein, serine/arginine-rich splicing factor, is essential for the function of the spliceosome, but is also involved in post-splicing activities, such as the selective degradation of RNA (Long and Caceres, 2009). Finally, a protein required for the ubiquitin-dependent degradation for a subset of cellular proteins, N-terminal asparagine amidohydrolase (236), is also part of this cluster of proteins, but it was not among the ten highest loadings (Figure 9; cluster IV). The abundance of these proteins was lower with normoxia in tidal in comparison to

subtidal mussels at 06:00, 12:00 and 06:00+. The trend was reversed for 18:00 tidal mussels.

In contrast, three of the isoforms with highest negative loadings were chaperones of the cytosol (two Hsp70 and T-complex protein 1 – TCP1), four of the mitochondrion (Hsp60, two Hsp78 and Stress-70), and one of the endoplasmic reticulum (Grp94)(Table 3A). Six of these chaperones group together in cluster I and two (Grp94 and TCP1) are found nearby in cluster II (Figure 9). Additional proteins were two isoforms of the major vault protein (MVP), which play an unspecified role in regulating (calcium) signaling pathways during stress (Berger et al., 2009). The abundance changes of these proteins showed the reverse pattern from those with positive loadings. This suggests that the earlier hours of the day (06:00 and 12:00 and to a large extent 06:00+) are characterized by generally higher abundances of elements of the ubiquitin-proteasome and lysosomal protein degradation pathways in subtidal mussels (Glickman and Ciechanover, 2001), while 18:00 showed higher abundances of chaperones, which mainly assist in the folding of newly translated proteins in the cytosol, mitochondrion and the ER (Kim et al., 2013). Tidal mussels displayed the opposite pattern.

Along PC2 (11.43%), the subtidal 06:00+ group (negative section) was separated the most from all other treatments. A majority of the negative loadings for PC2 were proteins involved in the ubiquitin-proteasome and lysosomal protein degradation pathways (Figure 9; cluster IV), which provides evidence that the subtidal 06:00+ group is characterized by the presence of proteolytic pathways. The high negative loading value of mitochondrial stress protein 70 (HspA9), which binds to poly A tails of mRNA and interacts with ubiquitin E3 ligases, suggests that mRNA may be sequestered instead of

being translated during high proteolytic activity (Peng et al., 2013; Castello et al., 2012; Davison et al., 2009). In addition, changes in the nuclear histone-binding protein RBBP7 suggest that subtidal mussels regulate transcription as part of the nucleosome remodeling and deacetylase (NuRD) complex in accordance with their role as histone chaperones (Kloet et al., 2015). Recent work has shown that such changes in the abundance of RBBP7 are following an endogenous circadian rhythm (Lehmann et al., 2015). Finally, three of the four proteasome subunits were overlapping with those with negative loadings from PC1 (cluster IV), otherwise there is no distinct pattern to how these proteins group.

The temporal separation of protein synthesis and degradation described here parallels the one for metabolic and antioxidant enzymes in subtidal *M. trossulus* experiencing normoxia (Figure 2A). Higher proteolytic activities and the stabilization of aggregation-prone proteins, including ETS enzymes, coincide with lower abundances of metabolic enzymes, while higher chaperoning activities coincide with higher abundances of metabolic enzymes.

M. trossulus- hypoxia

Emersion-induced hypoxia showed a separation of the tidal (negative) and subtidal (positive section) groups of *M. trossulus* with the exception of tidal 12:00, which groups with the subtidal mussels, along PC1 (20.36%; Figure 8B). The loading values of PC1 indicate a separation between tidal and subtidal mussels experiencing hypoxia based on higher abundances of proteolytic proteins, especially those belonging to the ubiquitin-proteasome pathway, in subtidal, and chaperones involved most generally in protein maturation in tidal mussels (Table 3B). Specifically, positive loadings of six isoforms of the proteasome, and cathepsin Z suggest that a higher proteolytic activity occurs in

subtidal mussels during hypoxia. Again, these proteins grouped together (Figure 9; cluster IV). Four of the proteasome subunits showed a three-way interaction, closely tracking acclimation and oxygen conditions and responding differently over time.

In contrast, higher abundances of seven cytosolic, mitochondrial and ER chaperones (Hsp70, Hsp78, Hsp60, and Grp94) suggest either higher levels of protein denaturation during hypoxia or, more likely, an increased capacity for protein translation and maturation in tidal mussels. These proteins are found either in cluster II or I (Figure 7C). Five of the ten proteins showed a three-way interaction, suggesting that protein maturation is affected by each of the different factor levels of the three-way ANOVA (Figure 9).

Based on the loading values and their subsequent patterns of protein abundance, the types of proteostasis proteins changing during hypoxia did not differ significantly from those contributing the most to the separation of *M. trossulus* during normoxia (Figure 8A; Table 3A). In fact, only two proteins differed between these two treatments. However, while normoxic conditions separated subtidal mussels into “dawn” (06:00, 12:00 and 06:00+) and “dusk” (18:00) groups, emersion-induced hypoxia disrupted this pattern. Instead, all time points of subtidal mussels grouped together along PC1, based on an increase in proteolytic proteins (Figure 8B). Put another way, time points of subtidal mussels exposed to normoxia “spread” across a wide range, under hypoxia, however, these mussels were instead characterized by a comparatively narrow range along PC1, mainly by moving the 18:00 mussels (Figure 8B). In comparison, tidal mussels time points distributed in a similar pattern to normoxia, especially when we exclude some outliers. Similar to the changes in metabolic enzymes, several three-way interactions

($P < 0.02$; three-way ANOVA) indicate that acclimation to different tidal regime affects the time course of the proteostasis response during emersion-induced hypoxia.

PC2 showed a separation of the early (06:00 and 12:00; positive) from the late (18:00 and 06:00+; negative) time points for both tidal and subtidal mussels along PC2 (11.31%), possibly indicating the effect of prolonged hypoxia (Figure 8B). Given that most of the mussels furthest separated from the positive section are the tidal 18:00 and 06:00+, the separation is heavily weighted toward their responses to hypoxia.

Two of the proteins, TCP10 and MVP, are represented with both positive and negative values (Table 3B), which indicates a role for post-translational modifications to modify the maturation of cytoskeletal proteins and signaling pathways, respectively.

The results indicate that the response of subtidal *M. trossulus* to hypoxia enhances the activities of the ubiquitin-proteasome and lysosomal protein degradation pathways while the proteomic response of tidal mussels is weighted towards cytosolic, mitochondrial and ER chaperones. Subtidally, more so than tidally acclimated *M. trossulus*, also responded to hypoxia by reducing the abundance of several metabolic enzymes representing the TCA cycle, the ETS and oxidative phosphorylation. Thus, the response to hypoxia may reflect the same separation between molecular chaperones and proteolysis we observed between time points under normoxia, possibly based on differences in the energetic state of the cell. In this context it is interesting that several of the chaperones, such as Hsp78 and Hsp60, repair damaged mitochondrial enzymes during stress (Juwono and Martinus, 2016; Schmitt et al., 1996; Leonhardt et al., 1993).

While there was substantial overlap (nine out of ten for both positive and negative loadings) among the proteins contributing to the loadings of PC1 between normoxia and hypoxia, the overlap for PC2 was limited to four (positive) and one (negative) out of ten (Tables 3A and B). Thus, while the separation along PC1 was based on a similar set of proteins during normoxia and hypoxia, the separation along PC2 was based on a largely different set of proteins during these treatments.

M. trossulus- summary of proteostasis

Changes in the distribution of time points along PC1 and the patterns of protein abundance in both PCAs indicate how tidal and subtidal *M. trossulus* responded to both normoxia (control) and hypoxia (Figures 8A and B). Tidal mussels showed a separation between early and late (18:00) groups along PC1 during normoxia and hypoxia. In comparison, subtidal *M. trossulus*, which also showed a separation of the 18:00 mussels under normoxia, although in reverse from the tidal mussels, showed greater overlap among all time points in response to hypoxia. This could indicate a disruption in endogenous rhythm of abundance changes in proteostasis proteins.

One of the key features of the separation of time points is based on the increase in the abundances of several cytosolic, mitochondrial and ER chaperones that facilitate protein maturation of newly translated proteins and can stabilize denaturing proteins while proteolytic proteins, especially subunits of the proteasome, lowered their abundances at the same time, mainly during the earlier day hours (06:00 and 12:00). While the reverse was the case during the “dawn” hour (18:00), suggesting that these processes were compartmentalized in time. Furthermore, overall lower levels of proteolytic proteins coincide with lower levels of metabolic enzymes in subtidal *M.*

trossulus during hypoxia, possibly indicating that ATP replete conditions are not required for high proteolytic activity. As a corollary, tidal mussels capable of maintaining ATP-producing enzymes at higher levels may gain greater tolerance towards hypoxic conditions through the stabilization of denaturing proteins, while subtidal mussels, with a greater degree of metabolic depression, are unable to maintain greater activities of chaperones during hypoxia, in part due to decreases in energy from metabolic depression.

M. galloprovincialis- normoxia

The PCA for *M. galloprovincialis* responding to normoxia showed a separation of the tidal and subtidal 06:00 and the 06:00+ (negative) from both the tidal and subtidal 12:00 and 18:00 groups (positive section), with only three mussels crossing the y-axis towards the opposite sign of PC1 (19.89%; Figure 11A). Subtidal mussels showed a greater degree of separation along PC1 than the tidal mussels despite the constant conditions.

Proteins with the highest positive loadings for PC1 are mostly chaperones, including the cytosolic Hsp70 and TCP1 and the mitochondrial Hsp78 and prohibitin (Table 4A). Additional proteins include two MVPs, the eukaryotic initiation factor 3 (eIF3 subunit I), the mitochondrial processing peptidase β and the E3 ubiquitin-protein ligase TRIM33. Proteins abundances are generally higher in the tidal and subtidal 12:00 and 18:00 than the 06:00 and 06:00+ groups. Eight of the ten proteins group together (Figure 9; cluster I).

The proteins with the highest negative loadings include eukaryotic translation initiation factor 3 subunit I (eIF3I), TCP1, TNF, MVP, proteasome α type 3, a protein

disulfide isomerase (PDI) and two sHsps and N-terminal asparagine amidohydrolase (NTAN) isoforms (Table 4A). This separation pattern, subsequent loading values and corresponding patterns of protein abundance suggest that “dawn” hours are characterized by translation factors, chaperones, holdases and proteolytic proteins, without a single category dominating. Specifically, NTAN is involved in the ubiquitin-proteasome proteolysis pathway by creating an unstable N-terminal residue known as an N-degron (Cantor et al., 2012; Hirai et al., 2006). Once marked with an N-degron signal, the protein is subsequently degraded by the 26S proteasome. The abundances of these proteins increased in the tidal and subtidal 06:00 and 06:00+ groups and changed in parallel (Figure 12, clusters II and III).

PC2 (10.95%) mainly showed a separation of the tidal (positive) from the subtidal (negative) mussels, with the tidal 06:00+ being the exception. Several isoforms of MVP and one of sHsp 24.1 showed both positive and negative loadings, indicating that PTMs may affect their abundances. Retinoblastoma binding protein 4 (RBB4), similar to the histone-binding protein (RBB7), and serine/arginine-rich splicing factor 1, indicate that tidal mussels regulate transcription by controlling the acetylation of lysine on nucleosomes and translation through the binding of mRNA to a splicing factor (Kloet et al., 2015; Long and Caceres, 2009). Two endoplasmic reticulum chaperones are homologs of Hsp90 and could play a role in regulating signaling pathways in tidal mussels. In addition, high negative loadings of eukaryotic translation initiation factor 3 (eIF3) indicate that subtidal mussels regulate cap-dependent translation differently than tidal mussels (Shah et al., 2016).

M. galloprovincialis- hypoxia

The PCA for *M. galloprovincialis* exposed to emersion-induced hypoxia showed a separation of the 06:00 and 6:00+ (negative) and the 12:00 and 18:00 (positive section) groups for tidal mussels along PC1, explaining 16.37% of the variation (Figure 11B). Subtidal mussels showed a reverse pattern along PC1. Thus, the separation is similar to normoxia for tidal, but is reversed for subtidal mussels. This is further supported by the observation that six and seven out of ten proteins presenting the highest positive and negative loadings are shared between the normoxia and hypoxia treatments, although their signs are reversed (Tables 4A and B). Our results thus suggest that hypoxia had a limited effect on *M. galloprovincialis* acclimated to tidal conditions, but subtidal mussels were shifting their timing.

Unique positive loadings of PC2 (11.83%), which may be showing higher levels at one time in tidal in comparison to subtidal *M. galloprovincialis* and that are common to both treatments include two endoplasmins (the ER chaperone Grp94, a Hsp90 homolog) and one phenylalanine-tRNA synthetase β isoform (Table 4A and B). One endoplasmin (05) showed higher levels at 06:00 and 06:00+ in tidal in comparison to subtidal mussels, while another one was higher at 18:00 in tidal mussels (Figure 13). One of the synthetases was higher at 12:00 in tidal mussels (Figure 13). A proteomic analysis on cells experiencing tunicamycin-induced ER stress up-regulated Grp94 and several aminoacyl-tRNA synthetases, suggesting that tidal *M. galloprovincialis* also experience ER stress in the morning (06:00) after high tide (Bull and Thiede, 2012).

The E3 ubiquitin-protein ligase TRIM33 isoforms shifted from a negative (154) under normoxia to a positive loading (20) under hypoxia (Allton et al., 2009). This resulted in higher abundances in subtidal mussels under normoxia at dusk and under hypoxia after 24 h of exposure to emersion (154), while another isoform showed higher abundances in tidal mussels at dusk during hypoxia (20) (Figure 13). TRIM33 function as a transcriptional repressor depends on its interactions with histones (Agricola et al., 2011). Furthermore, it is involved in repairing DNA damage through poly (ADPribose) polymerase (PARP) (Kulkarni et al., 2013). It is unclear which of these functions is most important during acclimation to different tidal conditions, but it may play a role in the regulation of transcription by controlling histones, similar to RBBP7.

Finally, the eukaryotic translation initiation factor 3 (subunit I) showed negative loadings under normoxia and hypoxia. Two of the three isoforms (152 and 174) showed higher abundances under hypoxia after 6 h of emersion in subtidal and tidal mussels, while levels of one of the two isoforms were highest at 18:00 under normoxia (Figure 13). This suggests that eIF3 subunit I is changing endogenously and that it increases in response to a 6 h exposure to emersion-induced hypoxia. Acclimation affected one of the isoforms (174). While several subunits of eIF3 are part of the general translation initiation complex (Hinnebusch, 2014), it facilitates an alternative cap-dependent translation mechanism for specific mRNAs (Lee et al., 2016). Several of these RNAs encode components of the ETS and changes in specific subunits of eIF3 have been shown to change the balance between respiration and glycolysis by promoting the translation of specific mRNAs (Shah et al., 2016). Although eIF3 subunit I is up-regulated in cancerous

cells, its specific role is not known (Hershey, 2015). Our results suggest that it may play a role in the response of mussels to hypoxia.

While tidal and subtidal mussels showed a common pattern of separating the 06:00 and 06:00+ from the 12:00 and 18:00 groups during normoxia, emersion-induced hypoxia reversed this order in subtidal but not tidal mussels. This suggests that the separation between “dawn” and “dusk” mussels under normoxia was disrupted in subtidal but not tidal mussels during emersion-induced hypoxia in *M. galloprovincialis*. This showed in the reversal of protein identities representing positive loadings under normoxia but negative ones under hypoxia, and reverse.

Comparing the congeners

The two species differed remarkably under both normoxia and hypoxia in their proteostasis response to acclimation and hypoxia treatment. First, while tidal and subtidal *M. trossulus* were showing reverse separations by time, based mainly on proteolytic pathways and molecular chaperones, both tidal and subtidal *M. galloprovincialis* showed the same separation between early and late time points. This points to a more stable endogenous rhythm of proteostasis protein abundances in *M. galloprovincialis* following acclimation. It is likely that one of the acclimation conditions disrupted the endogenous rhythm in *M. trossulus*.

Second, the separation along the first component was based on a shared (8 out of 20) and a different (12 out of 20) set of proteins in both species during normoxia (Tables 3A and 4A). The species shared the Hsp70s, Hsp78, MVPs, prohibitin, sHsp and one proteasome subunit. However, the majority of proteins with high variation in the *M. trossulus* PCAs were unique proteasome subunits, which were contributing very little in

M. galloprovincialis (out of the five subunits of the former species were also identified but showed no significant differences in *M. galloprovincialis*).

Third, tidal mussels of both species maintained the separation they showed under normoxia also under hypoxia. In contrast, subtidal *M. trossulus* showed a greater degree of overlap or a shrinking of the range of separation under hypoxia relative to normoxia. Subtidal *M. galloprovincialis* showed a “switch” in the separation of time points to show a reverse order of time points to the tidal mussels, similar to the patterns seen in normoxic *M. trossulus*.

Fourth, PC2 showed a separation of only specific time points, different ones, in tidal and subtidal *M. trossulus*, while it generally separated the tidal from the subtidal *M. galloprovincialis* during both normoxia and hypoxia, similar to PC1 in *M. trossulus*. However, while the same set of proteins were associated with a high amount of variation for the two acclimation groups in *M. trossulus*, the protein identities of the PC2 loadings differed between normoxic and hypoxic conditions in *M. galloprovincialis*. Thus, acclimation to tidal conditions highlighted a trade-off between proteolytic pathways and molecular chaperones, while histone chaperones and transcriptional repressors as well as cap-dependent translation processes specific to metabolic enzymes distinguished tidal and subtidal *M. galloprovincialis*. Tidal *M. galloprovincialis* are also characterized by higher levels of ER stress proteins, which represents higher constitutive levels and possible greater ER stress tolerance.

Cytoskeletal proteins

M. trossulus- normoxia

The *Mytilus trossulus* Normoxia PCA showed a separation of the subtidal 06:00 and 12:00 groups (negative section) from the 18:00 and 06:00+ groups along PC1 (16.55%; Figure 11A). For tidal mussels, the 06:00 and 18:00 groups were separated from the 12:00 and 06:00+ groups along the same axis. Moreover, there is no clear separation of any of the groups along PC2 (13.21%) and we won't consider this component any further.

The proteins with high positive loading values are several α/β -tubulin and actin isoforms, nacre domain containing protein and cell division control protein homolog 42 (cdc42) (Table 5A). Levels of the α/β -tubulin and actin isoforms remain relatively consistent throughout each of the different subtidal and tidal groups (Figures 16). Tubulin contributes to the microtubules of cilia and flagella of the gill (Marks et al., 2009; Nowak, 2013; Gosling, 2003). Cdc42 levels increased with time during normoxia in subtidal but not in tidal mussels, even during hypoxia (Figure 16). Cdc42 is a member of the Rho family of small G-proteins that is involved in the organization of the actin cytoskeleton by enhancing the actin-nucleating activity of the ARP (also known as α -centractin) complex. Most of the proteins contributing positive loadings to PC1 clustered together (cluster I), with the exception of cdc42 (cluster III; Figure 16). Cdc42 showed a significant two-way interaction effect for ToD and acclimation (Figure 16).

Proteins with high negative loadings include the signaling proteins ERK2 and Rho GDP dissociation inhibitor 1 (Rho GDI1), as well as several isoforms of actin and

the actin-binding proteins fascin, actin-interacting protein 1 and neuroglian (Table 5A). Levels of ERK2 decrease at 12:00 and 06:00+ during normoxia in tidal and subtidal mussels, respectively. Hypoxia is causing an increase at the same time in subtidal mussels (see below). In its role as protein kinase ERK2 phosphorylates several cytoskeletal proteins (Canagarajah et al., 1997). Rho GDI1 decreases with time during normoxia in both mussels (Figures 16). During hypoxia Rho GDI decreases in tidal mussels only. Importantly, Rho GDI1 can modulate the activities of Rho proteins such as cdc42 by inhibiting nucleotide exchange and prevent proteins from moving to cell membranes (Burrige and Wennerberg, 2004). Thus, high levels of Rho GDI1 in subtidal 06:00 and 12:00 mussels could explain the low levels of cdc42 at the same times.

The levels of fascin and neuroglian are similar across time points in subtidal and tidal mussels (Figure 16). The two isoforms of actin-interacting protein 1 (AIP1) are higher in the 06:00 and 12:00 groups compared to the 18:00 and 06:00+ groups in both mussels. Fascin is an actin-binding protein that organizes F-actin into well-ordered tightly packed bundles (Marks et al., 2009). AIP1 is involved in the turnover of actin by promoting the depolymerization of actin filaments (Konzok et al., 1999). This allows for the reorganization of actin, as well as increases the pool of actin monomers for polymerization. This is done through an association with the actin binding protein cofilin (Ono, 2003). Neuroglian is a cell adhesion protein that transmits positional information directly to the protein ankyrin to polarize cells (Debreuil et al., 1996). This facilitates the formation of tight junctions within cells to regulate diffusion within cells (Dubreuil et al., 1996). The proteins can be found in each of the three clusters (Figure 15), with the

majority being located in cluster II. The abundances of these proteins are influenced by both ToD and acclimation (Figure 15).

Based upon these loadings and their subsequent patterns of abundance it appears that more actin polymerization and severing of existing actin filaments occurs in the subtidal 06:00 and 12:00 groups and the tidal 06:00 and 18:00 groups. Actin polymerization may be linked to increased feeding activity and aerobic capacity during high tide, which preceded the tidal 06:00 and 18:00 groups.

M. trossulus- hypoxia

In the *M. trossulus* Hypoxia PCA (Figure 14B) there was a grouping of subtidal 06:00 and 06:00+ with the tidal 12:00 mussels along the positive axis of PC1 (16.16%). The tidal 06:00 and 06:00+ groups on the other hand grouped together along the negative axis of PC1 while the tidal 18:00 and subtidal 12:00 and 18:00 were centered around the middle of the axis (Figure 14B).

Proteins with both the highest positive and negative loadings for PC1 include eight cytoskeletal proteins that are part of cilia and flagella, such as tektin A1, α/β -tubulin and radial spoke head and four extracellular matrix proteins, such as short-chain collagen, laminin receptor and nacre domain containing protein (Table 5B). None of these proteins showed a significant pairwise comparison. Given the separation of 06:00 and 06:00+ groups for both tidal (negative) and subtidal (positive) mussels, we infer that the timing of cytoskeletal modifications shifted with acclimation and that emersion-induced hypoxia did not disturb these processes, as both 06:00 time points overlapped

even after 24 of emersion. This suggest a robust cytoskeletal response to hypoxia in *M. trossulus*.

All tidal mussel groups except one are within the negative section of PC2 (10.06%; Figure 14B). While the subtidal 06:00 and 12:00 groups are placed within the positive, the 06:00 group is entirely placed in the negative section. The subtidal 18:00 is in-between. Thus, since 18:00 is the first time point after experiencing emersion instead of immersion for tidal mussels and 18:00 and 06:00+ are similarly time points of prolonged hypoxia exposure for subtidal mussels, positive and negative loadings indicate shifts in abundance with prolonged hypoxia, more so in tidal than subtidal mussels.

Positive loadings represent proteins of cilia and flagella, extracellular proteins and two Rho GDI isoforms that specifically showed a drastic reduction in abundance in tidal 18:00 mussels exposed to hypoxia in comparison to normoxia (Figure 16E; Table 5B). Two β -tubulin isoforms (97 and 104) were first higher at 12:00 and then lower at 06:00+ in response to hypoxia in subtidal mussels only. Finally, the actin-bundling protein fascin (92) showed an increase in response to 24 h of hypoxia in subtidal mussels only.

Among the proteins with negative loadings, the actin-binding protein profilin first decreased dramatically at 12:00 in subtidal mussels only and then increased at 06:00+ in both mussels (Figure 16C; Table 5B). A similar pattern was seen for two actin depolymerizing actin-interacting 1 proteins for both, but only with a subsequent non-significant increase in subtidal mussels. Finally, the small G-protein Cdc42 showed an increase with time under both oxygen treatments, however, tidal mussels showed a decrease at 06:00 under hypoxic in comparison to normoxic conditions. Most of these

proteins grouped together (Figure 15, clusters II and III).

M. trossulus- summary of cytoskeleton

The control groups or the normoxic groups appeared to show less separation between the subtidal groups and the tidal groups. In addition, it appeared that the tidal groups were more heavily influenced by the presence of low tide, a trend that was not observed in the energy metabolism or the proteostasis proteins. In fact, levels of specific cytoskeletal proteins were more influenced by the presence of low tide rather than the time of day. The grouping of the tidal 06:00 and 18:00 groups was at first perplexing and did not match the patterns observed in both the energy metabolism and the proteostasis PCAs, which showed a pattern of separation between the early time points (06:00 and 12:00) and the late time points (18:00). A possible explanation for this could be that cytoskeletal proteins are more sensitive to low tide compared to energy metabolism and proteostasis proteins. However, the separation of the early time points from the late time points was observed in the subtidal groups.

In terms of hypoxia, a tidal acclimation appears to have prepared the tidal groups for hypoxic exposure, based upon the potential to form stress fibers and prevent aggregation of G actin. Conversely, the subtidal groups appear to only try and bolster the structure of the actin cytoskeleton through the use of fascin, while inhibiting Rho proteins that may promote actin depolymerization for the purpose of actin rearrangement. It could be that levels of stress in the subtidal groups are too high to allow for large amounts of actin rearrangement. However, after 24 hours of hypoxic exposure, it appears that the subtidal groups are now able to form stress fibers that were observed in the tidal groups.

M. galloprovincialis- normoxia

In the *M. galloprovincialis* normoxia PCA (Figure 17A) the subtidal 06:00 and 06:00+ groups are separated along the positive axis of PC1 (19.32%) along with the tidal 6:00 group. The subtidal 12:00 and 18:00 groups are clustered together along the negative axis of PC1 along with the tidal 18:00 group. There is no clear pattern of separation of the tidal 12:00 and 06:00+ groups, which are located in between the positive and negative axes of PC1. In addition, there is a pattern of separation along PC2 between only the 06:00 and 18:00 groups. The tidal 06:00 and 18:00 groups are separated along the positive axis of PC2 (10.39%) while the subtidal 06:00 and 18:00 groups are separated along the negative axis of PC2.

The proteins with the highest positive loading values for PC1 include cdc42, profilin, ADP-ribosylation factor (ARF) 2, Rho GDI1, translationally controlled tumor protein (TCTP) and α -centractin (also known as Arp2/3) (Table 6A). Abundance levels of profilin, Rho GDI1, cdc42, and TCTP appear to have the highest levels in the subtidal 06:00 and 06:00+ groups and the tidal 06:00 group when compared to the other tidal and subtidal groups. The levels of ARF and α -centractin remain relatively the same throughout the tidal and subtidal groups; however, ARF does have a decrease in abundance in the tidal 18:00 group (Figure 19). Most of the proteins are grouped together in clusters II and III, with the majority in cluster III (Figure 18).

Proteins with the highest negative loading values include several isoforms of actin and several isoforms of tubulin (Table 6A). All of these proteins are clustered together in cluster I of the hierarchical map (Figure 18).

Based upon the positive and negative loading values of PC1 and the corresponding patterns of protein abundance it appears that there is more actin filament and microtubule restructuring occurring in the tidal and subtidal 06:00 and 06:00+ (subtidal only) groups compared to the 12:00 and 18:00 groups (subtidal only). This is based upon the high amounts of actin-interacting proteins that have high loading values along the positive axis of PC1, and their corresponding abundance levels being higher in the tidal and subtidal 06:00 groups. Moreover, it appears that less re-structuring is occurring in the tidal and subtidal 12:00 and 18:00 groups as there are more free actin and tubulin monomers with high loading values, with no signaling proteins or actin-interacting proteins with high negative loading values (Table 6A). Thus, it could be then at the 12:00 and 18:00 time points there is higher amounts of depolymerization; however, due to a lack of presence of actin-depolymerizing proteins, no definitive conclusion can be drawn. More rearrangement of actin filaments and potentially microtubules may be necessary following periods of high aerobic activity in order to increase cytoskeletal rearrangement due to an increase in the need for protein trafficking due to an increase in protein translation. This rearrangement may be kick started by cdc42, and is also supported by the presence of ARF, a signaling protein that is involved in the formation of COPI and clathrin coated vesicles that are used to traffic newly synthesized proteins (Marks et al., 2009). Moreover, the presence of TCTP, a tubulin binding protein, may also influence the activity of tubulin rearrangement for vesicle trafficking. The presence of Rho GDI1 may suggest that Rho protein activity is inhibited; however, this inhibition may be more for the inhibition of cytoskeletal formation rather than rearrangement for protein trafficking.

The proteins with the highest positive loading values for PC2 include Rho GDI1, gelsolin, actin-interacting protein 1 and several isoforms of actin and tubulin (Table 6A). Levels of gelsolin, Rho GDI1, and actin-interacting protein 1 are relatively the same across all groups in the tidal and subtidal groups (Figure 18). Proteins with the highest positive loading values are not uniformly grouped together in any one cluster, as they are located in each of the three clusters (Figure 18).

Proteins with the highest negative loading values for PC2 include G protein (β subunit), tektin A1, gelsolin, cofilin and ARF (Table 6A). Levels of cofilin, gelsolin, and tektin A1 are relatively the same throughout the tidal and subtidal groups. Levels of the G protein are higher in the 06:00 and 18:00 subtidal groups, and are relatively the same in the tidal groups. And finally, ARF is the highest in the 06:00 and 06:00+ groups in the subtidal and tidal groups, with an extremely large decrease in abundance in the tidal 18:00 group (Abundance profiles, Figure 19). Most of these proteins are clustered together in cluster III with some also found in cluster I and cluster II (Figure 18).

Based upon the proteins with the highest loading values for PC2 it appears that more actin depolymerization is occurring in the tidal 06:00 and 18:00 group based upon the presence of gelsolin and Rho GDI1. The subtidal 06:00 and 18:00 groups also appear to have high amounts of depolymerization based upon the presence of gelsolin and cofilin. Both gelsolin and cofilin are actin-depolymerizing proteins that are used to increase actin depolymerization to increase the amount of free actin for actin rearrangement or restructuring (Marks et al., 2009). However, the presence of tektin A1 and ARF also indicate that more protein trafficking is occurring in the subtidal 06:00 and

18:00 groups. This was also observed in PC1 in the 06:00 group; however, the presence of this pattern in the subtidal 18:00 group does not line up with the analysis for PC1.

M. galloprovincialis- hypoxia

The cytoskeletal hypoxia PCA (Figure 17B) exhibits very minimal separation between the tidal and subtidal groups along either PC axis. However, there is some separation within PC1 (20.42%) which groups together the subtidal 06:00 and 0600+ groups along the positive axis. The tidal 06:00 and 0600+ groups are grouped together along the negative axis of PC1. The subtidal and tidal 12:00 and 18:00 groups fall in the center of the two axes of PC1. There is no clear separation between the hypoxia groups along either axis of PC2, which accounts for 10.34% of the variation.

The proteins with the highest positive loading values for PC1 include two isoforms of TCTP, cdc42, tubulin, Rho GDI1, cofilin, AIP1, and profilin (Table 6B). One isoform of TCTP (240) has relatively the same abundance levels in the subtidal groups with a large increase occurring at the 06:00+ group. The tidal groups have lower levels of TCTP in the 06:00 and 06:00+ groups. The other isoform of TCTP (241) has relatively the same levels of abundance throughout both the subtidal and tidal groups. Abundance levels of ARF are also relatively the same throughout the subtidal and tidal groups as well. Rho GDI1 expression levels are relatively high throughout each of the subtidal groups relative to the tidal groups. The expression levels of Rho GDI1 in the tidal groups, however, are high in the 06:00 group but then start to decrease at the 12:00, 18:00 and 0:600+ groups with the initial drop in abundance at the 12:00 group. Cofilin has relatively high abundance levels in the subtidal hypoxic groups relative to the tidal groups while the tidal groups also have relatively constant abundance levels that are

lower when compared to the subtidal hypoxic groups. AIP1 has abundance levels that increase after the 06:00 group in the subtidal hypoxic groups while in the tidal hypoxic groups the abundance levels are relatively the same until 0600+ group where abundance levels decrease. And finally, profilin has relatively the same high abundance levels across all subtidal groups. For the tidal groups, the abundance of profilin is high in the 06:00, 18:00 and 06:00+ groups while experiencing a decrease during the 12:00 group. These proteins are grouped together in clusters II and III, with most clustering in cluster III (Figure 18).

The proteins with the highest negative loading values for PC1 are several isoforms of tubulin, actin, gelsolin and Rho GDI1 (Table 6B). Abundance levels of the protein gelsolin remain relatively the same in the subtidal groups with a drop in abundance at the 0600+ group but an increase at 06:00+ in the tidal groups (Figure 19). Abundance levels of Rho GDI1 have lower levels in each of the subtidal hypoxic groups compared to the tidal groups. Levels of Rho GDI1 remain relatively the same throughout the tidal hypoxic groups but experience a huge increase at the 0600+ group. All of these proteins are grouped together in cluster I (Figure 18).

Based upon the abundance levels of the proteins with the highest loading values in the positive and negative axes of PC1, it appears that the *M. galloprovincialis* tidal 06:00 and 06:00+ hypoxic groups are experiencing higher levels of depolymerization of the actin cytoskeleton which is indicated by the high abundance levels of gelsolin. Gelsolin is an actin-severing protein that promotes the depolymerization of old actin filaments in order to promote the assembly of new filament structures (Marks et al., 2009). Thus, the activity of gelsolin could be influencing actin turnover to increase the

development of new actin-based structures or reinforce existing structures that have incurred damage due to, hypoxic stress. However, after 24 hours of hypoxic exposure, at the 0600+ group, there is a drop in gelsolin levels and an increase in Rho GDI1 levels. This could indicate that levels of actin rearrangement or rebuilding are decreasing due to stress. It must be then at the 0600+ group levels of ROS are high enough that they are starting to cause damage to the actin cytoskeleton. Thus, it would make sense to decrease levels of the actin severing protein gelsolin. Moreover, Rho GDI1 would then be needed in order to inhibit the formation of new actin cytoskeletal structures that could incur damage from the high levels of ROS that appear to be forming after 24 hours of hypoxic exposure.

In contrast, the subtidal groups may be undergoing larger amounts of actin polymerization possibly to increase the amount of protein trafficking. This increased need for protein trafficking could be a response to hypoxic stress. Hypoxic stress may be activating the cellular stress response (discussed in chapter 1). This cellular stress response may require an increased need for protein translation in order to produce the proteins needed to mitigate the effects of hypoxic stress. This increase in translation would also increase the need for protein trafficking networks. This increase in translation is supported by the activity of the two isoforms of TCTP and the increase in protein trafficking is supported by the activity of ARF. Moreover, the activity of cofilin, AIP1 and profilin suggests large amounts of actin turnover in order to restructure the actin network in order to allow for increased protein trafficking. Moreover, the presence of *cdcd42* also indicates actin restructuring that may be necessary to accommodate the increase in translation.

M. galloprovincialis- summary of cytoskeleton

Based upon the results of the normoxia PCA (Figure 17A) *M. galloprovincialis* mussels under control conditions have increased activity of actin-rearranging enzymes. This increase in rearrangement may be to accommodate for an increase in protein trafficking in order to accommodate a potential increase in translation. Moreover, it appeared that more depolymerization or less actin rearrangement was occurring in the later time points (12:00 and 18:00). However, a lack of actin binding proteins did not allow for any definitive conclusions to be drawn. It could be then that the increase in actin and tubulin monomers could be a product of decreased actin rearrangement coupling steady levels of actin depolymerization.

In response to hypoxia minimal differences were noted between the control groups and the hypoxic groups. In fact, the same groups of proteins that were identified in the normoxia PCA with high loading values were also identified as having high loading values in the hypoxia PCA. In the subtidal groups more actin rearrangement and restructuring appeared to be occurring through the use of cdc42, cofilin, AIP1 and profilin. This rearrangement and restructuring may have been to accommodate an increased need for protein trafficking. This increased need for protein trafficking may be to accommodate an increase in translation in order to produce the proteins necessary for the cellular stress response. Conversely, the tidal groups did not have the same approach. In fact, actin restructuring may have been at a minimum, as indicated by the high levels of Rho GDI1 inhibiting the activity of Rho proteins promoting actin restructuring. In addition, high levels of gelsolin may also be causing high levels of actin

depolymerization. This depolymerization, however, does not seem favorable as hypoxic stress could also be causing high levels of depolymerization as well.

Comparing the congeners

In terms of normoxic or control conditions, *M. trossulus* mussels are more heavily affected by the presence of a low tide compared to *M. galloprovincialis*. In fact, tidally acclimated *M. trossulus* mussels proceeding high tide events (tidal 06:00 and 18:00 groups) had higher levels of actin rebuilding through an increase in the free pool of actin monomers. This did not match the same trend that was observed in the subtidally acclimated *M. trossulus* mussels. *M. trossulus* mussels experienced increased cytoskeletal rebuilding during the early time points, while cytoskeletal rearrangement occurred in the later 18:00 time point. However, in the later subtidal time points it appears the formation of stress fibers was occurring through the activity of cdc42. In contrast, *M. galloprovincialis* mussels had increased levels of actin cytoskeletal rebuilding occurring in the early time points (06:00 and 06:00+). This increased restructuring and rearrangement may have been in response to increased translation. An increase in translation is typically accompanied by an increase in protein trafficking for protein modification (Marks et al., 2009). Thus, it could be then that more actin rearrangement was occurring for protein trafficking rather than in response to low tide as a stress or periods of increased aerobic activity, as was observed in the *M. trossulus* mussels.

In terms of the response to hypoxia *M. trossulus* subtidal groups experienced a disruption in actin depolymerization in order to decrease actin rearrangement. This decrease in actin rearrangement may be in response to hypoxic stress, as hypoxic stress may overwhelm the cell as actin rearrangement requires some level of actin

depolymerization in order to provide a pool of actin for the building of new structures (Marks et al., 2009). In contrast, subtidally acclimated *M. galloprovincialis* appear to increase actin rearrangement possibly to accommodate an increase for protein trafficking in order to allow for a robust cellular stress response. Tidally acclimated *M. trossulus* mussels do not have a clear strategy; however, it appears through the use of cdc42 that tidally acclimated *M. trossulus* mussels rely on the formation of stress fibers as a response to hypoxic stress. This formation of stress fibers is also accompanied by the prevention of aggregation of G actin fibers through the activity of profilin. Conversely, tidally acclimated *M. galloprovincialis* mussels are experiencing increased depolymerization during hypoxic exposure through the activity of gelsolin. However, this increase in actin depolymerization does not have a clear purpose. It could be to increase the pool of free actin for cytoskeletal rearrangement, possibly for the formation of stress fibers as in the tidally acclimated *M. trossulus* mussels; however, the presence of Rho GDI1 contradicts this theory.

Conclusions

Overall, there were many similarities between the control and the experimental groups in terms of patterns of protein abundance in each of the three functional categories. One major similarity that was unexpected was the potential presence of an endogenous rhythm. It appeared that in each of the three functional groups an endogenous rhythm had an influence on protein abundance changes, based upon the Tukey's *post hoc* protein abundance profiles. The influence of endogenous rhythms has been detected in species of marine mussels such as *Mytilus californianus* in a transcriptomics study conducted by Connor and Gracey (2011). The study acclimated

mussels to a diurnal and tidal rhythm. The results of their study yielded a total of 2,756 transcripts. Of those 2,756 transcripts only 236 oscillated with a tidal period of 12 hr while 2,365 oscillated with a circadian rhythm of 24 hr. This study indicated that, at the mRNA level or the level of transcriptal regulation, an endogenous circadian rhythm has a large influence. However, the results of the present study indicate that at the protein level, the tidal rhythm may actually have a stronger influence. However, this was only the case with certain groups of proteins.

In both species, the subtidal control (normoxic) appeared to be influenced by an endogenous rhythm in each of the three functional categories. This was based upon the separation of the groups in the normoxia PCAs (Figures 2A, 5A, 8A, 11A, 14A and 17A). In *M. trossulus*, the general trend of the subtidal normoxic groups was that the daytime groups (06:00 and 12:00) were typically grouped together and separated from the night time group (18:00) in each of the three functional categories. However, one limitation in this observation was the fact that the 06:00+ group, which has the same conditions as the 06:00 groups, did not typically match up with the 06:00 and 12:00 groups. In fact, in the energy metabolism PCA (Figure 2A), the 06:00+ group was grouped together with the 18:00 group. This observation may have been a result of another variable that was out of control for this type of experiment. In *M. galloprovincialis*, the general trend of the subtidal normoxic groups was a grouping of the 06:00 and 06:00+ groups separated from the 12:00 and the 18:00 group in each of the three functional categories. This differs from the subtidal *M. trossulus* normoxic groups as only the early morning groups (06:00 and 06:00+) are grouped together while the midday (12:00) and later evening (18:00) groups

are grouped together. This gives some evidence for a difference in physiologies between the two species as an endogenous rhythm effects each species differently.

In the tidal control or normoxic groups, the presence of a tidal cycle appeared to disrupt the influence of the endogenous groups in both species. However, the level of disruption varied between species as well as between functional groups. In the normoxic group energy metabolism PCA (Figure 2A) for *M. trossulus*, the 06:00 and 12:00 groups were separated from the 18:00 group; however, the degree of separation was smaller when compared to the subtidal groups. In the normoxic group cytoskeletal PCA (Figure 14A), the 06:00 group was not grouped together with the 12:00 group; instead, the 06:00 group was grouped together with the 18:00 group. This separation may have been caused by the presence of high tide. Both 06:00 and 18:00 groups follow a period of high tide. Thus, we see the influence of the tidal cycle on mostly the cytoskeletal proteins with also some influence in the energy metabolism groups. However, this was not the case for the proteostasis proteins. In the normoxic group proteostasis PCA (Figure 8A), the 06:00, 12:00 and 06:00+ groups are grouped together and separated from the 18:00 group. Thus, we see the influence of a tidal cycle only in the cytoskeletal proteins and the energy metabolism proteins, with very little influence in the proteostasis proteins. The proteostasis proteins were still disrupted to a certain extent by the presence of the tidal cycle as the groups did not match up with the subtidal groups. In fact, the subtidal 18:00 group was grouped together with the tidal 06:00, 12:00 and 06:00+ groups. Thus, we see that in *M. trossulus*, the addition of a tidal cycle, even in normoxic conditions, acts as an added stress, more so in the cytoskeletal and to some extent the energy metabolism protein groups. For the tidal *M. galloprovincialis* normoxic groups, there was some level

of disruption with the presence of a tidal cycle; however, the disruption was not as severe as in the *M. trossulus* normoxic tidal groups. In fact, in each of the three functional groups there was a grouping of the 06:00 and 06:00+ groups and a grouping of the 12:00 and 18:00 groups; however, the degree of separation between the groups was diminished in the tidal groups when compared to the subtidal groups. Thus, we see yet another difference. It appears that *M. trossulus* mussels are more sensitive towards a tidal cycle when compared to *M. galloprovincialis*.

In terms of the strategies used for individual functional categories, there were some similarities and differences between *M. trossulus* and *M. galloprovincialis* in both normoxic and hypoxic conditions. In fact, the strategies used in normoxic conditions appeared to be quite similar in each of the functional groups, with some differences. However, most of the differences were observed in the hypoxic groups. Both species appeared to have very different strategies utilized to mitigate the effects of hypoxic stress in each of the three functional categories.

In terms of energy metabolism during control conditions, both *M. trossulus* and *M. galloprovincialis* had higher levels of proteins associated with aerobic energy metabolism later in the day (18:00 in *M. trossulus*; 12:00 and 18:00 in *M. galloprovincialis*) with more antioxidant activity and glutathione production earlier in the day (06:00 and 12:00 in *M. trossulus*; 06:00 and 06:00+ in *M. galloprovincialis*). Another similarity was the oxidation of the branched chain amino acid valine, which could be fed into the TCA cycle (see Figure 21) in both species. One of the major differences between the two species was the higher activity of reactive aldehyde scavenging proteins such as α -amino adipic semialdehyde dehydrogenase and aldehyde dehydrogenase in *M. trossulus*

but not in *M. galloprovincialis*. It could be that higher amounts of lipid peroxidation occur in *M. trossulus*. In addition, there was also higher amounts of signaling proteins such as N(G), N(G), dimethylarginine dimethylaminohydrolase present in *M. galloprovincialis* when compared to *M. trossulus*. Thus, it appears that *M. galloprovincialis* uses the activity of signaling proteins to regulate protein activity.

During hypoxic conditions, there are differences between both species and acclimation conditions. However, it appears that tidal acclimation, in both species, better prepares the marine mussels for prolonged aerial exposure. In each of the four groups, metabolic depression was observed, a strategy that is consistent with other studies (Fields et al., 2014; Müller et al., 2012; Grieshaber et al., 1994). However, the mechanism of metabolic depression differed between species. *M. trossulus* appeared to decrease the activity of aconitase isoforms contributing to the TCA cycle (Chandel, 2015). Conversely, *M. galloprovincialis* decreased citrate synthase in order to decrease metabolic activity through the TCA cycle (Chandel, 2015) and utilized the signaling molecule H₂S in order to also decrease the activity of the ETC (Banerjee, 2011; Kabil and Banerjee, 2014). In terms of other strategies used to mitigate the effects of aerial exposure the congeners also differed. Subtidal *M. trossulus* mussels had the least effective strategy which relied on metabolic depression in order to “wait out” the stress as well as an increase mainly in reactive aldehyde scavenging proteins. Tidal *M. trossulus*, however, switched to anaerobic metabolism which involves the use of SDHB as well as propionyl-CoA carboxylase and ATP synthase (Müller et al., 2012). This strategy can produce more energy which can be used to maintain cellular structures that are damaged as a result of hypoxic stress. Both subtidal and tidal *M. galloprovincialis* mussels

appeared to have a decrease in TCA cycle but not as much as was observed in *M. trossulus* mussels (especially subtidal mussels). A difference between the subtidal and tidal *M. galloprovincialis* mussels was the utilization of glycolytic pathways for energy production as well as the use of nitrogen metabolism for energy production (subtidal). Conversely, the tidal *M. galloprovincialis* mussels had a similar strategy to the tidal *M. trossulus* mussels. It appears the tidal *M. galloprovincialis* mussels were maintaining branched-chain amino acid oxidation in order to produce and accumulate succinate and eventually propionate (Müller et al., 2012). However, unlike their *M. trossulus* counterparts, *M. galloprovincialis* did not utilize ATP synthase for anaerobic metabolism. Thus, the strategy utilized by *M. galloprovincialis* mussels may be better suited for energy production while also avoiding acidosis by not utilizing ATP synthase activity (Müller et al., 2012; Fields et al., 2014). In addition, tidal *M. galloprovincialis* mussels increased glutathione production using the signaling molecule H₂S which also decreases the activity of the ETC and helps to maintain redox balance (Kabil and Bannerjee, 2014). Nitric oxide or NO was also used by tidal *M. galloprovincialis* mussels to increase the antioxidant response to hypoxia (Kimoto et al., 1993).

During normoxic conditions, the proteostasis proteins utilized differed greatly between species and between acclimation conditions. During normoxic conditions in *M. trossulus* mussels, subtidally acclimated mussels have higher activity of proteins involved in the ubiquitin-proteasome and lysosomal protein degradation pathways during the earlier hours of the day (06:00, 12:00 and 06:00+), while the later 18:00 time point showed a higher abundance of chaperone proteins that assist in the folding of newly translated proteins in the cytosol, mitochondrion, and the ER (Kim et al., 2013). The

pattern was the opposite in tidally acclimated mussels. Thus, in *M. trossulus* mussels we see a trade-off between higher proteolytic activities and the stabilization of aggregation-prone proteins coincide with lower abundances of metabolic enzymes while higher chaperoning activities coincide with higher abundances of metabolic enzymes. In *M. galloprovincialis*, there appears to be higher levels of protein editing in the early time points (06:00 and 06:00+) in both the tidal and subtidal groups. This is based upon the presence of NTAN which is involved in marking unstable proteins for degradation. In the later time points, it appears that higher amount of translation are occurring based upon the presence of eIF3, which is involved in the initiation step of translation (Marks et al., 2009), E3 ubiquitin ligase, which has been known to have involvement in transcription (Weng et al., 2015) and chaperone proteins such as HSP70, which are known to assist in the folding of newly synthesized proteins. Thus, it appears that translation increases during the later time points when aerobic metabolism is at its peak. This makes sense as gene expression is an energetically costly process (Marks et al., 2009). After this period of increased expression, protein editing occurs in the early time points when metabolic depression occurs and antioxidant activity is high.

During hypoxic conditions in the proteostasis proteins, there was a difference between the strategies utilized by *M. trossulus* and *M. galloprovincialis*. In *M. trossulus* mussels, it was interesting to note that the same group of proteostasis proteins contributing to the separation in the normoxia PCA (Figure 8A; Table 3A) were the same proteins found to be contributing to the separation in the hypoxia PCA (Figure 8B; Table 3B). However, a major difference was that the protein abundance patterns separated groups by time of day (early morning together separated from later time of day) hypoxia

disrupted this pattern. Subtidal *M. trossulus* mussels utilized a strategy that involved an enhancement of the ubiquitin-proteasome and lysosomal protein degradation pathways. This differed from the tidal *M. trossulus* mussels which utilized higher levels of cytosolic, mitochondrial and ER chaperones. It is possible that the increase in chaperone activity is used for increased protein translation and maturation; however, it could also be for increased protein denaturation which occurs during hypoxia. As was observed in the energy metabolism analysis, there was less metabolic depression and an increase in the use of anaerobic metabolism occurring in tidal *M. trossulus* mussels which could provide the energy needed to power chaperone activity. Moreover, there is a large activity of mitochondrial specific chaperones, which could indicate that higher levels of damage are occurring to the respiratory machinery in the mitochondria. This differs greatly from *M. galloprovincialis* mussels which seemed to be minimally affected by hypoxic conditions in terms of proteostasis proteins. In fact, it appeared that hypoxia had a limited effect on tidally acclimated mussels while subtidal mussels appeared to be shifting their timing. This was based upon the fact that the same pattern of separation of the 06:00 and 06:00+ groups from the 12:00 and 18:00 groups was maintained in both the tidal and subtidal groups even during hypoxia. This same pattern was also observed in tidal *M. trossulus* but to a lesser degree. Tidal *M. galloprovincialis* mussels had higher levels of ER stress proteins, which indicates a higher ER stress tolerance. In addition, there appeared to be higher amounts of transcriptional control in the tidal *M. galloprovincialis* mussels compared to the subtidal mussels, which were utilizing eIF3 for the initiation of translation. It could be then that tidal mussels utilized more chaperone activity while conserving energy by moving away from gene expression to mitigate the effects of

hypoxic stress while subtidal mussels are utilizing translationally-associated mechanisms possibly to mount a more robust stress response.

Cytoskeletal proteins were the most sensitive towards the presence of low tide or aerial-emersion during the experimental treatment. In the control groups for *M. trossulus*, it appears more actin polymerization and severing of existing actin filaments occurs during the subtidal 06:00 and 12:00 groups and the tidal 06:00 and 18:00 groups. This increase may be associated with increased feeding patterns. In *M. galloprovincialis*, there is an increase in actin filament restructuring in the early time points in both the tidal and subtidal groups. This restructuring may be similar to the increase in actin polymerization and severing of existing actin filaments seen in *M. trossulus*. It is possible then that *M. galloprovincialis*, during the early times of day have an increase in feeding during this time of day.

During hypoxia, there appears to be a robust response exhibited by tidally acclimated *M. trossulus* against hypoxia. This was based upon the fact that the 06:00 and 06:00+ groups were grouped together in the hypoxia PCA (Figure 14B), even though hypoxia had been experienced for 24 hours in the 06:00+ groups. Moreover, it appears that the tidal groups may be forming stress fibers in order to maintain the architecture of the gill tissue during this prolonged hypoxia. This differed from the subtidal groups which appeared to try and bolster existing structures rather than form stress fibers. In *M. galloprovincialis*, there were minimal differences in cytoskeletal activity between the normoxic and hypoxic groups. The hypoxic tidal groups appeared to have increased depolymerization. This increase in depolymerization may be necessary to increase the pool of free actin to form other structures such as stress fibers. In the subtidal groups,

there appears to be an increase actin polymerization possibly to accommodate an increase in vesicular trafficking. This increase in vesicular trafficking may be necessary to increase the proteins needed for a cellular stress to mitigate the effects of hypoxic stress.

Overall, this study highlighted some similarities and differences between both species. It appears that *M. trossulus* is much more sensitive to hypoxic exposure compared to *M. galloprovincialis*. These differences are at the physiological level and are based upon the differing strategies used by each of the functional groups. In all cases it appears that the tidal cycle better prepared the mussels for hypoxic exposure. It may be then that stress hardening, a process that introduces small doses of stress at non-lethal doses, is necessary to prepare these organisms for stress exposure (Kültz, 2005). This study also highlighted the important of a circadian and tidal rhythm in the protein expression patterns of these marine bivalves. Future studies would look into taking more time points to try and map out the influence of a subtidal acclimation and a tidal accliamtion to really map out the effects of a diurnal and tidal rhythm on protein expression.

Materials and Methods

Animal Collection, maintenance and experimental design

Two species of blue mussels that inhabit the Pacific West Coast of the U.S., *Mytilus trossulus* and *Mytilus galloprovincialis*, were used for experimentation. *M. galloprovincialis* was collected from Santa Barbara, CA, USA (34°24'15"N, 119°41'30"W) while *M. trossulus* mussels were collected from Newport, OR, USA (44°38'25"N, 124°03'10"W). These sites were chosen according to the observations made

by Lockwood et al. (2010), and Braby and Somero (2006a), where it is surmised that *M. galloprovincialis* is dominant in the southern coastline off of California, while *M. trossulus* appears to be the dominant species North of Bodega Bay, CA with a hybrid zone existing from San Francisco Bay to Monterey Bay, CA. Mussels species were verified using genetic identification. Briefly, mussels from each location were dissected and gill tissue was collected for DNA extraction (Qiagen, Hilden, Germany). Two nuclear loci were targeted for PCR, Glu-5' and ITS. The GLU-5 locus amplifies different sized fragments in *M. trossulus* and *M. galloprovincialis* due to an insertion in the DNA of *M. galloprovincialis*. The ITS locus amplifies similar fragment sizes in the 2 species. In order to make a comparison, the restriction enzyme that recognizes the site HhaI was used to cut the PCR product for the ITS locus and the different sized fragments produced were observed as the recognition sites differ in the 2 species.

Experimental mussels were transported to the California Polytechnic State University (Cal Poly) Center for Coastal Marine Studies in Avila Beach, California, where they were evenly distributed and maintained in four different 30 gallon tanks, filled with raw sea water (12-18°C). All tanks were equipped with aquarium lighting to control for circadian rhythms (12h light: 12 h dark). Two tanks were equipped with automated drain valves that controlled tidal height within the tank and simulated daily tidal cycles (6 h high: 6 h low tide).

Mussels were acclimated for 3 weeks in July 2013 to one of two tidal cycle regimes: subtidal (constant submersion) or tidal (6 h high: 6 h low tide). Following acclimation, mussels were exposed to one of two hypoxia conditions: normoxia or aerally-induced hypoxia. Normoxia exposed mussels experienced a continuation of their

acclimation regimes while hypoxia conditions were created by simulating a prolonged low tide exposure. Mussels were dissected and gill tissue collected after 0, 6, 12 and 24 h of experimental exposure.

Samples were transported to the Environmental Proteomics laboratory (Cal Poly, San Luis Obispo, CA, USA) on dry ice for proteomic analysis. The preparation of gill tissue and the exploratory statistical analyses following published protocols and procedures (Fields et al., 2012b; Tomanek and Zuzow, 2010; Tomanek et al., 2012; Tomanek et al., 2011) are described below. We analyzed tissues from N=5-6 animals per treatment. There was some mortality recorded during acclimation as well as during hypoxic exposure.

Homogenization

Gill tissue was lysed in ground glass homogenizers in a 1:4 ratio of homogenization buffer [7M Urea, 2M Thiourea, 1%, ASB (amidodisulfobetaine)-14, 40mM Tris-base, 0.002% Bromophenol Blue, 40mM dithiothreitol, MiliQ water, and 0.5% immobilized pH 4-7 gradient (IPG) buffer], solubilized at 20°C for 1h, and then centrifuged at 16,100 g for 30 min at 20°C. Proteins were precipitated from the supernatant using a 1:4 ratio of ice-cold 10% trichloroacetic acid in acetone with an overnight incubation at -20°C. The samples were centrifuged at 18,000 g for 15 min at 4°C, after which the supernatant was discarded and the protein pellet washed with ice-cold acetone. Samples were again centrifuged according to previous parameters and the supernatant discarded. After briefly drying in air, the protein pellets were re-suspended in rehydration buffer [7M Urea, 2M Thiourea, 2% CHAPS

(cholamidopropyltrimethylammonio-propanesulfonic acid), 2% Nonidet P (nonylphenoxypolyethoxylethanol)-40, 0.002% Bromophenol Blue, 100mM dithioerythritol and 0.5% IPG buffer pH 4-7] and solubilized at 20°C for 1 h. To determine protein concentration in each sample, a concentration assay was performed using the 2-D QuantKit (GE Healthcare) according to manufacturer's instructions.

Two-dimensional gel electrophoresis

The proteins from each sample are separated based upon 2 dimensions: charge and size. The first dimension is based upon isoelectric points (pI), using IPG gel strips (pH 4-7, 11cm; GE Healthcare). Protein samples were diluted to a concentration of 2 µg/µL in rehydration buffer and 200 µL (400 µg) of rehydration buffer and precipitated protein were loaded onto each IPG gel strip in one well of an isoelectric focusing cell (Bio-Rad, Hercules, CA, USA). Protein solution was absorbed into each IPG gel strip over 5h of passive rehydration followed by 12h of active rehydration at 50V. Separation and focusing of proteins was performed by running gel strips at 500V for 1h, 1000V for 1h and then 8000V for 2.5h (maximum current 50 µA; all voltage changes occurred in rapid mode). After isoelectric focusing, gel strips were frozen at -80°C for at least one hour. After freezing, gel strips were prepared for second dimension SDS-PAGE by incubation in equilibration buffer [375mM Tris-base, 6M Urea, 30% glycerol, 2% SDS and 0.002% bromophenol] at room temperature (24°C) for two 15 minute periods. Each period had equilibration buffer mixed with first 65M dithiothreitol and then 135M iodoacetamide. Once equilibrated, the strips were placed over polyacrylamide gels of 11.8% and then treated with molten agarose of 1.2% for stability. The samples were run (Criterion Dodeca; Bio-Rad) at 200V for 55 minutes at a constant temperature of 10°C.

Once the run had completed, the gels were stained using colloidal Coomassie Blue (G-250) overnight and then destained through repeated washing with milliQ water over 48h. The gel images were scanned into the computer based using an EPSON 1280 transparency scanner (Epson, Long Beach, CA, USA).

Analysis of 2D Gel Images

Digitized 2D gel images were analyzed using Delta 2D (version 3.6; Decodon, Greifswald, Germany)(Berth et al., 2007). Protein spots were detected by multiplexing gels from all treatment groups into a single fused image. The spot boundaries detected on the fused image were transferred back to the individual gels, establishing protein spot parameters for each gel. Following background subtraction, the relative spot volume of each protein was quantified by normalization against the total spot volume of all proteins in the gel image.

Mass Spectrometry

Gel plugs extracted from gels were prepared for analysis using mass spectrometry (MS) according to previously published methods (Fields et al., 2012; Tomanek and Zuzow 2010). Briefly, peptide mass fingerprints (PMFs) were obtained using a matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-ToF-ToF) mass spectrometer (UltraFlex II; Bruker Daltonics, Inc., Billerica, MA, USA). To obtain *b*- and *y*- ion parameters, at least six peptides were selected for tandem MS. Spectral analysis of peptides followed previously published methods (Fields et al., 2012; Tomanek and Zuzow 2010). FlexAnalysis (version 3.0; Bruker Daltonic, Inc.) was used to detect

peptide peaks, using a signal-to-noise ratio of 6 for MS and 1.5 for MS/MS. Internal mass calibration was performed using porcine trypsin.

Proteins were identified using Mascot (version 2.2; Matrix Science, Inc., Boston, MA). PMFs and tandem mass spectra were combined and searched against 2 databases. One database is an EST library that initially contained approximately 26,000 entries, which represented 12,961 and 1688 different gene sequences for *Mytilus californianus* and *M. galloprovincialis*, respectively (Lockwood et al., 2010). The other one Swiss-Prot (last update: June 2009) with 17,360 molluscan protein sequences. Oxidation of methionine and carbamidomethylation of cysteine were the only variable modifications. The search allowed one missed cleavage during trypsin digestion. For tandem MS the precursor ion mass tolerance was set to 0.6 Da. The molecular weight search (MOWSE) score higher than 43 and 24 significant ($P < 0.05$) in a search in the *Mytilus* EST and Swiss-Prot database, respectively. For results with a significant MOWSE score, only positive identifications that included two matched peptide sequences were accepted.

Exploratory statistical analysis

Protein abundances between different samples utilized hierarchical clustering with average linking using a Pearson correlation metric in Delta 2D (version 3.6; Decodon, Greifswald, Germany). Normalized spot volumes, determined by using Delta 2D, were used to represent the response variable which is protein abundance. These normalized spot volumes were analyzed by using a three-way nonparametric permutation ANOVA ($P < 0.02$) comparing tidal acclimation versus acute aerial-emersion versus time of acute aerial emersion effects using JMP Pro (version 12; SAS, Cary, North Carolina, USA).

This three-way ANOVA was run on each individual protein. In order to provide the clearest picture of the cellular mechanisms occurring at the proteomic level, a Bonferroni adjustment was not used. This was to ensure that the highest number of proteins were incorporated for interpretation; however, we do recognize that the Type I error rate for this analytical approach is extremely high. Due to the high mortality rate in the *M. trossulus* mussels, only one zero hour time point collection was utilized. Thus, for the three-way ANOVA, the zero hour time point was used for both the zero hour normoxia group and the zero hour hypoxia group, as both would have the same proteomes and would have no significant difference between the proteomes as this time point occurs directly after acclimation before any experimental treatment has been applied.

In addition, two separate three-way ANOVAs had to be run for each species because a number of gel regions were difficult to match between species. In addition, for any proteins that did overlap, it was not clear whether the proteins were orthologous or paralogous homologs. In order to compare treatments on specific proteins of interest following the three-way ANOVA, *post-hoc* comparisons was conducted using Tukey's analysis ($P < 0.02$) using Minitab (version 17; Minitab Inc., State College, PA, USA). In order to determine which proteins contributed the most to the changing proteome in response to aerial-emersion hypoxia, principal component analysis was utilized within Delta 2D. For reasons similar to those concerning the three-way ANOVA used, the PCA was conducted within each species and compared afterwards. The results of the PCAs were examined using loading plots, which took into account the proteins that contributed the most to each component.

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Figure A.

Figure A. Global distribution pattern of the three main members of the genus *Mytilus*: *Mytilus trossulus*, *M. galloprovincialis* and *M. edulis*, with hybrid zones from geographic range overlaps (Hillbish et al., 2000).

Figure B.

Figure B. Map of the biogeographical ranges of *M. galloprovincialis*, *M. trossulus*, and their hybrids in the eastern North Pacific along the coast of both California and Oregon, USA. Figure was taken from Lockwood and Somero, 2011.

Figure 1A.

Figure 1B.

Figure 1. Fused gel image (proteome map) of all gels (N=5-6) from all 14 treatments showing detected protein spots from gill tissue of the blue mussels species *Mytilus trossulus* and *M. galloprovincialis*. (A) *M. trossulus* fused gel image depicting 323 protein spots (B) *M. galloprovincialis* fused gel image depicting 270 protein spots. The proteome map represents normalized spot volumes, which is calculated from the average pixel volumes for each protein spot. Each number represents an individual protein that had the potential to be identified using tandem mass spectrometry (for identification information, see the protein ID table in supplementary material Table S1).

Figure 2A.

Figure 2B.

Principal Component 1: 15.11%

Principal Component 2: 9.04%

Figure 2. *M. trossulus* energy metabolism protein principal component analysis of treatments based on proteins that showed a significant acclimation, time of day (ToD), oxygen, two-way interaction and three-way interaction effects (based on a three-way permutation ANOVA; $P < 0.02$). (A) Principal component analysis (PCA) composed using proteins significant for any factor level combination identified using a three-way nonparametric permutation ANOVA. These PCAs were also composed using only normoxia tidal and subtidal acclimation groups. (B) Principal component analysis (PCA) composed using proteins significant for any factor level combination identified using a three-way nonparametric permutation ANOVA. These PCAs were also composed using only hypoxia tidal and subtidal acclimation groups.

Table 1A. *M. trossulus*- Loadings (top ten positive and negative scores) for principal components 1 and 2 for normoxia group significant energy metabolism proteins.

Component Loading Rank	Principal Component 1		Principal Component 2	
	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
	Positive Loadings for <i>M. trossulus</i> Normoxia group (significant energy metabolism proteins)			
1	Aconitase, mitochondrial, partial (26)	2.1321	Malate dehydrogenase, cytosolic (202)	2.25627
2	Isocitrate dehydrogenase (NADP dependent), mitochondrial (124)	1.69041	Malate dehydrogenase, mitochondrial (217)	2.11255
3	Succinate dehydrogenase subunit B, mitochondrial (47)	1.51632	Isocitrate dehydrogenase (NADP dependent), mitochondrial (124)	1.70394
4	ATP Synthase β subunit, partial (108)	1.28723	Aldehyde dehydrogenase, mitochondrial (66)	1.22238
5	Ubiquinol-Cytochrome C reductase complex (137)	1.18821	S-formylglutathione hydrolase (242)	1.1969
6	Aconitase, partial (24)	1.14738	Glutathione S –transferase A (280)	1.05618
7	Malate dehydrogenase, cytosolic (209)	0.92993	ATP synthase β subunit (108)	0.94425
8	Arginase (198)	0.83801	Aconitase, mitochondrial (24)	0.87006
9	Aldehyde dehydrogenase, mitochondrial (102)	0.80876	Enoyl-CoA hydratase, mitochondrial (261)	0.86693
10	4-hydroxyphenylpyruvate dioxygenase (156)	0.63022	Citrate synthase, mitochondrial (143)	0.82991
	Negative Loadings for <i>M. trossulus</i> Normoxia group (significant energy metabolism proteins)			
1	Alpha-aminoadipic semialdehyde dehydrogenase (116)	-2.19945	S-methyl-5'-thioadenosine phosphorylase (275)	-2.33877
2	Isocitrate dehydrogenase (NADP dependent), mitochondrial (145)	-2.09697	4-hydroxyphenylpyruvate dioxygenase (156)	-2.28775
3	Isocitrate dehydrogenase (NADP dependent), mitochondrial (185)	-1.80339	3-hydroxyisobutyrate dehydrogenase, mitochondrial (214)	-2.2093
4	4-hydroxyphenylpyruvate dioxygenase (165)	-1.79879	ADP-ribose pyrophosphatase, mitochondrial (188)	-1.48156
5	Glutamine synthetase (173)	-1.66708	ATP synthase β subunit, partial (132)	-1.44291
6	Cystathionine γ -lyase (176)	-1.64102	Phosphates-binding periplasmic protein (256)	-1.17487
7	Alpha-aminoadipic semialdehyde dehydrogenase (113)	-1.50432	Malate dehydrogenase, cytosolic (209)	-1.13876
8	ATP synthase β subunit, partial (141)	-1.33271	S-methyl-5'-thioadenosine phosphorylase (263)	-1.13446
9	UDP-N-acetylhexosamine pyrophosphorylase (56)	-1.05161	Arginase type I-like protein (204)	-1.13443
10	Methylmalonate-semialdehyde dehydrogenase, mitochondrial (93)	-1.01098	Glycogenin, isoform B (170)	-0.91265

Table 1B. *M. trossulus*- Loadings (top ten positive and negative scores) for principal components 1 and 2 for hypoxia group significant energy metabolism proteins.

Component Loading Rank	Principal Component 1		Principal Component 2	
	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
	Positive Loadings for <i>M. trossulus</i> Hypoxia group (significant energy metabolism proteins)			
1	Malate dehydrogenase, mitochondrial, partial (223)	1.89042	Propionyl-CoA carboxylase α chain, mitochondrial (38)	1.75584
2	Aconitase, mitochondrial (29)	1.82786	Phosphates-binding periplasmic protein (256)	1.55755
3	Cystathionine γ -lyase (176)	1.61889	ATP synthase β subunit (108)	1.27661
4	Heavy metal binding protein (203)	1.60962	Aldehyde dehydrogenase, mitochondrial (102)	1.11282
5	Phosphates-binding periplasmic protein (256)	1.48197	ATP synthase β subunit (132)	1.10233
6	Isocitrate dehydrogenase, (NADP dependent), mitochondrial (162)	1.46595	Citrate synthase, mitochondrial (143)	0.96028
7	Arginase (198)	1.44126	UDP-N-acetylhexosamine pyrophosphorylase (56)	0.84827
8	3-hydroxyisobutyrate dehydrogenase, mitochondrial (214)	1.30795	Isocitrate dehydrogenase (NADP dependent), mitochondrial (145)	0.77909
9	Cu/Zn super oxide dismutase (304)	1.04407	Aconitase, mitochondrial, partial (29)	0.65199
10	S-methyl-5'-thioadenosine phosphorylase (275)	1.03385	ATP synthase β subunit (141)	0.6361
	Negative Loadings for <i>M. trossulus</i> Hypoxia group (significant energy metabolism proteins)			
1	ATP synthase β subunit, partial (108)	-1.68385	Phosphoglycerate kinase 1 (149)	-2.39138
2	Succinate dehydrogenase subunit B, mitochondrial (47)	-1.603	S-methyl-5'-thioadenosine phosphorylase (275)	-2.24099
3	ATP synthase β subunit, partial (141)	-1.57108	Methylmalonate-semialdehyde dehydrogenase, mitochondrial (93)	-1.70231
4	Succinate dehydrogenase, subunit B, mitochondrial (44)	-1.53817	Cu/Zn super oxide dismutase (304)	-1.67197
5	Succinate dehydrogenase, subunit B, mitochondrial (46)	-1.25796	Arginase (198)	-1.64283
6	Alpha-aminoadipic semialdehyde dehydrogenase (116)	-1.1019	4-hydroxyphenylpyruvate dioxygenase (156)	-1.55142
7	Methylmalonate-semialdehyde dehydrogenase, mitochondrial (93)	-1.09149	3-hydroxyisobutyrate dehydrogenase, mitochondrial (214)	-1.43149
8	Isocitrate dehydrogenase (NADP dependent), mitochondrial (124)	-1.08801	Iron dependent peroxidase (237)	-1.36304
9	Ubiquinol-cytochrome C reductase complex (137)	-0.93705	Enoyl-CoA hydratase, mitochondrial (261)	-1.28924
10	Aldehyde dehydrogenase, mitochondrial (66)	-0.86702	Succinate dehydrogenase, subunit B, mitochondrial (45)	-1.27787

Figure 3.

Figure 3. Hierarchical clustering of abundance changes of energy metabolism proteins, using Pearson correlation, in response to either subtidal or tidal acclimation regimes followed by subsequent aerial-emersion hypoxic exposure from gill tissue in *M. trossulus*. Blue coloring represents a decrease in average protein abundance (standardized values normalized volumes), yellow coloring represents an increase in average protein abundance, and black coloring represents no change in the average protein abundance. Each column represents an individual gill tissue sample from a single mussel, grouped together by treatment (N=5-6 for each treatment). Rows represent standardized abundance values of proteins, organized by clusters of similar abundance changes using Pearson correlation. Protein spot number and subsequent ID are listed to the right. Next to the protein ID is whether or not a protein is present in the loading tables for either the nomoxia or the hypoxia PCAs. Significances for an acclimation, ToD, oxygen, two-way interaction and three-way interaction effect are listed based upon a three-way nonparametric permutation ANOVA ($P < 0.02$).

Figure 4A. TCA cycle proteins

Aconitase, mitochondrial, partial (26)

Aconitase, partial (24)

Aconitase, mitochondrial (29)

Citrate synthase, mitochondrial (143)

**Figure 4A. TCA cycle proteins
(continued).**

Isocitrate dehydrogenase (NADP-dependent), mitochondrial (124)

Isocitrate dehydrogenase (NADP-dependent), mitochondrial (145)

Isocitrate dehydrogenase (NADP-dependent), mitochondrial (162)

Isocitrate dehydrogenase (NADP-dependent), mitochondrial (185)

**Figure 4A. TCA cycle proteins
(continued).**

Succinate dehydrogenase, flavoprotein subunit B, mitochondrial (45)

Succinate dehydrogenase, flavoprotein subunit B, mitochondrial (47)

Succinate dehydrogenase, flavoprotein subunit B, mitochondrial (44)

Succinate dehydrogenase, flavoprotein subunit B, mitochondrial (46)

**Figure 4A. TCA cycle proteins
(continued).**

Malate dehydrogenase, mitochondrial, partial (217)

Malate dehydrogenase, mitochondrial, partial (223)

Figure 4B. Electron transport chain and oxidative phosphorylation proteins.

Ubiquinol-cytochrome C reductase complex (137)

ATP synthase, β subunit (132)

ATP synthase, β subunit, partial (108)

ATP synthase, β subunit, partial (141)

Figure 3C. Branched-chain amino acid oxidation proteins

Methylmalonate-semialdehyde dehydrogenase, mitochondrial (93)

3-hydroxyisobutyrate dehydrogenase, mitochondrial (214)

Enoyl-CoA hydratase, mitochondrial (261)

Propionyl-CoA carboxylase, α chain, mitochondrial (38)

Figure 4D. Reactive-aldehyde scavenging proteins.

Aldehyde dehydrogenase, mitochondrial isoform 1 precursor (102)

Aldehyde dehydrogenase, mitochondrial (66)

α -aminoadipic semialdehyde dehydrogenase (116)

α -aminoadipic semialdehyde dehydrogenase (113)

Figure 4E. Nitrogen metabolism associated proteins.

Figure 4F. Glutathione-producing and NADPH-producing proteins.

Figure 4F. Glutathione-producing and NADPH-producing proteins. (continued)

Figure 4G. Antioxidant proteins.

Figure 4. Protein abundance profiles for significant energy metabolism proteins (three-way permutation ANOVA; $P < 0.02$) identified in *M. trossulus*. Protein abundances were subsequently compared using Tukey's *post hoc* pairwise comparison test ($P > 0.02$). Proteins are grouped together based upon the following groupings: **(A)** tricarboxylic acid (TCA) cycle, **(B)** Electron transport chain (ETC) and oxidative phosphorylation proteins, **(C)** Branched-chain amino acid oxidation proteins, **(D)** Reactive-aldehyde scavenging proteins, **(E)** Nitrogen metabolism associated proteins, **(F)** Glutathione-producing and NADPH-producing proteins and **(G)** Antioxidant proteins.

Figure 5A.

Figure 5B.

Figure 5. *M. galloprovincialis* energy metabolism protein principal component analysis of treatments based on proteins that showed a significant acclimation, time of day (ToD), oxygen, two-way interaction and three-way interaction effects (based on a three-way permutation ANOVA; $P \leq 0.02$). (A) Principal component analysis (PCA) composed using proteins significant for any factor level combination identified using a three-way nonparametric permutation ANOVA. These PCAs were also composed using only normoxia tidal and subtidal acclimation groups. (B) Principal component analysis (PCA) composed using proteins significant for any factor level combination identified using a three-way nonparametric permutation ANOVA. These PCAs were also composed using only hypoxia tidal and subtidal acclimation groups.

Table 2A. *M. galloprovincialis*- Loadings (top ten positive and negative scores) for principal components 1 and 2 for normoxia group significant energy metabolism proteins.

Component Loading Rank	Principal Component 1		Principal Component 2	
	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
	Positive Loadings for <i>M. galloprovincialis</i> Normoxia group (significant energy metabolism proteins)			
1	Cu/Zn super oxide dismutase (244)	2.23558	Malate dehydrogenase, mitochondrial (163)	1.57719
2	Macrophage galactose N-acetyl-galactosamine specific lectin 2 (216)	2.18329	Arginase (165)	1.43994
3	Malate dehydrogenase, mitochondrial (159)	1.88284	Calcyphosin-like protein (242)	1.42915
4	3-hydroxyisobutyryl-CoA hydrolase, mitochondrial (143)	1.85162	Aconitase, mitochondrial (172)	1.34015
5	Enoyl-CoA hydratase, mitochondrial (224)	1.83485	Glutamine synthetase (126)	1.32004
6	3-hydroxyisobutyrate dehydrogenase, mitochondrial (166)	1.76972	EP protein precursor (141)	1.29702
7	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 (156)	1.66758	Peroxiredoxin-5, mitochondrial (243)	1.2861
8	3-hydroxyisobutyrate dehydrogenase, mitochondrial (147)	1.53716	Carbonyl reductase [NADPG] 3 (195)	1.21943
9	EP protein precursor (141)	1.46852	S-methyl-5'-thioadenosine phosphorylase (214)	1.16905
10	Aconitase, mitochondrial (16)	1.45899	3-hydroxyanthranilate 3,4-dioxygenase (171)	1.07538
	Negative Loadings for <i>M. galloprovincialis</i> Normoxia group (significant energy metabolism proteins)			
1	Succinate dehydrogenase, subunit B, mitochondrial (28)	-1.68454	3-hydroxyanthranilate 3,4-dioxygenase (77)	-2.41981
2	Citrate synthase, mitochondrial precursor (68)	-1.56306	EF-hand domain-containing protein 1 (21)	-2.38873
3	Arginase (165)	-1.4947	Isocitrate dehydrogenase (NADP dependent) (248)	-2.13472
4	Citrate synthase, mitochondrial precursor (36)	-1.47835	UDP-N-acetylhexosamine pyrophosphorylase (263)	-1.91026
5	Ubiquinol-cytochrome C reductase complex (104)	-1.4366	Glutamate dehydrogenase 1, mitochondrial (78)	-1.70994
6	O-methyltransferase mdmC (206)	-1.1828	Amine oxidase (72)	-1.55594
7	Ornithine aminotransferase, mitochondrial (93)	-1.05052	Dihydrolipoyl dehydrogenase, mitochondrial (246)	-1.37223
8	S-adenosylhomocysteine hydrolase (249)	-1.01752	UDP-N-acetylhexosamine pyrophosphorylase (58)	-1.3006
9	Glutamate dehydrogenase 1, mitochondrial (13)	-0.99737	3-hydroxyisobutyryl-CoA hydrolase, mitochondrial (143)	-1.08226
10	Malate dehydrogenase, mitochondrial (163)	-0.82908	Cystathionine γ -lyase (257)	-1.06488

Table 2B. *M. galloprovincialis*- Loadings (top ten positive and negative scores) for principal components 1 and 2 for hypoxia group significant energy metabolism proteins.

Component Loading Rank	Principal Component 1		Principal Component 2	
	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
	Positive Loadings for <i>M. galloprovincialis</i> Hypoxia group (significant energy metabolism proteins)			
1	Succinate dehydrogenase, subunit B, mitochondrial (31)	1.9301	Citrate synthase, mitochondrial (68)	1.8248
2	Cystathionine γ -lyase (26)	1.9212	Citrate synthase, mitochondrial (36)	1.70063
3	N(G), N(G)-dimethylarginine dimethylaminohydrolase 1 (156)	1.89948	Macrophage galactose N-acetyl-galactosamine specific lectin 2 (216)	1.39868
4	Protein ETHE1, mitochondrial (213)	1.79948	Glutamate dehydrogenase 1, mitochondrial (13)	1.39135
5	EF hand domain-containing protein 1 (21)	1.72181	Ubiquinol-cytochrome C reductase complex (104)	1.37734
6	Cystathionine γ -lyase (257)	1.69851	Malate dehydrogenase, mitochondrial (163)	1.36124
7	Amine oxidase (72)	1.40566	Malate dehydrogenase, cytosolic (151)	1.21517
8	Malate dehydrogenase, mitochondrial (164)	1.35953	Amine oxidase (72)	1.16261
9	Glutathione S-transferase A (218)	1.31118	Arginase (165)	1.11474
10	Iron dependent peroxidase (180)	1.27725	Procollagen-proline dioxygenase β subunit (84)	0.87907
	Negative Loadings for <i>M. galloprovincialis</i> Hypoxia group (significant energy metabolism proteins)			
1	S-methyl-5'-thioadenosine phosphorylase (215)	-2.03148	Dihydropteridine reductase (210)	-2.21081
2	Thyroglobulin (90)	-1.92585	Triosephosphate isomerase (202)	-1.89588
3	ATP synthase β subunit, partial (268)	-1.62718	Malate dehydrogenase, mitochondrial (159)	-1.74
4	Triosephosphate isomerase (223)	-1.59266	Aconitase, mitochondrial (16)	-1.7132
5	Triosephosphate isomerase (202)	-1.51564	3-hydroxyisobutyrate dehydrogenase, mitochondrial (166)	-1.65038
6	Ubiquinol-cytochrome C reductase complex (104)	-1.04263	Triosephosphate isomerase (223)	-1.55729
7	Citrate synthase, mitochondrial (68)	-1.03316	S-methyl-5'-thioadenosine phosphorylase (214)	-1.42309
8	Aconitase, mitochondrial (172)	-1.03238	O-methyltransferase mdmC (206)	-1.40855
9	Cu/Zn superoxide dismutase (244)	-1.0239	3-hydroxyisobutyrate dehydrogenase, mitochondrial (147)	-1.37016
10	Ornithine aminotransferase, mitochondrial (93)	-0.97448	Glutamine synthetase (126)	-1.36355

Figure 6.

Figure 6. Hierarchical clustering of abundance changes of energy metabolism proteins, using Pearson correlation, in response to either subtidal or tidal acclimation regimes followed by subsequent aerial-emersion hypoxic exposure from gill tissue in *M. galloprovincialis*. Blue coloring represents a decrease in average protein abundance (standardized values normalized volumes), yellow coloring represents an increase in average protein abundance, and black coloring represents no change in the average protein abundance. Each column represents an individual gill tissue sample from a single mussel, grouped together by treatment (N=5-6 for each treatment). Rows represent standardized abundance values of proteins, organized by clusters of similar abundance changes using Pearson correlation. Protein spot number and subsequent ID are listed to the right. Next to the protein ID is whether or not a protein is present in the loading tables for either the normoxia or the hypoxia PCAs. Significances for an acclimation, ToD, oxygen, two-way interaction and three-way interaction effect are listed based upon a three-way nonparametric permutation ANOVA ($P < 0.02$).

Figure 7A. Glycolytic proteins.

Figure 7B. TCA cycle proteins.

Aconitase, mitochondrial, partial (172)

Aconitase (16)

Citrate synthase, mitochondrial precursor (68)

Citrate synthase, mitochondrial precursor (36)

**Figure 7B. TCA cycle proteins.
(continued)**

Isocitrate dehydrogenase (NADP-dependent) (248)

Malate dehydrogenase, mitochondrial, partial (164)

Malate dehydrogenase, mitochondrial, partial (163)

Malate dehydrogenase, mitochondrial, partial (159)

Figure 7B. TCA cycle proteins.
(continued)

Figure 7C. Electron transport chain and oxidative phosphorylation proteins.

Ubiquinol-cytochrome C reductase complex (104)

ATP synthase, β subunit (268)

Figure 7D. Branched-chain amino acid (valine) oxidation proteins.

3-hydroxyisobutyryl-CoA hydratase, mitochondrial (143)

Enoyl-CoA hydratase, mitochondrial (224)

3-hydroxyisobutyrate dehydrogenase, mitochondrial (166)

3-hydroxyisobutyrate dehydrogenase, mitochondrial (147)

Figure 7E. Nitrogen metabolism proteins.

Ornithine aminotransferase, mitochondrial (93)

Glutamate dehydrogenase 1, mitochondrial, partial (13)

Glutamate dehydrogenase 1, mitochondrial, partial (78)

Figure 7F. Glutathione-producing and NADPH-producing proteins.

**Figure 7F. Glutathione-producing and NADPH-producing proteins.
(continued)**

Figure 7G. Antioxidant proteins.

Peroxiredoxin-5, mitochondrial (243)

Cu/Zn superoxide dismutase (244)

Carbonyl reductase [NADPG] (195)

Glutathione S-transferase A (218)

Figure 7H. Signaling proteins.

N(G), N(G)-dimethylarginine dimethylaminohydrolase (156)

Calcyphosin-like protein (242)

EP protein precursor (141)

EF hand-domain containing protein 1 (21)

Figure 7H. Signaling proteins.
(continued)

Figure 7. Protein abundance profiles for significant energy metabolism proteins (three-way permutation ANOVA; $P < 0.02$) identified in *M. galloprovincialis*. Protein abundances were subsequently compared using Tukey's *post hoc* pairwise comparison test ($P > 0.02$). Proteins are grouped together based upon the following grouping: **(A)** Glycolytic proteins, **(B)** Tricarboxylic acid (TCA) cycle, **(C)** Electron transport chain (ETC) and oxidative phosphorylation proteins, **(D)** Branched-chain amino acid (valine) oxidation proteins, **(E)** Nitrogen metabolism associated proteins, **(F)** Glutathione-producing and NADPH-producing proteins, **(G)** Antioxidant proteins, and **(H)** Signaling proteins.

Figure 8A.

Figure 8B.

Figure 8. *M. trossulus* proteostasis protein principal component analysis of treatments based on proteins that showed a significant acclimation, time of day (ToD), oxygen, two-way interaction and three-way interaction effects (based on a three-way permutation ANOVA; $P \leq 0.02$). (A) Principal component analysis (PCA) composed using proteins significant for any factor level combination identified using a three-way nonparametric permutation ANOVA. These PCAs were also composed using only normoxia tidal and subtidal acclimation groups. (B) Principal component analysis (PCA) composed using proteins significant for any factor level combination identified using a three-way nonparametric permutation ANOVA. These PCAs were also composed using only hypoxia tidal and subtidal acclimation groups.

Table 3A. *M. trossulus*- Loadings (top ten positive and negative scores) for principal components 1 and 2 for normoxia group significant proteostasis proteins.

Component Loading Rank	Principal Component 1		Principal Component 2	
	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
	Positive Loadings for <i>M. trossulus</i> Normoxia group (significant proteostasis proteins)			
1	Small heat shock protein 24.1 (290)	1.52173	Peptidylprolyl isomerase protein, partial (268)	1.93784
2	Proteasome subunit α type-5 (273)	1.3782	Prohibitin (272)	1.90169
3	Proteasome subunit α type-6 (248)	1.26012	Procollagen-proline dioxygenase β subunit (101)	1.82451
4	Eukaryotic translation initiation factor 5a-1 (306)	1.23869	T-complex protein 1 subunit θ (72)	1.77069
5	Proteasome subunit α type-3 (259)	1.23248	DnaJ-like protein subfamily B member 11 (172)	1.5769
6	Proteasome subunit β type-3 (271)	1.22096	Major vault protein (19)	1.40757
7	Serine/arginine-rich splicing factor 1 (233)	1.11104	26S proteasome non-ATPase regulatory subunit 14 (230)	1.17057
8	Prohibitin (272)	1.09695	Major vault protein (22)	0.98449
9	Cathepsin Z, partial (238)	1.06147	Proteasome subunit β type-3 (271)	0.9734
10	Proteasome subunit β type-2 (295)	1.06004	T-complex protein 1 subunit β (78)	0.95569
	Negative Loadings for <i>M. trossulus</i> Normoxia group (significant proteostasis proteins)			
1	Heat shock protein 78 (31)	-1.6276	Stress-70 protein, mitochondrial (48)	-1.6754
2	Heat shock protein 78 (28)	-1.5545	Histone-binding protein RBBP7 (105)	-1.5546
3	Heat shock protein 60 (74)	-1.496	Heat shock protein 70 (37)	-1.2136
4	Major vault protein (14)	-1.2715	Proteasome subunit α type-6 (267)	-1.1785
5	Heat shock protein 70 (37)	-1.2498	Proteasome subunit β type-2 (295)	-1.1576
6	Major vault protein (17)	-1.2332	Proteasome subunit α type-4 (269)	-1.0996
7	Stress-70 protein, mitochondrial (48)	-1.1808	Heat shock protein 70 (32)	-0.966
8	Heat shock protein 70 (32)	-1.0187	Peptidylprolyl isomerase protein, partial (277)	-0.9278
9	Glucose related protein 94 (heat shock protein) (09)	-1.008	Proteasome subunit α type-3 (259)	-0.7449
10	T-complex protein 1 subunit β (78)	-0.9008	Eukaryotic translation initiation factor 5a-1 (306)	-0.6115

Table 3B. *M. trossulus*- Loadings (top ten positive and negative scores) for principal components 1 and 2 for hypoxia group significant proteostasis proteins.

Component Loading Rank	Principal Component 1		Principal Component 2	
	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
	Positive Loadings for <i>M. trossulus</i> Hypoxia group (significant proteostasis proteins)			
1	Proteasome subunit α type-4 (269)	1.5223	Phenylalanyl-tRNA synthetase β subunit (59)	1.32992
2	Proteasome subunit β type-3 (271)	1.39326	T-complex protein 1 subunit θ (72)	1.20913
3	Small heat shock protein 24.1 (290)	1.32533	Proteasome subunit β type-3 (271)	1.19558
4	Proteasome subunit α type-6 (248)	1.32526	Major vault protein (15)	1.18039
5	Cathepsin Z, partial (238)	1.28349	Eukaryotic translation initiation factor 5a-1 (306)	1.12885
6	Proteasome subunit β type-2 (295)	1.13347	Chaperonin-containing T-complex polypeptide subunit zeta (94)	1.1211
7	Eukaryotic translation initiation factor 5a-1 (306)	1.11139	Major vault protein (22)	1.07401
8	Proteasome subunit α type-5 (273)	1.05476	Heat shock protein 78 (28)	1.06789
9	Prohibitin (272)	1.01827	Glucose related protein 94 (heat shock protein) (09)	1.04727
10	Proteasome subunit α type-3 (259)	0.98344	Prohibitin (272)	1.03296
	Negative Loadings for <i>M. trossulus</i> Hypoxia group (all significant proteins)			
1	Heat shock protein 78 (31)	-1.8587	Serine-arginine-rich splicing factor 1 (233)	-2.1708
2	Stress-70 protein, mitochondrial (48)	-1.473	DnaJ-like protein subfamily B member 11 (172)	-1.8805
3	Major vault protein (14)	-1.3754	T-complex protein 1 subunit θ (80)	-1.7589
4	Heat shock protein 60 (74)	-1.3431	40S ribosomal protein SA (177)	-1.7474
5	Heat shock protein 78 (28)	-1.2844	Proteasome subunit α type-6 (267)	-1.1675
6	Heat shock protein 70 (37)	-1.2803	Major vault protein (17)	-1.0746
7	Heat shock protein 70 (32)	-1.2285	T-complex protein 1 subunit β (78)	-0.975
8	Major vault protein (17)	-1.2261	26S proteasome non-ATPase regulatory subunit 14 (230)	-0.9461
9	Glucose-related protein 94 (heat shock protein) (09)	-1.0593	Major vault protein (14)	-0.9411
10	Tryptophanyl-tRNA synthetase, cytoplasmic (127)	-0.8927	Proteasome subunit α type-5 (273)	-0.7081

Figure 9.

Figure 9. Hierarchical clustering of abundance changes of proteostasis proteins, using Pearson correlation, in response to either subtidal or tidal acclimation regimes followed by subsequent aerial-emersion hypoxic exposure from gill tissue in *M. trossulus*. Blue coloring represents a decrease in average protein abundance (standardized values normalized volumes), yellow coloring represents an increase in average protein abundance, and black coloring represents no change in the average protein abundance. Each column represents an individual gill tissue sample from a single mussel, grouped together by treatment (N=5-6 for each treatment). Rows represent standardized abundance values of proteins, organized by clusters of similar abundance changes using Pearson correlation. Protein spot number and subsequent ID are listed to the right. Next to the protein ID is whether or not a protein is present in the loading tables for either the normoxia or the hypoxia PCAs. Significances for an acclimation, ToD, oxygen, two-way interaction and three-way interaction effect are listed based upon a three-way nonparametric permutation ANOVA ($P < 0.02$).

Figure 10A. Ubiquitin-proteasome proteolytic pathway associated proteins

**Figure 10A. Ubiquitin-proteasome proteolytic pathway associated proteins.
(continued)**

**Figure 10A. Ubiquitin-proteasome proteolytic pathway associated proteins.
(continued)**

Figure 10B. Chaperone proteins

**Figure 10B. Chaperone proteins
(continued)**

**Figure 10B. Chaperone proteins
(continued)**

Stress-70 protein (heat shock protein A9) (48)

T-complex protein 1 subunit β (78)

T-complex protein 1 subunit θ (72)

T-complex protein 1 subunit θ (80)

**Figure 10B. Chaperone proteins
(continued)**

Chaperonin-containing T-complex polypeptide subunit zeta (94)

Glucose-related protein 94 (heat shock protein) (09)

Figure 10C. Transcriptionally and translationally-related protein

Serine-arginine-rich splicing factor 1 (233)

Tryptophanyl-tRNA synthetase, cytoplasmic (127)

Phenylalanyl-tRNA synthetase, β subunit (59)

Histone binding protein 7 (RBBP7) (105)

Figure 10C. Transcriptionally and translationally-related protein (continued)

Figure 10C. Transcriptionally and translationally-related protein (continued)

Figure 10D. Major vault proteins

**Figure 10D. Major vault proteins.
(continued)**

Figure 10. Protein abundance profiles for significant proteostasis proteins (three-way permutation ANOVA; $P < 0.02$) identified in *M. trossulus*. Protein abundances were subsequently compared using Tukey's *post hoc* pairwise comparison test ($P > 0.02$). Proteins are grouped together based upon the following groupings: **(A)** Ubiquitin proteolytic proteasome pathway associated proteins, **(B)** chaperone proteins, **(C)** transcriptionally and translationally-related proteins, and **(D)** major vault proteins.

Figure 11A.

Principal Component 1: 19.89%
Principal Component 2: 10.95%

PC1

PC2

Figure 11B.

Principal Component 1: 16.37%

Principal Component 2: 11.83%

Figure 11. *M. galloprovincialis* proteostasis protein principal component analysis of treatments based on proteins that showed a significant acclimation, time of day (ToD), oxygen, two-way interaction and three-way interaction effects (based on a three-way permutation ANOVA; $P \leq 0.02$). (A) Principal component analysis (PCA) composed using proteins significant for any factor level combination identified using a three-way nonparametric permutation ANOVA. These PCAs were also composed using only normoxia tidal and subtidal acclimation groups. **(B)** Principal component analysis (PCA) composed using proteins significant for any factor level combination identified using a three-way nonparametric permutation ANOVA. These PCAs were also composed using only hypoxia tidal and subtidal acclimation groups.

Table 4A. *M. galloprovincialis*- Loadings (top ten positive and negative scores) for principal components 1 and 2 for normoxia group significant proteostasis proteins.

Component Loading Rank	Principal Component 1		Principal Component 2	
	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
	Positive Loadings for <i>M. galloprovincialis</i> Normoxia group (significant proteostasis proteins)			
1	Heat shock protein 70 (30)	1.75906	Major vault protein, partial (14)	2.46857
2	Prohibitin (122)	1.6224	Small heat shock protein 24.1 (183)	1.79177
3	Major vault protein, partial (10)	1.61664	Serine/arginine-rich splicing factor 1 (193)	1.53549
4	Major vault protein, partial (08)	1.569	Endoplasmin (heat shock protein 90) (05)	1.5084
5	Heat shock protein 78 (269)	1.40007	Endoplasmin (heat shock protein 90) (270)	1.32421
6	T-complex protein 1 subunit eta (63)	1.39682	Phenylalanyl-tRNA synthetase β chain (40)	1.31039
7	Eukaryotic translation initiation factor 3 subunit I (152)	1.2618	Heat shock protein 70 (24)	1.26734
8	Heat shock protein 70 (53)	1.17745	Retinoblastoma binding protein 4, (RBBP4) (85)	1.20571
9	Peptidase (mitochondrial processing) β (106)	0.88668	Major vault protein, partial (04)	1.18902
10	E3 ubiquitin-protein ligase TRIM33 (154)	0.87958	Heat shock protein 70 (42)	0.9939
	Negative Loadings for <i>M. galloprovincialis</i> Normoxia group (significant proteostasis proteins)			
1	Eukaryotic translation initiation factor 3 subunit I (174)	-1.53506	Proteasome subunit α type-6 (211)	-1.5752
2	T-complex protein 1 subunit β (55)	-1.37126	Eukaryotic translation initiation factor 3 subunit (174)	-1.48008
3	TNF ligand-like 1 (186)	-1.33729	Peptidase (mitochondrial processing), β (106)	-1.29643
4	Major vault protein (09)	-1.27837	Major vault protein, partial (06)	-1.28379
5	Proteasome subunit α type-3 (204)	-1.24861	Protein disulfide-isomerase A3, partial (140)	-1.11934
6	Protein disulfide-isomerase A3, partial (140)	-1.18497	Small heat shock protein 24.1 (177)	-1.11229
7	Small heat shock protein 24.1 (201)	-1.07614	Major vault protein, partial (10)	-1.04504
8	Protein N-terminal asparagine amidohydrolase (181)	-1.04372	Major vault protein, partial (110)	-0.99666
9	Small heat shock protein 24.1 (177)	-0.98663	E3 ubiquitin-protein ligase TRIM33 (154)	-0.92183
10	Protein N-terminal asparagine amidohydrolase (200)	-0.985	Proteasome subunit α type-3 (204)	-0.71169

Table 4B. *M. galloprovincialis*- Loadings (top ten positive and negative scores) for principal components 1 and 2 for hypoxia group significant proteostasis proteins.

Component Loading Rank	Principal Component 1		Principal Component 2	
	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
	Positive Loadings for <i>M. galloprovincialis</i> Hypoxia group (significant proteostasis proteins)			
1	T-complex protein 1 subunit θ (57)	1.5293	Phenylalanyl-tRNA synthetase β chain (37)	1.9936
2	T-complex protein subunit β (55)	1.48569	Stress-70 protein, mitochondrial (32)	1.7905
3	Eukaryotic translation initiation factor 3 subunit I (174)	1.41736	Heat shock protein 78 (17)	1.62633
4	Protein disulfide isomerase A2, partial (140)	1.30991	E3 ubiquitin-protein ligase TRIM33 (20)	1.54551
5	Proteasome subunit α type-3 (204)	1.30435	Endoplasmic (heat shock protein 90) (270)	1.54482
6	Proteasome subunit α type-6 (211)	1.20023	Heat shock protein 70 (33)	1.50082
7	Small heat shock protein 24.1 (201)	1.07558	Phenylalanyl-tRNA synthetase β chain (40)	1.49337
8	Small heat shock protein 24.1 (188)	1.06278	T-complex protein 1 subunit η (63)	1.29097
9	DnaJ-like protein subfamily B member 11 (129)	0.99569	Endoplasmic (heat shock protein 90) (05)	1.16742
10	Small heat shock protein 24.1 (177)	0.99248	Major vault protein, partial (14)	1.04464
	Negative Loadings for <i>M. galloprovincialis</i> Hypoxia group (all significant proteins)			
1	Heat shock protein 78 (269)	-2.03436	Eukaryotic translation initiation factor 3 subunit I (152)	-1.97344
2	Heat shock protein 70 (30)	-1.62926	Heat shock protein 70 (53)	-1.43192
3	Major vault protein, partial (10)	-1.57257	Eukaryotic translation initiation factor 3 subunit I (173)	-1.29685
4	Prohibitin (122)	-1.55814	Proteasome subunit α type-6 (211)	-1.25828
5	Major vault protein, partial (14)	-1.32266	Heat shock protein 70 (30)	-1.16262
6	Endoplasmic (heat shock protein 90) (05)	-1.2672	Small heat shock protein 24.1 (188)	-1.07796
7	Heat shock protein 70 (53)	-1.2426	Protein N-terminal asparagine amidohydrolase, partial (200)	-0.98346
8	Heat shock protein 70 (33)	-1.08041	Major vault protein, partial (08)	-0.77232
9	Major vault protein, partial (08)	-0.93574	Major vault protein, partial (10)	-0.74853
10	T-complex protein 1 subunit η (63)	-0.80712	Peptidase (mitochondrial processing) β (106)	-0.61931

Figure 12.

Figure 12. Hierarchical clustering of abundance changes of proteostasis proteins, using Pearson correlation, in response to either subtidal or tidal acclimation regimes followed by subsequent aerial-emersion hypoxic exposure from gill tissue in *M. galloprovincialis*. Blue coloring represents a decrease in average protein abundance (standardized values normalized volumes), yellow coloring represents an increase in average protein abundance, and black coloring represents no change in the average protein abundance. Each column represents an individual gill tissue sample from a single mussel, grouped together by treatment (N=5-6 for each treatment). Rows represent standardized abundance values of proteins, organized by clusters of similar abundance changes using Pearson correlation. Protein spot number and subsequent ID are listed to the right. Next to the protein ID is whether or not a protein is present in the loading tables for either the nomoxia or the hypoxia PCAs. Significances for an acclimation, ToD, oxygen, two-way interaction and three-way interaction effect are listed based upon a three-way nonparametric permutation ANOVA ($P < 0.02$).

Figure 13A. Ubiquitin-proteasome proteolytic pathway associated proteins

Protein N-terminal asparagine amidohydrolase, partial (200)

Protein N-terminal asparagine amidohydrolase, partial (181)

Proteasome subunit α type-3 (204)

Proteasome subunit α type-6 (211)

Figure 13A. Ubiquitin-proteasome proteolytic pathway associated proteins (continued)

E3 ubiquitin-protein ligase TRIM33 (20)

E3 ubiquitin-protein ligase TRIM33 (154)

Figure 13B. Chaperone proteins

**Figure 13B. Chaperone proteins
(continued)**

**Figure 13B. Chaperone proteins
(continued)**

T-complex protein 1 subunit θ (57)

Prohibitin (122)

Endoplasmic (heat shock protein 90) (05)

Endoplasmic (heat shock protein 90) (270)

**Figure 13B. Chaperone proteins
(continued)**

Small heat shock protein 24.1 (188)

Small heat shock protein 24.1 (201)

Small heat shock protein 24.1 (183)

Stress-70 protein, mitochondrial (heat shock protein A9) (32)

Figure 13C. Transcriptionally and translationally-related protein.

Eukaryotic translation initiation factor 3 subunit I (eIF3) (152)

Eukaryotic translation initiation factor 3 subunit I (eIF3) (173)

Eukaryotic translation initiation factor 3 subunit I (eIF3) (174)

Protein disulfide isomerase A3, partial (140)

**Figure 13C. Transcriptionally and translationally-related protein.
(continued)**

Phenylalanyl-tRNA synthetase, β chain (37)

Phenylalanyl-tRNA synthetase, β chain (40)

Retinoblastoma binding protein 4 (RBBP4) (85)

Serine-arginine-rich splicing factor 1 (193)

Figure 13D. Major vault proteins.

**Figure 13D. Major vault proteins.
(continued)**

Figure 13. Protein abundance profiles for significant proteostasis proteins (three-way permutation ANOVA; $P < 0.02$) identified in *M. galloprovincialis*. Protein abundances were subsequently compared using Tukey's *post hoc* pairwise comparison test ($P > 0.02$). Proteins were grouped together based upon the following groups: **(A)** ubiquitin-proteasome proteolytic degradation pathway, **(B)** chaperone proteins, **(C)** Transcriptionally and translationally-related proteins, and **(D)** major vault proteins.

Figure 14A.

Figure 14B.

Figure 14. *M. trossulus* cytoskeletal protein principal component analysis of treatments based on proteins that showed a significant acclimation, time of day (ToD), oxygen, two-way interaction and three-way interaction effects (based on a three-way permutation ANOVA; $P \leq 0.02$). (A) Principal component analysis (PCA) composed using proteins significant for any factor level combination identified using a three-way nonparametric permutation ANOVA. These PCAs were also composed using only normoxia tidal and subtidal acclimation groups. **(B)** Principal component analysis (PCA) composed using proteins significant for any factor level combination identified using a three-way nonparametric permutation ANOVA. These PCAs were also composed using only hypoxia tidal and subtidal acclimation groups.

Table 5A. *M. trossulus*- Loadings (top ten positive and negative scores) for principal components 1 and 2 for normoxia group significant cytoskeletal proteins.

	Principal Component 1		Principal Component 2	
Component Loading Rank	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
	Positive Loadings for <i>M. trossulus</i> Normoxia group (significant cytoskeletal proteins)			
1	β -tubulin, partial (97)	1.63115	β -1-tubulin, partial (88)	1.94198
2	β -tubulin (104)	1.6056	α -tubulin, partial (71)	1.82162
3	Actin (138)	1.56432	α -tubulin, partial (89)	1.44375
4	Short chain collagen C4 (206)	1.52337	Galectin (65)	1.31314
5	Nacre c1q domain-containing protein 1 (197)	1.45653	Actin-interacting protein 1, partial (39)	1.16
6	Nacre c1q domain-containing protein 1 (200)	1.44151	Actin (82)	1.11485
7	Actin (163)	1.38985	Na ⁺ /H ⁺ exchange regulatory cofactor NHE-RF2 (62)	1.05364
8	Actin (82)	1.38782	Actin-interacting protein 1, partial (64)	1.0371
9	α -tubulin, partial (71)	1.37612	Flagellar protofilament ribbon protein rib 74 (25)	1.03146
10	Cdc 42, partial (300)	1.33616	β -tubulin, partial (121)	0.97304
	Negative Loadings for <i>M. trossulus</i> Normoxia group (significant cytoskeletal proteins)			
1	Fascin (92)	-1.6594	Sperm-associated antigen 8 (278)	-1.9996
2	ERK2 (164)	-1.6181	G protein beta subunit (211)	-1.8845
3	Actin (169)	-1.5326	Rho GDP dissociation inhibitor 1 (292)	-1.8475
4	Actin-interacting protein 1, partial (64)	-1.4602	Radial spoke head (166)	-1.6615
5	Rho GDP dissociation inhibitor 1 (293)	-1.4178	Short chain collagen C4 (228)	-1.5514
6	Fascin (103)	-1.3324	Rho GDP dissociation inhibitor 1 (293)	-1.4297
7	Collagen alpha-5 chain (146)	-1.1785	Actin (167)	-1.3785
8	Neuroglian (05)	-1.1771	Laminin receptor (194)	-1.3039
9	Actin-interacting protein 1 (39)	-1.0473	Actin (168)	-1.0334
10	Actin (167)	-0.9516	Short chain collagen C4 (219)	-1.0235

Table 5B. *M. trossulus*- Loadings (top ten positive and negative scores) for principal components 1 and 2 for hypoxia group significant cytoskeletal proteins.

	Principal Component 1		Principal Component 2	
Component Loading Rank	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
	Positive Loadings for <i>M. trossulus</i> Hypoxia group (significant cytoskeletal proteins)			
1	Short chain collagen C4 (228)	1.6127	Rho GDP dissociation inhibitor 1 (293)	2.22517
2	G protein β subunit (211)	1.58129	Rho GDP dissociation inhibitor 1 (292)	2.04586
3	Sperm-associated antigen 8 (278)	1.40967	β -tubulin (104)	1.82088
4	Short chain collagen C4 (219)	1.38193	Fascin (84)	1.67444
5	Actin (161)	1.17779	β -tubulin, partial (97)	1.58054
6	Tektin A1 (111)	1.15674	Radial spoke head (166)	1.2057
7	Laminin receptor (194)	1.15028	Collagen α -5 chain (146)	1.1172
8	β -tubulin (117)	1.06423	β -tubulin, partial (95)	1.09047
9	Nacre c1q domain containing protein 1 (224)	1.04731	Laminin receptor (194)	1.0753
10	Radial spoke head (166)	1.00007	Short chain collagen C4 (245)	1.0031
	Negative Loadings for <i>M. trossulus</i> Hypoxia group (significant cytoskeletal proteins)			
1	β -1-tubulin (88)	-2.114	Profilin (318)	-1.9473
2	Actin (163)	-2.1025	Flagellar protofilament ribbon protein rib74 (25)	-1.5455
3	α -tubulin, partial (71)	-1.8694	Short chain collagen C4 (231)	-1.4861
4	α -tubulin, partial (89)	-1.7475	Actin-interacting protein 1, partial (64)	-1.4214
5	β -actin (159)	-1.6677	Actin-interacting protein 1, partial (39)	-1.2496
6	Non-neuronal cytoplasmic intermediate filament protein (69)	-1.5246	Cdc 42, partial (300)	-1.1671
7	Actin (138)	-1.2922	Neuroglian (01)	-1.1314
8	β -tubulin, partial (95)	-1.2089	Actin (82)	-1.1302
9	Actin (171)	-1.204	Short chain collagen C4 (228)	-1.0833
10	β -1-tubulin, partial (121)	-1.1899	Actin (169)	-0.9867

Figure 15.

Figure 15. Hierarchical clustering of abundance changes of cytoskeletal proteins, using Pearson correlation, in response to either subtidal or tidal acclimation regimes followed by subsequent aerial-emersion hypoxic exposure from gill tissue in *M. trossulus*. Blue coloring represents a decrease in average protein abundance (standardized values normalized volumes), yellow coloring represents an increase in average protein abundance, and black coloring represents no change in the average protein abundance. Each column represents an individual gill tissue sample from a single mussel, grouped together by treatment (N=5-6 for each treatment). Rows represent standardized abundance values of proteins, organized by clusters of similar abundance changes using Pearson correlation. Protein spot number and subsequent ID are listed to the right. Next to the protein ID is whether or not a protein is present in the loading tables for either the normoxia or the hypoxia PCAs. Significances for an acclimation, ToD, oxygen, two-way interaction and three-way interaction effect are listed based upon a three-way nonparametric permutation ANOVA ($P < 0.02$).

Figure 16A. Actin-binding proteins.

**Figure 16A. Actin-binding proteins.
(continued)**

Actin-interacting protein 1, partial (64)

Actin-interacting protein 1 (39)

Figure 16C. Cell signaling proteins.

**Figure 16C. Cell signaling proteins.
(continued)**

Figure 16. Protein abundance profiles for significant cytoskeletal proteins (three-way permutation ANOVA; $P < 0.02$) identified in *M. trossulus*. Protein abundances were subsequently compared using Tukey's *post hoc* pairwise comparison test ($P > 0.02$). Proteins were grouped together based upon the following groupings: **(A)** tubulin protein isoforms, **(B)** actin protein isoforms, **(C)** actin-binding proteins, **(D)** tubulin-binding proteins, and **(E)** cell signaling proteins.

Figure 17A.

Figure 17B.

Figure 17. *M. galloprovincialis* cytoskeletal protein principal component analysis of treatments based on proteins that showed a significant acclimation, time of day (ToD), oxygen, two-way interaction and three-way interaction effects (based on a three-way permutation ANOVA; $P \leq 0.02$). (A) Principal component analysis (PCA) composed using proteins significant for any factor level combination identified using a three-way nonparametric permutation ANOVA. These PCAs were also composed using only normoxia tidal and subtidal acclimation groups. (B) Principal component analysis (PCA) composed using proteins significant for any factor level combination identified using a three-way nonparametric permutation ANOVA. These PCAs were also composed using only hypoxia tidal and subtidal acclimation groups.

Table 6A. *M. galloprovincialis*- Loadings (top ten positive and negative scores) for principal components 1 and 2 for normoxia group significant cytoskeletal proteins.

Component Loading Rank	Principle Component 1		Principle Component 2	
	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
	Positive Loadings for <i>M. galloprovincialis</i> Normoxia group (significant cytoskeletal proteins)			
1	Cell division cycle 42, partial (239)	1.88199	Flagellar protofilament ribbon protein rib74 (11)	2.19879
2	Profilin (245)	1.72145	Rho GDP dissociation inhibitor 1 (12)	2.01116
3	ADP-ribosylation factor 2, isoform CRA_b (259)	1.44165	α -tubulin, partial (65)	1.70917
4	Laminin receptor (128)	1.28181	Gelsolin (107)	1.30447
5	C1q domain-containing protein (235)	1.16091	Collagen-like protein 8, partial (49)	1.29602
6	α -tubulin 1a, partial(148)	1.1406	Laminin receptor (128)	1.14476
7	Rho GDP-dissociation inhibitor 1 (233)	1.13699	Short chain collagen C4 (179)	1.11567
8	Translationally-controlled tumor protein (241)	1.13545	nacre c1q domain-containing protein 1, partial (136)	1.09305
9	α -centractin (109)	1.10632	Actin-interacting protein 1, partial (45)	1.01603
10	C15orf26 (178)	0.97657	β -tubulin, partial (94)	0.98801
	Negative Loadings for <i>M. galloprovincialis</i> Normoxia group (significant cytoskeletal proteins)			
1	Actin (119)	-1.81745	G protein β subunit (162)	-1.96745
2	α -tubulin, partial (60)	-1.75075	Tektin A1 (95)	-1.74771
3	Actin (125)	-1.73351	Actin (18)	-1.70264
4	Actin (64)	-1.60976	Gelsolin (101)	-1.55433
5	α -tubulin, partial (65)	-1.46202	Short chain collagen C4 (160)	-1.39739
6	β -tubulin, partial (94)	-1.44413	Severin (112)	-1.33215
7	α -tubulin (62)	-1.42858	Cofilin/-tropomyosin type actin-binding protein (260)	-1.19891
8	Tubulin, β chain (81)	-1.40739	Radial spoke head 9 homolog-like (170)	-1.17384
9	Collagen-like protein 2 (67)	-1.3733	ADP-ribosylation factor 2, isoform CRA_b (259)	-1.08135
10	Actin (18)	-1.32372	Actin (64)	-0.98511

Table 6B. *M. galloprovincialis*- Loadings (top ten positive and negative scores) for principal components 1 and 2 for hypoxia group significant cytoskeletal proteins.

Component Loading Rank	Principal Component 1		Principal Component 2	
	Protein (Spot ID)	Loading Value	Protein (Spot ID)	Loading Value
	Positive Loadings for <i>M. galloprovincialis</i> Hypoxia group (significant cytoskeletal proteins)			
1	Tubulin, β chain (217)	1.64822	Rab GDP dissociation inhibitor β , partial (265)	1.90034
2	Translationally-controlled tumor protein (240)	1.52377	Flagellar protofilament ribbon protein rib74 (264)	1.56632
3	Translationally-controlled tumor protein (241)	1.34165	Actin-interacting protein 1, partial (45)	1.49137
4	Cell division cycle 42, partial (239)	1.32885	Collagen α (IV) chain (145)	1.40866
5	Rho GDP-dissociation inhibitor 1 (233)	1.31806	Rho GDP-dissociation inhibitor 1 (12)	1.22264
6	ADP-ribosylation factor 2, isoform CRA_b (259)	1.28892	Flagellar protofilament ribbon protein rib74 (11)	1.20448
7	Cofilin/tropomyosin-type actin-binding protein (260)	1.27163	α centractin (109)	1.16071
8	C1q-domain containing protein (235)	1.01081	Protein singed (83)	0.96493
9	Profilin (245)	0.82474	Cell division cycle 42, partial (239)	0.94486
10	Actin-interacting protein 1, partial (191)	0.82983	Actin (27)	0.8964
	Negative Loadings for <i>M. galloprovincialis</i> Hypoxia group (significant cytoskeletal proteins)			
1	Actin (18)	-1.84147	Nacre apextrin-like protein 1 (228)	-1.93972
2	α -tubulin, partial (60)	-1.80921	Gelsolin (101)	-1.91881
3	Actin (64)	-1.76411	C15orf26 (178)	-1.53444
4	Gelsolin (107)	-1.76201	Actin (119)	-1.51325
5	α -tubulin (62)	-1.66839	Tektin A1 (95)	-1.47394
6	α -tubulin, partial (65)	-1.59859	Tubulin, β chain (81)	-1.46705
7	Actin (125)	-1.52463	Actin (125)	-1.43291
8	Actin (119)	-1.45517	G protein β subunit (162)	-1.38745
9	Tubulin, β chain (81)	-1.38645	Actin (120)	-1.37153
10	Rho GDP dissociation inhibitor 1 (12)	-1.26166	Profilin (245)	-1.33569

Figure 18.

Figure 18. Hierarchical clustering of abundance changes of cytoskeletal proteins, using Pearson correlation, in response to either subtidal or tidal acclimation regimes followed by subsequent aerial-emersion hypoxic exposure from gill tissue in *M. galloprovincialis*. Blue coloring represents a decrease in average protein abundance (standardized values normalized volumes), yellow coloring represents an increase in average protein abundance, and black coloring represents no change in the average protein abundance. Each column represents an individual gill tissue sample from a single mussel, grouped together by treatment (N=5-6 for each treatment). Rows represent standardized abundance values of proteins, organized by clusters of similar abundance changes using Pearson correlation. Protein spot number and subsequent ID are listed to the right. Next to the protein ID is whether or not a protein is present in the loading tables for either the nomoxia or the hypoxia PCAs. Significances for an acclimation, ToD, oxygen, two-way interaction and three-way interaction effect are listed based upon a three-way nonparametric permutation ANOVA ($P < 0.02$).

Figure 19A. Actin binding proteins.

**Figure 19A. Actin-binding proteins.
(continued)**

**Figure 19A. Actin binding proteins.
(continued)**

Figure 19C. Cell Signaling proteins.

Rho GDP dissociation inhibitor 1 (12)

Rho GDP dissociation inhibitor 1 (233)

Rab GDP dissociation inhibitor 1 (265)

Cell division cycle 42, partial (239)

**Figure 19C. Cell Signaling proteins.
(continued)**

Figure 19. Protein abundance profiles for significant cytoskeletal proteins (three-way permutation ANOVA; $P < 0.02$) identified in *M. galloprovincialis*. Protein abundances were subsequently compared using Tukey's *post hoc* pairwise comparison test ($P > 0.02$). Proteins were grouped together based upon the following groupings: **(A)** tubulin protein isoforms, **(B)** actin protein isoforms, **(C)** actin-binding proteins, **(D)** Tubulin binding proteins, and **(E)** cell signaling proteins.

Figure 20.

Synthesis of glutathione (GSH) from the metabolism of methionine

Figure 20. Proposed mechanism of methionine metabolism for the eventual formation of the antioxidant glutathione. Pathway highlights the steps that start with the amino acid methionine and leads to the formation of the amino acid cysteine, which is then incorporated into glutathione. This pathway is also connected to the TCA cycle which provides a source of α -ketoglutarate, which is converted into glutamate to be used by γ -glutamylcysteine synthase. Orange circles represent products that are formed from the activity of enzymes. Yellow boxes are enzymes that have been identified in the present study using MALDI-TOF/TOF-based proteomics. Pink boxes are enzymes that have been identified by Dilly et al. (2012) to contribute to the formation of GSH but were not identified in the current study. The green boxes represent products and substrates that are formed as a result of the activity of the TCA cycle. Pathway is based upon the findings of Dilly et al. (2012).

Valine Oxidation Pathway and its Relation to the TCA cycle

Figure 21.

Figure 21. Proposed mechanism of oxidation of the branched-chain amino acid valine for the eventual formation of the TCA cycle intermediate succinyl-CoA. Pathway highlights the steps that start with the branched-chain amino acid valine, which leads to the formation of propionyl-CoA. In a series of three steps propionyl-CoA is converted into succinyl-CoA, which is then shuttled to the TCA cycle. The three enzymes involved in the conversion of propionyl-CoA to succinyl-CoA are unknown. Orange circles represent products that are formed from the activity of enzymes. Yellow boxes are enzymes that have been identified in the present study using MALDI-TOF/TOF-based proteomics. Pink boxes are enzymes that have been identified by Wanders et al. (2012) that contribute to this pathway but were not identified in the current study. 3-hydroxyisobutyryl-CoA hydratase was identified only in the *M. galloprovincialis* proteome not the *M. trossulus* proteome in the current study. Pathway is based upon the findings of Wanders et al. (2012).

APPENDIX B: SUPPLEMENTAL INFORMATION

Table S1. List of identified proteins, predicted and estimated molecular weight (kDa) and isoelectric point (pI) from Delta2D or Expasy Protein Parameters, GenBank ID and NCBI accession number, and MASCOT protein ID score, peptide matches, and sequence coverage for *Mytilus trossulus*.

Spot ID	Protein ID	MW (kDa) Estimated	pI Estimated	MW (kDa) Predicted	pI Predicted	GenBank ID	Maxent Score	Peptide Matches	Sequence Coverage	Functional Category
1	Neurofilin	17536.78	5.10	10800.00	6.01	BC24451.1	88	2	4	cytoskeleton/integral membrane glycoprotein, cell adhesion
2	Neurofilin, partial	113809.83	5.35	97000.00	6.07	FM48253.1	53	2	4	cytoskeleton/integral membrane glycoprotein, cell adhesion
5	Neurofilin	115306.78	5.10	99000.00	6.14	BC24451.1	87	2	4	cytoskeleton/integral membrane glycoprotein, cell adhesion
9	glucose related protein 94 (heat shock protein)	95566.51	4.83	93000.00	5.12	BAF5637.1	156	2	10	proteostasis/chaperone protein
13	major vault protein	95865.70	5.64	93000.00	5.87	NP_001118989.1	77	3	3	proteostasis/cell signaling protein/nuclear transport/proteostasis
14	major vault protein	95865.70	5.64	90000.00	5.92	NP_001118989.1	157	3	3	proteostasis/cell signaling protein/nuclear transport/proteostasis
15	major vault protein	95865.70	5.64	93000.00	6.10	NP_001118989.1	85	2	2	proteostasis/cell signaling protein/nuclear transport/proteostasis
17	major vault protein	95865.70	5.64	90000.00	5.86	NP_001118989.1	189	4	4	proteostasis/cell signaling protein/nuclear transport/proteostasis
19	major vault protein	95865.70	5.64	93000.00	6.16	NP_001118989.1	69	2	2	proteostasis/cell signaling protein/nuclear transport/proteostasis
21	major vault protein	95865.70	5.64	93000.00	6.22	NP_001118989.1	78	3	3	proteostasis/cell signaling protein/nuclear transport/proteostasis
24	aconitate hydratase, mitochondrial	84344.20	8.36	85000.00	6.79	EFNA1350.1	226	4	4	energy metabolism
25	flagellar protofilament ribbon protein rib74	85553.34	8.15	80000.00	6.69	NP_595826.1	121	2	2	cytoskeleton/vesicle transport
26	aconitate hydratase, mitochondrial, partial	84344.20	8.36	84000.00	6.76	EFNA1350.1	213	4	4	energy metabolism
28	heat shock protein 70	70225.76	4.99	83000.00	5.11	HAH02536.1	158	4	5	proteostasis/chaperone
29	aconitate hydratase, mitochondrial, partial	84344.20	8.36	84000.00	6.73	EFNA1350.1	183	3	3	energy metabolism
31	heat shock protein 78	70225.76	4.99	83000.00	5.14	HAH02536.1	127	3	4	proteostasis/chaperone
32	HSP70	71164.32	5.13	77000.00	5.58	AAH52766.1	183	3	6	proteostasis/chaperone
37	HSP70	71164.32	5.13	77000.00	5.53	AAH52766.1	147	3	6	proteostasis/chaperone
38	propionyl-CoA carboxylase alpha chain, mitochondrial	79940.09	6.65	77000.00	5.65	NP_062203.1	95	2	2	energy metabolism/fatty acid metabolism (mitochondrial)
39	Actin-interacting protein 1, partial	66369.39	5.92	83000.00	6.57	KYN61415.1	101	2	1	cytoskeleton/actin binding protein
44	succinate dehydrogenase (ubiquinone) flavoprotein subunit B, mitochondrial	83855.47	6.28	77000.00	6.08	BC148201.1	103	2	2	energy metabolism/ETC
45	succinate dehydrogenase (ubiquinone) flavoprotein subunit B, mitochondrial	83855.47	6.28	77000.00	6.10	BC148201.1	117	4	4	energy metabolism/ETC
46	succinate dehydrogenase flavoprotein subunit B, mitochondrial	83855.47	6.28	77000.00	5.86	BC148201.1	94	3	3	energy metabolism/ETC
47	succinate dehydrogenase flavoprotein subunit B, mitochondrial	83855.47	6.28	77000.00	5.94	BC148201.1	48	2	2	energy metabolism/ETC
48	stress-70 protein, mitochondrial	73684.78	5.87	76000.00	5.46	NP_001233756.1	115	3	2	proteostasis/chaperone
52	Phenylalanyl-tRNA synthetase beta chain	82638.32	5.95	75000.00	6.32	BC118179.1	71	2	2	proteostasis/attaches L-phenylalanine to the terminal adenosine of tRNA
53	NA+/H+ exchange regulatory cofactor NHE-RF2	39140.85	5.53	77000.00	5.05	AC010339.1	109	2	3	cytoskeleton/scaffold protein, connects plasma membrane proteins to ezrin/moesin/radixin family (link to actin cytoskeleton)
56	UDP-N-acetylhexosamine pyrophosphorylase	53882.99	5.95	75000.00	6.40	H053227.1	122	2	7	energy metabolism/hexosamine pathway
59	Phenylalanyl-tRNA synthetase beta subunit	82612.62	5.96	75000.00	6.24	NP_001007769.1	100	4	4	proteostasis/attaches L-phenylalanine to the terminal adenosine of tRNA
62	NA+/H+ exchange regulatory cofactor NHE-RF2	39140.85	5.53	76000.00	5.13	AC010339.1	59	3	18	cytoskeleton/scaffold protein, connects plasma membrane proteins to ezrin/moesin/radixin family (link to actin cytoskeleton)
64	Actin-interacting protein 1, partial	66369.39	5.92	75000.00	6.57	KYN61415.1	77	2	2	cytoskeleton/actin binding protein
65	galactin	63278.22	4.99	75000.00	5.21	AAH40777.1	84	2	2	cytoskeleton/mediation of cell-cell interactions/cell-matrix adhesion/transmembrane signaling
66	mitochondrial aldehyde dehydrogenase	56384.06	8.10	73000.00	6.67	AB118712.1	71	2	3	energy metabolism
69	non-neuronal cytoplasmic intermediate filament protein	69345.89	5.38	74000.00	5.72	BC118158.1	74	2	3	cytoskeleton/structural support
71	alpha tubulin, partial	10814.38	4.80	65000.00	5.34	AAH36066.1	142	2	27	cytoskeleton/vesicle transport
72	T-complex protein 1 subunit theta	59144.69	5.86	68000.00	6.38	NP_001033347.1	110	2	10	proteostasis/chaperone
74	heat shock protein 60	61078.85	5.18	68000.00	5.42	AQ22379.1	102	3	5	proteostasis/chaperone
76	flagellar associated protein, partial	45951.96	5.94	69000.00	5.81	NP_001702394.1	127	3	15	cytoskeleton/mucous production
78	T-complex protein 1 subunit beta	57448.06	6.03	68000.00	6.05	BC127629.1	81	2	3	proteostasis/chaperone
80	T-complex protein 1 subunit theta	59089.67	5.86	69000.00	5.90	BC127636.1	94	2	2	proteostasis/chaperone
82	actin	42719.73	5.46	65000.00	5.54	AAH40314.1	172	3	10	cytoskeleton
84	fascin	57376.55	6.34	66000.00	6.44	EFNA6987.1	116	2	3	cytoskeleton/actin binding protein
88	beta2-tubulin	50113.12	4.74	63000.00	5.14	ABH5592.1	111	3	3	cytoskeleton/vesicle transport
89	alpha tubulin, partial	10814.38	4.80	65000.00	5.23	AAH36066.1	182	2	27	cytoskeleton/vesicle transport
92	fascin	57376.55	6.34	65000.00	6.27	EFNA6987.1	116	2	3	cytoskeleton/actin binding protein
93	methylmalonate semialdehyde dehydrogenase (acylating), mitochondrial	56888.55	8.55	67000.00	6.06	MF33741.1	103	2	4	energy metabolism/fatty acid metabolism
94	chaperonin containing T-complex polypeptide subunit zeta	58420.98	6.53	68000.00	6.79	AF29045.1	67	2	6	proteostasis/chaperone protein
95	beta tubulin, partial	18103.75	4.78	63000.00	5.03	AAH36067.1	109	2	11	cytoskeleton/vesicle transport
97	beta tubulin, partial	18103.75	4.78	63000.00	4.99	AAH36067.1	126	2	15	cytoskeleton/vesicle transport
99	aldehyde dehydrogenase, mitochondrial isoform 1 precursor	56345.62	6.63	65000.00	5.79	NP_000688.2	94	2	2	energy metabolism
101	procollagen-proline dihydrogenase beta subunit	55659.61	4.53	64000.00	4.71	AAH08917.1	124	2	4	proteostasis
102	aldehyde dehydrogenase, mitochondrial isoform 1 precursor	56345.62	6.63	65000.00	5.69	NP_000688.2	152	4	5	energy metabolism
103	fascin	57376.55	6.34	65000.00	6.36	EFNA6987.1	200	3	3	cytoskeleton/actin binding protein
104	beta tubulin	50017.00	4.74	64000.00	4.97	K012182.1	93	2	6	cytoskeleton/vesicle transport
105	histone-binding protein RBBP7	47722.11	4.71	63000.00	4.91	NP_591775.1	68	2	6	proteostasis/nuclear protein, cell proliferation
108	ATP synthase beta subunit, partial	46231.01	4.95	62000.00	5.07	AAH06148.1	293	4	13	energy metabolism
110	sperm-associated antigen 6	55343.27	6.16	62000.00	6.84	K012654.1	48	2	2	cytoskeleton/ciliary activity
111	tektin A1	51770.84	5.30	63000.00	5.38	AC115284.1	111	3	3	cytoskeleton/vesicle transport/ciliary activity
113	Alpha-aminoadipic semialdehyde dehydrogenase	55182.15	5.85	63000.00	6.42	BC142665.1	48	2	2	energy metabolism/osmotic regulation/chemical chaperone/betaine metabolism
116	Alpha-aminoadipic semialdehyde dehydrogenase	55182.15	5.85	63000.00	6.54	BC142665.1	134	2	5	energy metabolism/osmotic regulation/chemical chaperone/betaine metabolism
117	beta tubulin	50004.01	4.73	62000.00	5.87	BA422382.1	104	3	8	cytoskeleton/vesicle transport

121	beta-1-tubulin, partial	4855.08	4.67	6200.00	5.22	APH0947.1	122	3	8	cytoskeletal/vesicle transport
122	C15orf26	32484.27	6.49	6000.00	6.15	AC00857.1	100	2	6	cytoskeletal/motility/dynein arm assembly
124	isocitrate dehydrogenase [NADP dependent], mitochondrial	50461.48	6.77	5800.00	6.61	AF56364.1	31	2	3	energy metabolism/Krebs cycle
126	peptidase (mitochondrial processing) beta	58548.01	5.72	6000.00	5.80	NP_00279134.1	122	3	7	proteostasis
127	tryptophanyl-DNA synthetase, cytoplasmic	46334.45	5.80	5800.00	6.42	BCIC2079.1	148	2	2	proteostasis
130	processing peptidase beta subunit, putative	59485.66	5.62	5900.00	5.78	NP_002407386.2	71	2	4	proteostasis
131	severin	48172.37	5.39	5700.00	6.34	BCIC2167.1	152	4	5	cytoskeletal/actin interacting protein
132	ATP synthase beta subunit, partial	46231.01	4.95	5900.00	5.01	AAT06148.1	206	4	12	energy metabolism
133	tubulin beta chain	50017.00	4.74	5800.00	5.67	NCI23182.1	88	3	11	cytoskeletal/vesicle transport
135	pyruvate dehydrogenase	48552.79	8.30	5700.00	6.51	EHK6764.1	77	2	5	energy metabolism/connection Glycolysis->TCA cycle
137	ubiquinol-cytochrome C reductase complex	47969.56	9.00	5800.00	5.59	NP_001008401.1	145	2	4	energy metabolism/ETC
138	actin	48719.73	5.46	5700.00	5.53	AAD49314.1	173	3	10	cytoskeletal
140	ubiquinol-cytochrome C reductase complex	47969.56	9.00	5800.00	5.48	NP_001008401.1	166	4	8	energy metabolism/ETC
141	ATP synthase beta subunit, partial	46231.01	4.95	5900.00	5.08	AAT06148.1	209	4	13	energy metabolism
143	citrate synthase, mitochondrial	58569.75	6.79	5600.00	6.72	EGN12509.1	72	2	8	energy metabolism/TCA cycle
145	isocitrate dehydrogenase [NADP dependent], mitochondrial	50461.48	6.77	5500.00	6.58	AF56364.1	120	3	5	energy metabolism/Krebs cycle
146	collagen alpha-5 chain	24744.14	5.94	5700.00	6.10	BCIC4033.1	99	2	8	cytoskeletal/extracellular matrix
147	mitochondrial-processing peptidase subunit beta	54379.15	6.00	5700.00	5.96	NCI18831.1	100	2	9	proteostasis
148	APF3 actin-related protein 3 homolog	47382.98	5.71	5800.00	6.02	AAT23583.1	110	2	1	cytoskeletal/actin binding protein
149	Phosphoglycerate kinase 1	48000.51	7.59	5600.00	6.89	BCIC2802.1	123	2	4	energy metabolism/glycolysis
151	ATP-binding cassette subfamily E member 1	46073.68	8.92	5500.00	6.37	NP_004303802.1	66	2	5	energy metabolism/ETC
153	isocitrate dehydrogenase	50461.48	6.77	5600.00	6.75	AF56364.1	128	3	5	energy metabolism/Krebs Cycle
155	actin	48719.73	5.46	5400.00	5.43	AAD49314.1	211	4	13	cytoskeletal
156	4-hydroxyphenylpyruvate dioxygenase	35215.10	6.72	5500.00	6.65	MT208655.1	124	2	19	energy metabolism/tyrosine catabolism/oxidative stress/dioxygenase
158	actin	48719.73	5.46	5400.00	5.34	AAD49314.1	119	4	12	cytoskeletal
159	beta-actin	48704.77	5.29	5400.00	5.63	AH065094.1	162	4	12	cytoskeletal
161	actin	48719.73	5.46	5400.00	5.75	AAD49314.1	104	3	10	cytoskeletal
162	isocitrate dehydrogenase, NADP dependent, mitochondrial	50461.48	6.77	5400.00	6.43	AF56364.1	128	3	5	energy metabolism/Krebs cycle
163	actin	48719.73	5.46	5400.00	5.55	AAD49314.1	117	2	7	cytoskeletal
164	ERN2	48412.17	6.42	5200.00	6.47	APPS7674.1	228	4	10	cytoskeletal/MAP kinase/cell signaling
165	4-hydroxyphenylpyruvate dioxygenase	211446.47	6.63	5300.00	6.71	BCIC3515.1	61	2	5	energy metabolism/degradation of tyrosine
166	radial spoke head	32138.60	4.34	5400.00	4.63	NP_00177084.1	167	4	28	cytoskeletal/mucous production
167	actin	48719.73	5.46	5400.00	5.89	AAD49314.1	154	3	10	cytoskeletal
168	actin	48719.73	5.46	5400.00	5.95	AAD49314.1	99	3	10	cytoskeletal
169	actin	48719.73	5.46	5400.00	6.12	AAD49314.1	120	3	10	cytoskeletal
170	glycogenin, isoform B	37751.88	5.27	5300.00	4.91	NP_755040.1	151	2	6	energy metabolism/glycogenin glucosyltransferase activity, glucose to glycogen
171	actin	48719.73	5.46	5400.00	5.52	AAD49314.1	152	3	12	cytoskeletal
172	DnaJ-like protein subfamily B member 11	153377.48	5.05	5200.00	6.05	BCIC0863.1	91	2	11	proteostasis/chaperone
173	glutamine synthetase	48344.87	5.63	5300.00	6.21	ADM9477.1	97	2	2	energy metabolism/metabolism of nitrogen
174	laminin receptor	34017.98	5.02	5300.00	4.99	APV23300.1	91	2	10	cytoskeletal
176	cystathione gamma-lyase	46902.73	6.57	5300.00	6.73	BCIC9931.1	99	2	1	energy metabolism
177	40S ribosomal protein S4	34036.95	4.81	5200.00	4.83	NP_001089106.1	62	2	10	proteostasis/translational control
178	actin	48719.73	5.46	5300.00	5.95	AAD49314.1	163	3	10	cytoskeletal
180	actin	48719.73	5.46	5200.00	6.10	AAD49314.1	158	3	10	cytoskeletal
183	actin	48719.73	5.46	5200.00	5.38	AAD49314.1	70	2	6	cytoskeletal
185	isocitrate dehydrogenase [NADP dependent], mitochondrial	50461.48	6.77	5200.00	6.59	AF56364.1	71	2	3	energy metabolism/Krebs cycle
188	ADP-ribose pyrophosphatase, mitochondrial	31550.85	6.19	4900.00	5.33	EFN66048.1	80	3	6	energy metabolism/protein glycosylating agent
194	laminin receptor	35202.54	4.84	4700.00	4.93	AB036169.1	158	3	20	cytoskeletal
197	nacre C1q domain-containing protein 1	26987.64	5.20	4400.00	5.16	AUC07857.1	119	2	10	cytoskeletal/shell formation
198	arginase	36786.91	5.47	4400.00	5.78	ADA68869.1	129	2	4	energy metabolism/catalytic activity, urea cycle
199	arginase	36786.91	5.47	4600.00	5.86	ADA68869.1	151	2	4	energy metabolism/catalytic activity, urea cycle
200	nacre C1q domain-containing protein 1	26987.64	5.20	4300.00	5.25	AUC07857.1	161	3	15	cytoskeletal/shell formation
202	cytosolic malate dehydrogenase	36370.59	6.02	4500.00	6.37	AAT79366.1	111	2	7	energy metabolism
203	heavy metal binding protein	26867.00	5.09	4400.00	5.08	CAE2197.1	102	2	10	energy metabolism/binds heavy metals, carrier of divalent cations in plasma
204	arginase type I-like protein	38141.07	5.24	4600.00	5.72	AER78965.1	118	2	4	energy metabolism/catalytic activity, forms urea and ornithine
206	short chain collagen C4	38729.83	8.99	4200.00	5.36	NCI17591.1	164	3	4	cytoskeletal
207	caspase 3/7-2	35126.68	5.62	4400.00	5.47	AUC24781.1	61	2	6	energy metabolism
208	serine/threonine-protein phosphatase alpha 1 isoform	46234.58	7.04	4500.00	6.47	NYG5663.1	75	3	3	energy metabolism/glycogen metabolism
209	cytosolic malate dehydrogenase	36370.59	6.02	4400.00	6.40	AAT79366.1	177	2	7	energy metabolism
211	G protein beta subunit	37385.95	5.62	4400.00	5.94	AAT53593.1	108	2	2	cytoskeletal
213	F-actin-capping protein subunit alpha	32658.12	5.45	4500.00	6.29	NC064843.1	105	3	10	cytoskeletal/actin binding protein
214	3-hydroxyisobutyrate dehydrogenase, mitochondrial	43883.32	8.00	4400.00	6.65	BCIC4123.1	130	2	4	energy metabolism/fatty acid metabolism (mitochondria)
215	Proteasome subunit alpha type-1	27725.88	6.07	4400.00	5.60	BCIC2932.1	163	3	12	proteostasis/proteolysis
217	malate dehydrogenase, mitochondrial, partial	34702.34	8.92	4300.00	6.59	HN07893.1	140	2	10	energy metabolism

218	beta tubulin	5004.01	4.73	43000.00	5.69	B442380.1	121	3	8	cytoskeleton/vesicle transport
219	short chain collagen C4	33729.63	8.99	42000.00	5.49	KX17590.1	85	2	3	cytoskeleton
223	malate dehydrogenase, mitochondrial, partial	34702.34	8.92	43000.00	6.76	KM7289.1	177	3	20	energy metabolism
224	nucleo c1q domain containing protein 1	26087.64	5.20	42000.00	5.39	KM7285.1	124	2	10	cytoskeleton
225	tubulin beta-2C chain	31959.63	4.31	42000.00	5.65	KM0871.1	119	2	19	cytoskeleton/vesicle transport
227	short chain collagen C4	33729.63	8.99	42000.00	4.97	KX17590.1	84	3	4	cytoskeleton/extracellular matrix
228	short chain collagen C4	33729.63	8.99	42000.00	5.61	KX17590.1	88	2	4	cytoskeleton/extracellular matrix
229	C15orf26	33484.27	6.49	42000.00	6.42	AC08857.1	138	3	13	cytoskeleton/motility/dynein arm assembly
230	26S proteasome non-ATPase regulatory subunit 14	34540.57	5.87	42000.00	6.65	EH6532.1	54	2	14	proteostasis
231	short chain collagen C4	33729.63	8.99	42000.00	5.15	KX17590.1	130	2	3	cytoskeleton
232	short chain collagen C4	33729.63	8.99	42000.00	5.06	KX17590.1	166	3	4	cytoskeleton
233	serine/arginine-rich splicing factor 1	38287.94	9.85	42000.00	5.91	KM7289.1	146	3	14	proteostasis
236	protein N-terminal asparagine amidohydrolase, partial	34753.53	5.74	42000.00	6.73	KM7254.1	139	3	13	proteostasis
237	iron dependent peroxidase	50419.45	6.47	42000.00	6.47	CD545573.1	85	2	2	energy metabolism/glutathione peroxidase/antioxidant
238	Cathepsin Z, partial	28865.52	5.75	42000.00	6.22	KF09377.1	41	2	3	proteostasis/protein degradation, lysosomal cysteine proteinase
241	small heat shock protein 24.1	28519.86	5.61	39000.00	5.68	AE92368.1	133	3	16	proteostasis/chaperone
242	S-formylglutathione hydrolase	31163.15	6.18	38000	6.56	EKC36313.1	63	2	15	energy metabolism/formation of glutathione/antioxidant
244	small heat shock protein 24.1	28519.86	5.61	39000	5.57	AE92368.1	46	2	6	proteostasis/chaperone
245	short chain collagen C4	33729.63	8.99	39000	5.27	KX17590.1	167	3	5	cytoskeleton/extracellular matrix
248	proteasome subunit alpha type-6	27354.8	6.34	38000	6.33	NP_001132918.1	85	2	8	proteostasis
256	phosphates-binding periplasmic protein	35356.22	6.47	37000	6.69	KP95396.1	138	3	19	energy metabolism
257	F-actin-capping protein subunit beta	30723.58	5.7	36000	5.32	NP_956229.1	48	2	2	cytoskeleton
259	proteasome subunit alpha type-3	28450.03	4.94	36000	5.18	A0027884.1	65	2	13	proteostasis/proteolysis
260	Proteasome subunit alpha type-6	27354.8	6.34	36000	6.40	NP_001132918.1	124	3	12	proteostasis/proteolysis
261	enoyl-CoA hydratase, mitochondrial	31232.13	8.8	36000	6.62	NP_001004529.1	96	2	13	energy metabolism/fatty acid beta oxidation
263	S-methyl-5'-thioadenosine phosphorylase	62706.5	6	35000	6.67	EKC30156.1	54	2	2	energy metabolism/antioxidant
264	O-methyltransferase mdmC	35936.27	8.34	35000	6.48	EKC12363.1	79	2	14	energy metabolism/2 carbon metabolism
265	phosphate ABC transporter substrate-binding protein	29825.47	5	35000	6.70	WP_044451513.1	64	3	19	energy metabolism/oxidative stress/phosphate import
267	proteasome subunit alpha type-6	27354.8	6.34	35000	6.33	NP_001132918.1	82	2	8	proteostasis
268	peptidyl(isomerase) protein, partial	14673.49	5.29	34000	6.80	AB727347.1	166	2	6	proteostasis/chaperone protein
269	proteasome subunit alpha type-4	29513.13	6.91	35000	6.19	NP_001279383.1	91	2	5	proteostasis
270	O-methyltransferase mdmC	35936.27	8.34	33000	6.53	EKC12363.1	210	3	19	energy metabolism/2 carbon metabolism
271	proteasome subunit beta type-3	22888.44	4.92	35000	5.38	KX25711.1	69	2	10	proteostasis
272	prohibitin	30552.78	5.39	34000	5.63	ETN62887.1	185	4	21	proteostasis/modulate transcriptional activity, inhibits DNA synthesis
273	proteasome subunit type alpha-5	26432.19	4.75	34000	4.97	NP_001134432.1	78	3	14	proteostasis
274	carbonic anhydrase	28397.81	5.87	34000	6.34	ALF62133.1	88	2	6	energy metabolism/acid/base balance
275	S-methyl-5'-thioadenosine phosphorylase	62706.5	6	34000	6.89	EKC30156.1	72	2	2	energy metabolism/catalytic activity, methionine metabolism
276	thiamine biosynthesis protein ThiJ	24865.02	8.41	33000	6.75	WP_02987607.1	121	2	6	energy metabolism
277	peptidyl(isomerase) protein, partial	14673.49	5.29	34000	6.57	AB727347.1	183	3	9	proteostasis
278	sperm-associated antigen 8	21866.86	5.62	33000	5.75	KX25973.1	58	2	8	cytoskeleton/regulator of activator of CREM in testis during spermatogenesis
280	Glutathione S-transferase A	26494.34	6.24	33000	6.68	EKC36795.1	108	2	5	energy metabolism/oxidative stress
281	6-phosphogluconolactonase, partial	28236.63	5.73	32000	6.19	KRM68417.1	101	2	10	energy metabolism/pentose phosphate pathway
286	growth factor receptor-bound protein	25237.53	5.5	30000	6.08	AD158029.1	76	2	3	cytoskeleton/ERK2 interact/adaptor protein, signal transduction/cell communication
288	NADH dehydrogenase [ubiquinone] iron-sulfur 8, mitochondrial	24022.75	5.89	30000	5.05	NP_659119.2	90	3	19	energy metabolism/ETC
290	small heat shock protein 24.1	28519.86	5.61	30000	5.98	AE92368.1	121	2	10	proteostasis/chaperone
291	thioredoxin peroxidase	21857.91	5.95	29000	6.71	ADM88074.1	301	4	13	energy metabolism/antioxidant/transcriptional induction/inhibitor of apoptosis
292	Rho GDP dissociation inhibitor 1	23556.01	5.15	29000	5.24	EKC31723.1	78	3	9	cytoskeleton/cell cycle signaling/vesicle transport
293	Rho GDP dissociation inhibitor 1	23556.01	5.15	29000	5.36	EKC31723.1	114	2	7	cytoskeleton/cell cycle signaling/vesicle transport
295	proteasome subunit beta type-2	22531.58	6.2	26000	6.21	NP_001110571.1	57	2	9	proteostasis/proteolysis
299	GTP-binding protein SAR1b	24875.47	7.1	24000	6.59	EKC29660.1	155	2	7	cytoskeleton/vesicle transport
300	cdk42, partial	19164.77	5.45	24000	6.73	CAQ64775.1	161	2	13	cytoskeleton/cell cycle signaling
302	ATPase	59236.88	6.79	22000	5.44	WP_026362159.1	157	3	9	energy metabolism/ETC
303	calcyphosin	21401.6	4.99	22000	5.49	ADO28065.1	90	4	6	energy metabolism/oxidative stress/calcium binding/ion binding regulation
304	Cu/Zn super oxide dismutase	15919.85	5.84	21000	6.33	AF52312.1	146	2	13	energy metabolism/oxidative stress
306	eukaryotic translation initiation factor 5a-1	16894.35	5.19	20000	5.52	NP_001187938.1	83	2	6	cytoskeleton/actin interacting protein
318	profilin	15272.48	6.1	11000	6.70	NP_995760.1	94	2	10	cytoskeleton

Table S2. List of identified proteins, predicted and estimated molecular weight (kDa) and isoelectric point (pI) from Delta2D or Expasy Protein Parameters, GenBank ID and NCBI accession number, and MASCOT protein ID score, peptide matches, and sequence coverage for *Mytilus galloprovincialis*.

Spot ID	Protein ID	MW (kDa) Estimated	pI Estimated	MW (kDa) Predicted	pI Predicted	UniProt ID	Mass Score	Peptide Matches	Sequence Coverage(%)	Functional Category
4	major vault protein, partial	31740.69	5.45	8900.00	6.17	A0D48063.1	84	2	8	proteostasis/cell signaling protein/nuclear transport
5	Endoplasmic	125136.91	4.88	88000.00	5.18	EKC38233.1	129	2	10	proteostasis/chaperone/HSP90
6	major vault protein, partial	31692.66	5.69	88000.00	5.97	ABK63640.1	88	2	10	proteostasis/cell signaling protein/nuclear transport
8	major vault protein, partial	95865.70	5.64	88000.00	5.97	NP_001169889.1	110	4	3	proteostasis/cell signaling protein/nuclear transport
9	major vault protein	95865.70	5.64	88000.00	6.01	NP_001169889.1	206	3	3	proteostasis/cell signaling protein/nuclear transport
10	major vault protein, partial	31692.66	5.69	88000.00	5.92	ABK63640.1	71	2	8	proteostasis/cell signaling protein/nuclear transport
11	flagellar protofilament ribbon protein rib74	75986.06	5.83	88000.00	5.32	NP_999628.1	112	3	5	cytoskeletal/ciliary movement/mucous production
12	Rho GDP-dissociation inhibitor 1	25596.01	5.15	88000.00	5.38	EKC31723.1	48	2	9	cytoskeletal/vesicle transport
13	Glutamate dehydrogenase 1, mitochondrial, partial	45008.86	7.32	87000.00	5.41	EMC79955.1	135	2	6	energy metabolism/nitrogen and glutamate metabolism
14	major vault protein, partial	31692.66	5.69	88000.00	5.45	ABK63640.1	112	2	8	proteostasis/cell signaling protein/nuclear transport
16	Aconitate hydratase, mitochondrial, partial	84344.20	8.36	83000.00	6.81	EFN81350.1	93	2	2	energy metabolism/catalyze citrate to isocitrate, TCA cycle
17	heat shock protein 78	75225.76	4.99	78000.00	5.17	A1N05256.1	106	2	11	proteostasis/chaperone
18	actin	41719.73	5.46	74000.00	5.57	AAD40314.1	141	3	10	cytoskeletal
19	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	79362.36	5.82	79000.00	5.64	NP_001007766.1	76	2	10	energy metabolism
20	E3 ubiquitin-protein ligase TRIM33	85992.13	8.51	77000.00	6.64	EKC18736.1	46	2	7	proteostasis/ubiquitin proteasome pathway, SMAD4 ubiquitination
21	EF-hand domain-containing protein 1	65194.03	5.84	77000.00	6.74	KJ08615.1	107	3	5	energy metabolism/calcium binding protein, ER
22	V-type proton ATPase catalytic subunit A	70805.63	5.2	76000.00	5.82	EKC18221.1	103	4	4	energy metabolism/ATPase
23	E3 ubiquitin-protein ligase TRIM33	85992.13	8.51	74000.00	6.81	EKC18736.1	77	2	7	proteostasis/ubiquitin proteasome pathway, SMAD4 ubiquitination
24	HSP70	71164.32	5.33	75000.00	5.69	AAW52766.1	136	3	5	proteostasis/chaperone
25	alpha-actinin	42572.89	6.29	75000.00	5.78	KJ212483.1	59	2	4	cytoskeletal/vesicle transport/cell cycle
26	cystathionine gamma-lyase	46901.73	6.57	78000.00	5.94	EKC29931.1	165	3	3	energy metabolism/glutathione synthesis/oxidative stress
27	actin	41719.73	5.46	76000.00	6.02	AAD40314.1	204	4	13	cytoskeletal
28	succinate dehydrogenase [ubiquinone] flavoprotein subunit 8, mitochondrial	83835.47	6.28	75000.00	5.89	EKC34020.1	268	4	4	energy metabolism/ETC
30	HSP70	71164.32	5.33	74000.00	5.62	AAW52766.1	240	3	5	proteostasis/chaperone
31	succinate dehydrogenase [ubiquinone] flavoprotein subunit 8, mitochondrial	83835.47	6.28	75000.00	6.07	EKC34020.1	87	3	3	energy metabolism/ETC
32	stress-70 protein, mitochondrial	75559.78	5.97	74000.00	5.47	XP_006180391.1	121	2	2	proteostasis/cell signaling/chaperone
33	HSP70	71164.32	5.33	74000.00	5.51	AAW52766.1	204	3	5	proteostasis/chaperone
35	cytosolic malate dehydrogenase	36370.59	6.02	73000.00	5.30	AAZ79366.1	213	3	11	energy metabolism
36	citrate synthase, mitochondrial precursor	51596.43	8.12	75000.00	5.73	NP_999441.1	123	3	5	energy metabolism/carbohydrate metabolism/TCA cycle
37	Phenylalanyl-tRNA synthetase beta chain	82638.32	5.95	74000.00	6.51	EKC18579.1	97	3	3	proteostasis/scaffold protein
40	Phenylalanyl-tRNA synthetase beta chain	82638.32	5.95	74000.00	6.42	EKC18579.1	65	2	2	proteostasis/scaffold protein
41	shell nacre collagen-like protein 1, partial	20688.74	8.99	72000.00	5.36	AKQ70836.1	192	2	10	cytoskeletal/shell formation/ECM
42	heat shock protein 70	70830.06	5.28	74000.00	6.26	AEP26301.1	81	2	4	proteostasis/chaperone
44	isocitrate dehydrogenase (NADP dependent)	58461.48	6.77	72000.00	5.07	AFI56364.1	115	2	3	energy metabolism/Krebs cycle, NADP dependent
45	actin-interacting protein 1, partial	66369.39	5.92	70000.00	6.71	KYN05415.1	81	2	1	cytoskeletal/actin binding protein
48	collagen-like protein-8, partial	24253.34	5.11	72000.00	5.09	AKS48186.1	250	2	10	cytoskeletal/ECM
49	collagen-like protein-8, partial	24253.34	5.11	73000.00	5.21	AKS48186.1	139	2	10	cytoskeletal/ECM
53	HSP70	71164.32	5.33	71000.00	5.62	AAW52766.1	199	3	5	proteostasis/chaperone
55	T-complex protein 1 subunit beta	57449.06	6.03	68000.00	6.06	EKC37629.1	75	2	3	proteostasis/chaperone
56	monamine oxidase	57794.70	8.89	69000.00	6.25	WP_036230585.1	66	2	3	energy metabolism/nitric oxide synthesis regulation
57	T-complex protein 1 subunit theta	83699.67	5.66	67000.00	5.93	EKC27336.1	141	2	9	proteostasis/chaperone/protein folding
58	UDP-N-acetylhexosamine pyrophosphorylase	52882.99	5.95	68000.00	6.32	KOC62272.1	53	2	7	energy metabolism/glycosaminoglycan pathway
60	alpha-tubulin, partial	41667.72	5.11	64000.00	5.30	ADK91434.1	103	2	8	cytoskeletal/vesicle transport
61	Titin	149800.99	4.53	65000.00	5.77	EKC20506.1	84	2	3	cytoskeletal/ECM/muscle contraction
62	alpha tubulin	40975.51	4.97	65000.00	5.21	AAA29138.1	175	3	12	cytoskeletal/vesicle transport
63	T-complex protein 1 subunit eta	59144.69	5.86	66000.00	5.42	NP_001032347.1	94	2	10	proteostasis/chaperone/protein folding
64	actin	41719.73	5.46	64000.00	5.56	AAD40314.1	159	4	13	cytoskeletal
65	alpha-tubulin, partial	41667.72	5.11	63000.00	5.37	ADK91434.1	129	2	8	cytoskeletal/vesicle transport
67	collagen-like protein-2	50105.97	7.10	62000.00	5.73	AKS48142.1	135	2	12	cytoskeletal/extracellular matrix
68	citrate synthase, mitochondrial precursor	51596.43	8.12	63000.00	5.63	NP_999441.1	86	2	3	energy metabolism/citric acid cycle
69	glutamine synthetase, partial	9747.91	6.94	65000.00	5.98	AAN46296.1	97	2	12	energy metabolism/nitrogen metabolism
71	T-complex protein 1 subunit eta	59144.69	5.86	66000.00	6.54	NP_001032347.1	110	2	10	proteostasis/chaperone
72	amine oxidase	52780.99	6.04	66000.00	6.64	KPL74011.1	94	3	5	energy metabolism
73	major vault protein	95865.70	5.64	65000.00	5.89	NP_001169889.1	206	3	3	proteostasis/signaling protein/nuclear transport
75	Protein disulfide-isomerase A3, partial	55983.12	5.61	64000.00	6.79	KFM67004.1	229	3	4	proteostasis/ER, protein oxidation
77	3-hydroxyanthranilate 3,4-dioxygenase, partial	32580.80	5.73	64000.00	6.62	KFV76246.1	72	2	5	energy metabolism
78	Glutamate dehydrogenase 1, mitochondrial, partial	45008.86	7.32	66000.00	6.88	EMC79955.1	135	2	6	energy metabolism/nitrogen and glutamate metabolism
79	aldehyde dehydrogenase 2 family (mitochondrial), isoform CRA_d	21999.97	6.35	63000.00	5.82	EAW97976.1	99	2	9	energy metabolism/alcohol metabolism
81	Tubulin beta chain	50225.49	4.72	61000.00	5.12	KJ214675.1	116	4	8	cytoskeletal/vesicle transport
82	Protein singed	57464.38	6.17	64000.00	6.2	KDR22994.1	83	2	3	cytoskeletal/actin bundling protein/fascin-like
83	Protein singed	57464.38	6.17	63000.00	6.38	KDR22994.1	155	2	3	cytoskeletal/actin bundling protein/fascin-like

84	procollagen-proline dihydrogenase beta subunit	5959.61	4.53	6200.00	4.76	AFM58917.1	236	4	8	energy metabolism/ECM
85	Retinoblastoma binding protein 4, like	47018.15	4.71	6200	4.96	AA54779.1	64	2	6	proteostasis/transcriptional control
86	Severin	43172.37	5.39	6800	5.86	EKC21617.1	132	3	4	cytoskeletal/actin binding protein
87	fascin	57376.55	6.34	6900.00	6.29	ETN68987.1	241	2	3	cytoskeletal/actin binding protein
90	Thyroglobulin	314273.12	8.59	6000	4.61	KXJ13128.1	174	4	18	energy metabolism/oxidative stress/iodine storage
93	Ornithine aminotransferase, mitochondrial	47419.63	7.95	61000.00	5.91	EKC25719.1	94	2	2	energy metabolism/nitrogen (proline) metabolism (feeds into arginine production), urea cycle
94	beta tubulin, partial	34112.39	4.81	61000.00	5.23	AAR3410.1	151	3	10	cytoskeletal/vesicle transport
95	tektin A1	51770.84	5.5	6000	6.01	AC3524.1	153	3	4	cytoskeletal/vesicle transport/ciliary movement/mucus production
100	Ornithine aminotransferase, mitochondrial	47419.63	7.95	56000.00	6.31	EKC25719.1	94	2	2	energy metabolism/nitrogen (proline) metabolism (feeds into arginine production), urea cycle
101	gelsoin	42001.88	5.22	57000.00	5.97	CAF21683.1	91	2	11	cytoskeletal/actin severing protein
102	peptidase (mitochondrial processing) beta	53549.01	5.72	58000.00	5.93	NP_001279114.1	146	3	8	proteostasis/protein cleavage
103	Severin	43172.37	5.39	5600	6.36	EKC21617.1	132	3	4	cytoskeletal/actin binding
104	Ubiquinol-cytochrome C reductase complex	47989.56	9	5800	5.63	NP_001084801.1	142	3	5	energy metabolism/complex III ETC
105	arginase	36706.91	5.47	58000.00	5.44	ADA68688.1	104	2	4	energy metabolism/urea cycle
106	peptidase (mitochondrial processing) beta	53549.01	5.72	58000.00	5.81	NP_001279114.1	116	3	8	proteostasis/protein cleavage
107	gelsoin	42071.70	5.22	58000.00	5.14	CAC37029.1	71	2	4	cytoskeletal/actin severing protein
109	alpha-centractin	42522.89	6.29	58000.00	6.87	KXJ23483.1	59	2	4	cytoskeletal/dynactin complex cellular functions (chromosome movement, spindle formation)
110	major vault protein, partial	31692.66	5.69	58000.00	5.08	ABK65840.1	75	3	11	proteostasis/cell signaling protein/nuclear transport
111	Actin-related protein 3	58279.61	6.87	57000.00	6.14	KKF17190.1	118	3	3	cytoskeletal
112	Severin	43172.37	5.39	5700	6.24	EKC21617.1	100	3	4	cytoskeletal/actin binding protein
113	Actin-related protein 3	58279.61	6.87	57000.00	6.06	KKF17190.1	193	3	2	cytoskeletal
115	Fumarylacetoacetase, partial	46741.61	7.83	56000.00	6.09	KFW17969.1	63	2	3	energy metabolism/urea cycle
116	phosphoglycerate kinase 1	43000.51	7.59	55000.00	6.91	EKC28824.1	146	2	5	energy metabolism/glycolysis
117	isocitrate dehydrogenase (NADP dependent)	50461.40	6.77	54000.00	6.86	AF56364.1	82	3	5	energy metabolism/Krebs cycle, NADP dependent
118	Actin-related protein 3	58279.61	6.87	56000.00	6.02	KKF17190.1	134	3	2	cytoskeletal
119	actin	41719.73	5.46	55000.00	5.47	AAD40314.1	219	4	13	cytoskeletal
120	actin	41719.73	5.46	54000.00	5.39	AAD40314.1	149	3	9	cytoskeletal
121	actin	41719.73	5.46	55000.00	5.28	AAD40314.1	118	3	10	cytoskeletal
122	prohibitin	30852.78	5.39	55000.00	5.66	ETN62887.1	258	4	18	proteostasis/cell signaling/cell proliferation
124	phosphoglycerate kinase 1	43000.51	7.59	55000.00	4.95	EKC28824.1	146	2	5	energy metabolism/glycolysis
125	actin	41719.73	5.46	55000.00	5.55	AAD40314.1	160	3	10	cytoskeletal
126	glutamine synthetase	41324.87	5.63	55000.00	6.26	ADM94277.1	126	2	2	energy metabolism/metabolism of nitrogen, catalyze glutamate to glutamine
128	raminin receptor	35302.54	4.84	51000.00	4.87	ABO2619.1	165	3	20	cytoskeletal
129	DnaJ-like protein subfamily B member 11	153377.48	5.05	52000.00	6.08	EKC30863.1	87	2	11	proteostasis/co-chaperone to HSPAs
131	isocitrate dehydrogenase	50461.40	6.80	55000.00	6.45	AF56366.1	109	2	3	energy metabolism/Krebs cycle, NADP dependent
132	ERK2	41421.27	6.42	52000.00	6.50	APP57674.1	266	4	10	cytoskeletal/MAPK/ERK cascade/cell growth or cell cycle/adhesion/small G protein
133	actin	41719.73	5.46	51000.00	5.41	AAD40314.1	144	3	9	cytoskeletal
136	nacre C1q domain-containing protein 1, partial	26087.64	5.20	46000.00	5.19	AKQ70857.1	117	2	7	cytoskeletal/ECM
140	Protein disulfide-isomerase A3, partial	55983.12	5.61	47000.00	4.97	KFM67004.1	229	3	4	proteostasis/ER/protein oxidation
141	EP protein precursor	26898.98	5.24	46000.00	5.11	AAQ63463.1	129	3	13	energy metabolism/oxidative stress/extracellular matrix/heavy metal binding protein
143	3-hydroxyisobutyryl-CoA hydrolase, mitochondrial, partial	22298.46	5.32	46000.00	5.79	ETE65884.1	49	2	2	energy metabolism/valine or AA catabolism
145	collagen alpha-4(VI) chain	314273.12	8.59	47000.00	6.85	KXJ13132.1	92	2	12	cytoskeletal/extracellular matrix, cell binding protein
146	EP protein precursor	26898.98	5.24	45000.00	5.27	AAQ63463.1	173	3	13	energy metabolism/oxidative stress/extracellular matrix/heavy metal binding protein
147	3-hydroxyisobutyrate dehydrogenase, mitochondrial	42863.32	8.00	47000.00	6.70	EKC24123.1	88	3	3	energy metabolism/fatty acid metabolism (mitochondria)
148	alpha-tubulin 1a, partial	30066.98	5.23	46000.00	5.76	AJ27191.1	111	2	13	cytoskeletal/vesicle transport
149	heparan sulfate proteoglycan-like protein-1	471546.93	4.96	46000.00	5.91	AKS48136.1	71	2	16	cytoskeletal/extracellular matrix/cell surface, cell signaling
151	cytosolic malate dehydrogenase	36399.59	6.02	45000.00	6.42	AAZ79367.1	177	2	7	energy metabolism/catalytic activity, NAD+ to NADH, citric acid cycle
151	cytosolic malate dehydrogenase	36399.59	6.02	45000.00	6.42	AAZ79367.1	165	2	7	energy metabolism/citric acid cycle
152	eukaryotic translation initiation factor 3 subunit I	36263.48	5.22	45000.00	6.37	NP_990355.1	164	3	20	proteostasis/initiation of protein synthesis/ribosome
154	E3 ubiquitin-protein ligase TRIM33	85902.13	8.51	44000.00	5.35	EKC18736.1	77	2	7	proteostasis/ubiquitin proteasome pathway, SMAD4 ubiquitination
156	NG1/NG2-dimethylarginine dimethylaminohydrolase 1	39513.50	6.29	44000.00	6.44	EKC44017.1	163	3	3	energy metabolism/nitric oxide synthesis regulation
157	small heat shock protein 24.1	28519.86	5.61	4300	5.4	AEP02968.1	196	4	14	proteostasis/chaperone
158	short-chain collagen C4	24588.56	6.81	4100	5.5	KXJ23128.1	90	2	14	cytoskeletal/ECM
159	malate dehydrogenase, mitochondrial, partial	34702.34	8.92	43000.00	6.80	KFM73959.1	208	3	20	energy metabolism
160	short-chain collagen C4	24588.56	6.81	4300	5.44	KXJ23128.1	120	2	14	cytoskeletal/ECM
161	Transcriptional activator protein Pur-alpha, partial	28100.26	8.96	4300	5.66	KFM76873.1	88	2	7	proteostasis/DNA replication control
162	G protein beta subunit	37385.95	5.62	44000.00	5.80	AGY52593.1	119	2	2	cytoskeletal/G protein/vesicle transport
163	malate dehydrogenase, mitochondrial, partial	34702.34	8.92	43000.00	5.74	KFM73959.1	251	3	20	energy metabolism
164	malate dehydrogenase, mitochondrial, partial q	34702.34	8.92	43000.00	6.63	KFM73959.1	231	3	20	energy metabolism
165	arginase	36706.91	5.47	43000.00	5.92	ADA68688.1	104	2	4	energy metabolism/catalytic activity, urea cycle
166	3-hydroxyisobutyrate dehydrogenase, mitochondrial	42863.32	8.00	43000.00	6.70	EKC24123.1	88	3	3	energy metabolism/fatty acid metabolism (mitochondria)
167	3-hydroxyanthranilate 3,4-dioxygenase, partial	32986.60	5.73	43000.00	6.76	KFY78246.1	111	2	6	energy metabolism
168	nacre apertin-like protein 1	24461.79	4.91	43000.00	5.55	AKQ70860.1	110	3	11	cytoskeletal/shell formation/ECM
170	radial spoke head 9 homolog-like	31210.75	4.97	4300	6.14	NP_001177389.1	139	2	6	cytoskeletal/ciliary action

171	3-hydroxyanthranilate 3,4-dioxygenase, partial	32980.60	5.73	43000.00	6.55	KFY70246.1	49	2	6	energy metabolism
171	3-hydroxyanthranilate 3,4-dioxygenase, partial	32980.60	5.73	43000.00	6.55	KFY70246.1	74	3	7	energy metabolism/NAD+ biosynthesis
172	Aconitate hydratase, mitochondrial, partial	84344.20	8.36	41000.00	5.66	EFN81390.1	93	2	2	energy metabolism/TCA cycle
173	eukaryotic translation initiation factor 3 subunit I	36263.48	5.22	42000.00	6.26	NP_998155.1	126	3	20	proteostasis/initiation of protein synthesis/ribosome
174	eukaryotic translation initiation factor 3 subunit I	36263.48	5.22	42000.00	6.29	NP_998155.1	128	2	13	proteostasis/initiation of protein synthesis/ribosome
177	small heat shock protein 24.1	28519.86	5.61	41000	6.08	AEP02968.1	147	3	14	proteostasis/chaperone
178	C15orf26	32484.27	6.49	41000.00	6.44	AC008957.1	124	2	6	cytoskeletal/motility/dynein arm assembly
178	C15orf26	32484.27	6.49	41000.00	6.44	AC008957.1	115	3	13	cytoskeletal/motility/dynein arm assembly
179	short-chain collagen C4	24088.56	6.81	41000	5.35	KXJ2128.1	47	2	14	cytoskeletal/ECM
180	iron dependent peroxidase	59419.45	6.47	39000.00	6.50	CDS45373.1	74	2	8	energy metabolism/glutathione peroxidase/antioxidant
181	protein N-terminal asparagine amidohydrolase, partial	34753.53	5.74	41000.00	6.77	KFM72254.1	91	2	8	proteostasis/polyubiquitination/arginylation/degradation
183	small heat shock protein 24.1	28519.86	5.61	40000	5.85	AEP02968.1	129	3	14	proteostasis/chaperone
186	TNF ligand-like 1	30084.75	5.5	39000	6.29	AJQ21535.1	70	2	18	proteostasis/apoptosis
188	small heat shock protein 24.1	28519.86	5.61	39000	5.92	AEP02968.1	153	3	14	proteostasis/chaperone
191	actin-interacting protein 1, partial	66089.39	5.92	39000.00	5.41	KYN05415.1	95	3	2	cytoskeletal
193	serine/arginine-rich splicing factor 1	27060.36	9.71	38000	5.79	KOC2108.1	116	2	10	proteostasis/mRNA splicing
195	carboxyl reductase [NAD(P)] 3	30752.61	5.59	38000.00	5.61	NP_001290936.1	110	2	8	energy metabolism/oxidative stress
197	dihydropteridine reductase	24949.60	5.64	38000.00	6.65	XP_001689487.1	49	2	4	energy metabolism/tyrosine metabolism
198	protein N-terminal asparagine amidohydrolase, partial	34753.53	5.74	38000.00	6.71	KFM72254.1	121	2	8	proteostasis/polyubiquitination/arginylation/degradation
200	protein N-terminal asparagine amidohydrolase, partial	34753.53	5.74	37000.00	5.42	KFM72254.1	91	2	8	proteostasis/polyubiquitination/arginylation/degradation
201	small heat shock protein 24.1	28519.86	5.61	36000	5.84	AEP02968.1	87	2	6	proteostasis/chaperone
202	triosephosphate isomerase	27218.95	7.57	37000	6.33	AEF33397.1	119	3	3	energy metabolism/sub pathway of glycolysis
204	proteasome subunit alpha type-3	29450.03	4.94	36000.00	5.23	ADQ27884.1	71	2	13	proteostasis
206	O-methyltransferase mdmC	35936.27	8.34	35000.00	5.25	EKC21343.1	46	2	8	energy metabolism/1 carbon metabolism
210	dihydropteridine reductase	24949.60	5.64	35000.00	6.61	XP_001689487.1	76	2	4	energy metabolism/tyrosine metabolism
211	proteasome subunit alpha type-6	27354.8	6.34	35000.00	6.41	NP_001132918.1	85	2	8	proteostasis
212	enoyl-CoA hydratase, mitochondrial	31232.12	8.80	35000.00	6.71	NP_001040529.1	79	2	13	energy metabolism/fatty acid beta-oxidation/lipid metabolism/Enolase
213	Protein ETHE1, mitochondrial	27755.16	7.05	35000.00	6.44	EKC2847.1	172	4	10	energy metabolism/sulfur metabolism
214	S-methyl-5'-thioadenosine phosphorylase	62706.5	6	34000	6.54	EKC30156.1	108	2	4	energy metabolism/1 carbon metabolism
215	S-methyl-5'-thioadenosine phosphorylase	30827.37	5.99	34000	6.74	NP_956848.1	73	4	4	energy metabolism/1 carbon metabolism
216	macrophage galactose N-acetyl-galactosamine specific lectin 2	38443.68	5.40	35000.00	5.20	NP_660118.1	101	2	8	energy metabolism/hexosamine pathway
217	tubulin beta chain	50817	4.74	35000	5.02	KXJ23102.1	126	3	13	cytoskeletal/vesicle transport
218	Glutathione S-transferase A	26494.34	6.24	33000.00	6.38	EKC36795.1	70	2	5	energy metabolism/oxidative stress proteins
219	Peptidylprolyl isomerase protein, partial	14673.49	5.29	33000.00	6.84	ABY27547.1	307	4	10	proteostasis/chaperone
223	triosephosphate isomerase	27218.95	7.57	33000	6.33	AEF33397.1	81	2	3	energy metabolism/sub pathway of glycolysis
224	enoyl-CoA hydratase, mitochondrial	30261.37	8.14	34000.00	6.65	KPP63749.1	104	3	4	energy metabolism/fatty acid beta-oxidation/lipid metabolism/Enolase
227	thiamine biosynthesis protein ThiJ	24863.02	8.41	32000	6.79	WP_026997607.1	109	2	9	energy metabolism/synthesis of thiamine
228	naeve appetrie-like protein 1	24461.79	4.91	32000.00	6.73	AKQ70860.1	70	2	16	cytoskeletal/shell formation/ECM
230	thioredoxin peroxidase 2	21857.91	5.95	29000	6.75	ADM88874.1	213	4	11	energy metabolism/antioxidant, transcriptional induction, inhibitor of apoptosis
232	C1q-domain-containing protein	18742.11	8.39	29000.00	6.70	CBH31055.1	124	2	11	cytoskeletal
233	Rho GDP-dissociation inhibitor 1	23556.01	5.15	29000	5.26	EKC31723.1	48	2	9	cytoskeletal/vesicle transport
235	C1q-domain-containing protein	18742.11	8.39	27000.00	6.70	CBH31055.1	159	2	11	cytoskeletal
236	thioredoxin peroxidase 2	21857.91	5.95	26000	5.88	ADM88874.1	104	3	11	energy metabolism/antioxidant
238	Ras-related protein Rab-2A, partial	23034.77	5.97	25000	6.55	KFM0634.1	130	3	2	cytoskeletal/vesicle transport between ER and golgi complex
239	cell division cycle 42, partial	19164.77	5.45	24000.00	6.78	CAQ64775.1	62	2	13	cytoskeletal/cell division
240	translationally-controlled tumor protein	19249.4	4.65	23000	4.97	AEQ76396.1	53	2	10	proteostasis/apoptosis, heat shock protein, chaperone
241	translationally-controlled tumor protein	19249.4	4.65	22000	4.71	AEQ76396.1	93	3	25	proteostasis/apoptosis, heat shock protein, chaperone
242	calyophosin-like protein	21401.60	4.99	22000.00	5.75	ADQ28065.1	172	4	15	energy metabolism/calcium binding/signaling
243	Peroxiredoxin-5, mitochondrial	16566.30	5.66	20000.00	6.58	EKC39599.1	92	2	8	energy metabolism/ROS scavenging
244	Cu/Zn super oxide dismutase	15919.85	5.84	16000.00	6.24	AFV52312.1	163	2	13	energy metabolism/oxidative stress proteins
245	profilin	15722.48	6.1	10000	6.74	NP_999780.1	111	2	10	cytoskeletal/actin binding protein
246	dihydropyridyl dehydrogenase, mitochondrial	57721.62	7.01	63000.00	6.56	KKJ22156.1	118	2	4	energy metabolism
248	isocitrate dehydrogenase (NADP dependent)	50461.48	6.77	64000.00	6.69	AFB6364.1	95	3	7	energy metabolism/Krebs cycle, NADP dependent
249	S-adenosylhomocysteine hydrolase	47498.05	5.98	56000	6.51	ACT13639.1	78	2	7	energy metabolism/antioxidant/oxidative stress/glutathione
250	citrate synthase, mitochondrial precursor	51596.43	8.12	56000.00	6.55	NP_999441.1	86	2	3	energy metabolism/TCA cycle
251	citrate synthase, mitochondrial precursor	51596.43	8.12	56000.00	6.60	NP_999441.1	123	3	5	energy metabolism/TCA cycle
253	isocitrate dehydrogenase	50464.49	6.80	54000.00	6.54	AFB6366.1	91	2	3	energy metabolism, Krebs Cycle, NADP dependent
254	isocitrate dehydrogenase	50464.49	6.80	54000.00	6.70	AFB6366.1	102	2	3	energy metabolism, Krebs Cycle, NADP dependent
255	cystathionine gamma-lyase	46901.73	6.57	52000.00	6.86	EKC29931.1	135	2	1	energy metabolism/glutathione synthesis/oxidative stress
257	cystathionine gamma-lyase	46901.73	6.57	53000.00	6.74	EKC29931.1	138	2	1	energy metabolism/glutathione synthesis/oxidative stress
258	profilin, partial	12038.02	4.99	12000	6.41	AFG4407.1	168	2	11	cytoskeletal/actin binding protein
259	ADP-ribosylation factor 2, isoform CRA_b	15819.25	6.14	18000.00	6.55	EDK34206.1	82	2	7	proteostasis/protein secretion regulation
260	Cofilin/tropomyosin-type actin-binding protein	15964.03	5.17	16000.00	6.57	XP_001581272.1	175	3	5	cytoskeletal
261	C1q-domain-containing protein	18742.11	8.39	13000.00	6.71	CBH31055.1	218	3	16	cytoskeletal
262	ERK2	41421.27	6.42	63000.00	6.47	APP57674.1	201	4	10	cytoskeletal/MAPK/ERK cascade/cell growth or cell cycle/adhesion/small G protein
263	UDP-N-acetylhexosamine pyrophosphorylase	52802.99	5.95	66000	6.46	KOC62272.1	104	2	7	energy metabolism/hexosamine pathway
264	flagellar protofilament ribbon protein rib74	73986.06	5.83	83000.00	6.72	NP_99828.1	96	3	5	cytoskeletal/ciliary movement/mucous production
265	Rab GDP dissociation inhibitor beta, partial	36618.51	5.26	57000.00	6.45	KFQ09796.1	98	2	15	cytoskeletal/vesicle transport
268	ATP synthase beta subunit, partial	40231.01	4.95	60000.00	5.08	AAT06148.1	296	4	13	energy metabolism/ETC
269	heat shock protein 78	73225.76	4.99	78000.00	5.19	AHN92526.1	119	2	11	proteostasis/chaperone
270	Endoplasmic	125136.91	4.88	88000.00	5.15	EKC38233.1	174	2	10	proteostasis/chaperone/HSP90