THE ROLE OF SIRTUIN INHIBITORS ON THE PROTEOMIC RESPONSES
OF THE MUSSELS *MYTILUS GALLOPROVINCIALIS* AND *MYTILUS*
TROSSULUS TO MENADIONE INDUCED OXIDATIVE STRESS

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TITLE: The Role of Sirtuin Inhibitors on the Proteomic Responses of the Mussels *Mytilus galloprovincialis* and *Mytilus trossulus* to menadione induced oxidative stress

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ABSTRACT

The Role of Sirtuin Inhibitors on the Proteomic Responses of the Mussels *Mytilus galloprovincialis* and *Mytilus trossulus* to Menadione Induced Oxidative Stress

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Global climate change imposes physiological constraints on marine ecosystems that can alter the distribution of intertidal organisms. In one such instance, the native cold-adapted mussel *Mytilus trossulus* is being replaced along its southern range by the invasive warm-adapted *Mytilus galloprovincialis*. These blue mussels occur throughout rocky intertidal zones where they are subjected to greatly varying environmental conditions known to induce oxidative stress. We hypothesize that while under acute stress, related *Mytilus* congeners undergo a shift in redox potential from NADH-fueled respiratory pathways to pathways producing NADPH as a way to decrease the production of reactive oxygen species (ROS) and provide reducing equivalents to detoxify ROS. Additionally, we hypothesize that sirtuins (SIRT; a family of NAD-dependent deacetylases) might be involved in the regulation of this metabolic transition. To test the latter, a discovery approach will be used to analyze the proteomic response of *M. galloprovincialis* and *M. trossulus* to the pro-oxidant menadione, and sirtuin-inhibitors nicotinamide and suramin. Menadione can induce oxidative stress by increasing endogenous peroxide and superoxide radicals, while suramin and nicotinamide both inhibit sirtuin activity. Organisms were exposed to

these compounds in filtered seawater for 8 h, followed by a 24.5 h recovery period under constant aeration. A multivariate analysis utilizing 2D-gel electrophoresis and protein identification via mass spectrometry showed that 18% and 17% of all identified protein spots detected demonstrated changes in abundance in *M. galloprovincialis* and *M. trossulus*, respectively. Using matrix-assisted laser desorption ionization (MALDI) tandem time-of-flight mass spectrometry, we were able to identify proteins with 32-41% success rate, depending on the species.

The two *Mytilus* congeners showed the greatest differences in changes of protein abundance for oxidative stress proteins (including NADP-dependent isocitrate dehydrogenase) and the common hemolymph protein heavy metal binding protein (*M. trossulus* only). Both congeners showed similar effects in response to simultaneous sirtuin inhibition and MIOS for proteins involved in protein degradation (proteasome), cytoskeletal modifications (actin and tubulin), proteins regulating actin filament growth (F-actin capping protein), amino acid metabolism and stress signaling (G-proteins, small G-proteins and MAPK). Results indicate that protein acetylation plays an important role in the oxidative stress response of *M. galloprovincialis*. More specifically this suggests that sirtuins play an important role in regulating the general stress response in *M. galloprovincialis* and thus contribute to the greater stress resistance of this species. Furthermore, the changes in the abundance of several molecular chaperones suggest a greater effect of sirtuins in regulating the cellular

response to heat stress, which could in part explain why this species is more heat-tolerant than the native *M. trossulus*.

Keywords: Proteomics, oxidative stress, sirtuins, *Mytilus*

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I. INTRODUCTION

Danish physiologist August Krogh set forth an idea, now coined “Korh’s Principle,” arguing that for any given biological question, there exists an ideal organism and study system best suited to answer that particular question (Krebs, 1975).

Considering this, congeners of the marine invertebrate *Mytilus*, which inhabit the rocky intertidal along the Pacific coast are model organisms to use for the identification of coping mechanisms induced by periods of environmental stress, as their sessile nature makes them completely dependent on ambient conditions for growth and survival (Campos et al., 2012). One stressor in particular, oxidative stress, is characterized by the increased production or insufficient degradation of endogenous reactive oxygen species (ROS) that result from extended periods of emersion and other accompanying stressors. Environmental stressors like oxidative stress are predicted to increase in the next century due to global climate change. Climate change will not only impact environmental conditions but has also been shown to alter species ranges and influence the spread of invasive species.

Therefore, in order to ascertain the impact of climactic changes on the distribution ranges of marine invasives, we have chosen to investigate molecular changes in two species of the genus *Mytilus*: *Mytilus galloprovincialis* and *Mytilus trossulus*. The genetic proximity and differences in distributional range allow us to directly compare molecular changes between the two species. These differences not only characterize individual species’ response to periods of oxidative stress, but also allow us to better examine the implications of global climate change and what governs the establishment of certain tolerance limits.

Recently proteomics has emerged as a powerful tool to describe cellular responses to oxidative stress, as it has already been successfully used to track fluctuations in protein abundance in various *Mytilus* congeners (Tomanek and Zuzow, 2010; Tomanek 2009; McDonagh and Sheehan, 2007; Campos et al., 2012). From protein synthesis, to posttranslational modifications, to expressional changes and degradation, proteomics allows for the identification of individual proteins involved in specific metabolic functions (Lovric, 2011). This methodology poses several advantages including that it makes no previous assumptions regarding the proteins involved in any given system. Instead, it provides a comprehensive and quantitative model with predictive abilities to better characterize the molecular mechanisms involved in these complex biological systems (Aggarwal, 2003; Tomanek and Zuzow, 2010). Thus, through comparative proteomics, closely related organisms, adapted to unique environments and stressors, can be compared to gather a more complete picture of their cellular stress response.

Previous studies show that while under acute stress, related *Mytilus* congeners undergo a shift in redox potential from NADH-fueled respiratory pathways to pathways producing NADPH as a way to decrease the production of reactive oxygen species (ROS) and provide reducing equivalents to detoxify ROS (Tomanek, 2012). Given that acetylation/deacetylation mediated control is an important way of influencing intracellular protein function, we hypothesize that sirtuins (a family of NAD-dependent deacetylases) might be involved in the regulation of this metabolic transition and studies have suggested that they may play an important role in stress responses of marine intertidal invertebrates (Tomanek and Zuzow, 2010).

The purpose of this study is to address the way organisms respond to periods of acute oxidative stress and whether sirtuins function in the stress response through simultaneous sirtuin inhibition at the level of the proteome. This will not only increase our understanding of the physiological tolerance limits of these marine invertebrates and their response to the imminent climate changes, but will also better articulate the specific effects of sirtuins' in the cell's ability to control and acclimate to periods of acute oxidative stress.

In this account, I will discuss each of the four main components of this project: (1) the *Mytilus* study system, (2) the impact of environmental stress on *Mytilus* (3) the role of sirtuins in regulating the metabolic response to periods of oxidative stress, and (4) the basic approach and tools utilized in proteomics. Results from this study will therefore help explain niche establishment and which physiological mechanisms govern tolerance limits allowing certain animals to grow and survive in the dynamic intertidal environment.

***Mytilus* as a Study Organism**

Natural History

Species of the mussel genus *Mytilus* are found worldwide from warm- to cold-temperate latitudes within the rocky intertidal and are a keystone species in a variety of coastal ecosystems (Braby and Somero, 2006; Fields et al., 2012; Tomanek and Zuzow, 2010; Tomanek et al., 2011). As one of the most abundant and invasive intertidal invertebrates, mussels play a fundamental role in establishing and maintaining local ecological dynamics by acting as ecological engineers (Braby and Somero, 2006). While certain species prefer protected bays, others thrive in the pounding surf and can be found attached in dense masses to wave-washed rocks from the high intertidal to the shallow sub-littoral zone (Nowak, 2013) and tidal location has been shown to influence shell thickness (Gosling, 2003).

Mytilus are suspension-feeding bivalves that filter ambient water for free-floating organic matter and plankton (Nowak 2013; Riisgard et al., 2011). Water is drawn into the branchial chamber through the incurrent siphon where bands of cilia on the interfilamentary canals of the gill facilitate water transport and subsequent separation of particles (Nowak 2013; Riisgard et al., 2011).

Cilia are of particular importance in *Mytilus* for nutrient acquisition across the gill epithelium (Moore 1971). Particles are strained out of the water by a mesh of finger-like extensions, cirri, which then transfer particles to frontal surfaces of the gill before being funneled to the mouth by the labial palps (Moore 1971; Nowak 2013). Cilia branch off each cirrus forming a feather-like network, increasing the particle retention, “stickiness,” created by both the cirri and mucus covering the gill epithelium (Moore 1971). However,

while studies show increased mucus production in response to high concentrations of suspended particles, the role of mucus in nutrient filtration across the gill epithelium is still debated (Riisgard et al., 2011). Wastewater and rejected particles are subsequently converted to feces prior to expulsion through the recurrent siphon (Nowak 2013). Therefore, the ability of gills to filter nutrients, mediate gas exchange and facilitate other metabolic processes makes them ideal tissues to use in this energetic study.

Assessment of Global Distribution and Demographic History

Globally, blue mussels exhibit species-selective distribution patterns, as a result of allopatric speciation upon the opening of the Bering Strait (Rawson and Harper, 2009). The opening of the Bering Strait was an important oceanographic event that resulted in the divergence of several populations of the same species, through which isolation has interfered with genetic interchange thereby resulting in three distinct blue mussel species, *M. trossulus*, *M. edulis* and *M. galloprovincialis* (Rawson and Harper, 2009).

The divergence of *M. trossulus* and *M. edulis* occurred when mussels, previously restricted to the Pacific, colonized the northern Atlantic following the opening of the Bering Strait (Lallias et al., 2009). Subsequent evolution of *M. galloprovincialis* resulted after a subpopulation of North Atlantic *M. edulis* became isolated in the Mediterranean Sea (Lallias et al., 2009). Thus, populations of two closely related sister taxa, *M. edulis* and *M. galloprovincialis*, and a more distantly related taxon, *M. trossulus* are now distributed throughout different regions of the globe (Rawson et al., 1999; Fig. 1).

Figure 1: Global distribution pattern of blue mussels, *M. galloprovincialis*, *M. trossulus*, and *M. edulis* (see Hillbish et al., 2000 for a detailed account of sampling sites).

In general, *M. trossulus* colonizes regions of the northwest Atlantic and northeast/northwest Pacific; *M. edulis* is found in the northeast Atlantic; and *M. galloprovincialis* resides in Mediterranean climates in southern Europe (Rawson and Harper, 2008; Craft et al., 2010; Nowak, 2013). In addition, as of recently, *M. galloprovincialis* also inhabits regions along the coasts of China, Korea, southeast Australia, Hawaii, Mexico, South Africa, and the west and east coast of Canada (Rawson and Harper, 2008; Craft et al., 2010; Nowak, 2013). Natural and anthropogenic migrations gave rise to populations now found at subtropical latitudes, as during this past century, the warm-adapted Mediterranean *M. galloprovincialis* successfully invaded and displaced the native cold-adapted congener, *M. trossulus* along the coast of California as far north as San Francisco Bay (Lallias et al., 2009; Tomanek and Zuzow, 2010; Braby and Somero, 2006; Fig. 2). The northward expansion of *M. galloprovincialis*' range is

causing a retraction in the biogeographic distribution range of *M. trossulus* to northern California and Oregon (Schneider, 2008). Conversely, its ability to outcompete and threaten the genetic integrity of native mussel species in certain regions designates *M. galloprovincialis* as an ecologically invasive species and a source of “genetic pollution” in certain localities (Nowak, 2013).

Figure 2: Biogeographic range distribution of *Mytilus trossulus* and *Mytilus galloprovincialis* and hybrid species along the Pacific coast of North America (Lockwood and Somero, 2011).

Genetic Diversity

Genetic differentiation between *Mytilus* congeners governs global distribution patterns and helps characterize why different populations are better adapted to their native environment (Braby and Somero, 2006). Genetic analyses using allozyme characters, electrophoretic assessment of proteins, and mtDNA markers confirm variation in allele frequency, reaffirming that despite morphological similarities, blue mussels are

not a single cosmopolitan species, but rather three genetically distinct species (McDonald et al., 1991; Suchanek et al., 1997).

Assessment of the genetic structure within and between populations of *M. trossulus* and *M. galloprovincialis* along the Pacific coast of North America indicates that when looking at the genome as a whole, as opposed to individual genes under high selection pressure, populations of *Mytilus* congeners appear genetically homogenous, even when found in hybrid regions (Sarver and Foltz, 1993). Despite that the high dispersion potential of planktonic larvae increases the potential for hybridization between species, *Mytilus* congeners maintain genetic cohesiveness within their biogeographic ranges (Koehn et al., 1976; Sarver and Foltz, 1993). Therefore, given the lack of genetic homogenization across populations and lack of hybrid dispersal to regions beyond where two congeners' distribution ranges overlap, it may be that there is strong selection acting against heterozygotes.

Instability of hybrid regions can be explained, in part, by outcrossing depression. Organisms are not experiencing panmixia, but rather there is evidence of assortative mating (Bierne et al., 2007). Assortative mating through gamete recognition and fertilization preference could explain selection patterns (Bierne et al., 2007). Perhaps gametes from separate species aren't recognizing each other due to cell membrane incompatibility between eggs and sperm of different species. While experimental evidence of this is lacking, mechanistically this could lead to a better understanding of what drives the speciation of blue mussels (Bierne et al., 2007).

However, in the case that gametes from separate species do recognize each other and fertilize, decreased hybrid viability during larval stages is indicative of

developmental genetic incompatibility, thus explaining lower hybrid fitness (Bierne et al., 2007). Lastly, influence of environmental factors such as salinity, vertical occurrence within the intertidal, and temperature could also play roles in the spatial distribution of alleles seen in hybrids (Bierne et al., 2007).

***Mytilus*' Response to Acute Environmental Stress**

While biotic interactions play an important role in successful invasions, the physiological processes that set tolerance limits to abiotic factors have become a key component in defining and predicting the invasiveness of a species (Somero, 2011; Lockwood and Somero, 2011). Therefore, due to its influence on these periods of environmental stress and inherent biological processes, the ubiquitous effects of climate change will exacerbate the impact of invasive species on native species' biodiversity and distribution (Mieszkowska et al., 2006; Jones et al., 2010; Doney et al., 2012).

The invasion of non-native species has the potential to alter the local community dynamics of an established marine ecosystem (Schneider, 2008). Literally hundreds of marine species invaded the Pacific coast over the last few decades and some of these species have become keystone species in their novel environment, e.g. the sea urchin *Centrostephanus rodgersii*, the sea star *Pisaster ochraceus*, and the mussel *Mytilus* (Sorte et al., 2010; Harley et al., 2006). However, the particular mechanisms and attributes that successful invasive organisms use to infiltrate a new region are largely unknown but could help explain the higher fitness observed compared to native species occupying a similar niche (Schneider, 2008). Thus, the physiological response of both native and invasive species to abiotic stress provides insight into the cellular processes that are

activated to resist and rebound from perturbations by the environment and assist in invasion success.

Differences in the abundances of *M. trossulus* and *M. galloprovincialis* in bays and estuaries along the California coast can be explained by the dynamic fluctuations in various stressors including: temperature, pH, oxygen availability, salinity, and CO₂ (Braby and Somero, 2006; Tomanek, 2011; Ivanina and Sokolova, 2013). These abiotic factors, in combination or individually, often force species to reallocate energy into coping mechanisms for defense and repair and can lead to the up-regulation of specific proteins with a direct impact on growth, reproduction and mortality (Petes et al., 2008; Finke et al., 2007).

For example, valve closure in bivalve mollusks aids in combating transient adverse environmental changes by minimizing desiccation during periods of emersion at low tide, depressing heart and respiratory rates, and protecting tissues from extreme changes in internal osmotic concentrations (Akberali and Trueman, 1985; Shumway, 1977). However, while sealing off the harsh environment temporarily alleviates the repercussions of sub-optimal conditions, valve closure doesn't confer long-term survival as the animal not only sacrifices feeding and mating opportunities, but also accumulates metabolic build-up (Akberali and Trueman, 1985). Therefore, in line with the notion that anticipated climate change will shift the local ecological balance and community dynamics of mussel populations, changes in both biotic and abiotic factors will have far reaching effects on many currently thriving ecosystems. This will be reflected through the persistence of organisms with the ability to withstand these permanent or recurrent deviations.

Thermal Stress Response

During periods of environmental stress (e.g. temperature stress), intertidal organisms exhibit a heat shock response, in which several isoforms of heat shock proteins (HSPs) are synthesized to facilitate proper protein folding after stress-induced denaturation (Freire et al., 2012; Tomanek, 2010; Buckley et al., 2001; Feder and Hofmann, 1999). Expression of HSPs corresponds to the vertical position of a given organism in the intertidal, with elevated expression levels characteristic of organisms inhabiting more exposed regions (Freire et al., 2012).

Intertidal organisms, such as mussels, induce this response frequently in response to dynamic intertidal temperature fluctuations, which occur as a result of tidal emersion (Tomanek, 2010; Buckley et al., 2001; Hofmann and Somero, 1996). However, mussels can only synthesize heat shock proteins at temperatures within a few degrees of their typical habitat and body temperatures, thus setting northern and southern distributional boundaries, e.g. a species-specific thermo-tolerance range (Tomanek, 2010; Feder and Hofmann, 1999).

Previous studies on heart rates, transcriptomics and proteomics all indicate that *M. galloprovincialis* is more heat-tolerant than *M. trossulus* (Braby and Somero, 2006; Lockwood and Somero, 2011; Schneider, 2008; Tomanek and Zuzow, 2010). Species-specific temperature dependent changes in HSP expression characterize thermo-tolerance differences between *M. galloprovincialis* and *M. trossulus*, as the presence of more stable HSP isoforms, increased expression of sHSP24, and higher onset temperatures of HSP synthesis in *M. galloprovincialis* could explain its success in warmer localities and hybrid

regions (Lockwood et al., 2010; Tomanek, 2010; Hofmann and Somero, 1996; Schneider 2008).

Beyond their thermo-tolerance limits, mussels are not able to effectively respond to variable temperatures, and as a consequence, may be driven to local extinction (Tomanek, 2010; Feder and Hofmann, 1999; Hofmann and Somero, 1996). Thus, given the anticipated trends in global climate change, *M. trossulus* will not likely recolonize the warmer regions of southern California without continuous reintroduction by ships, as the shifting currents are unlikely to disperse larvae enough to reestablish populations of *M. trossulus* in these regions (Suchanek et al., 1997). Therefore, further contraction of *M. trossulus*' species range is anticipated in response to current global warming trends.

Osmotic Stress Response

Marine organisms in the intertidal region also encounter daily salinity fluctuations due to rain, freshwater run-off and tidal rhythms, especially in regions where freshwater and seawater mix, e.g. estuaries, tidepools, and river mouths (Shumway, 1977). Moreover, these salinity fluctuations are predicted to become more severe with climate change due to the increased frequency of heavy precipitation events, thereby subjecting the native biota to considerable osmotic stress (Rhein and Rintoul, 2013). Sessile organisms, like marine bivalves, are unable to escape these dramatic changes in osmotic conditions and are thus among the most affected by periods of hyposaline stress.

In response, mussels have developed various behavioral patterns and physiological responses to salinity fluctuations to protect their internal chemistry and minimize energy consumption (Shumway, 1977). For example, mussels often control the

degree of openness of shell valves, e.g. gaping behavior, to facilitate aerobic metabolism and provide access to food and has been shown to occur in response to periods of decreased salinity (Lockwood and Somero, 2011). Not only has gaping behavior been observed during hypo-osmotic conditions, but also during oxygen limitation (Dowd and Somero, 2013) and following exposure to thermal extremes which can cause oxidative damage (Dowd and Somero, 2013).

Additionally, in order to maintain a state where the total concentrations of solutes in their internal fluids match that of the environment, mussels constantly regulate fluxes in mantle and hemolymph osmotic and ionic concentrations (e.g. Na^+ , Mg^{2+} , and Ca^{2+}) as perturbations in cellular ion composition would otherwise result from periods of acute salinity changes (Shumway 1977; Koehn and Siebenaller, 1980). Maintaining osmolarity is crucial for the proliferation and survival of mussels, as deviation from osmotic homeostasis has the potential to compromise the activity of endogenous enzymes that function in regulating natural physiological processes. While *M. galloprovincialis* is the more heat tolerant of the two congeners, the native *M. trossulus* is a euryhaline, cold-adapted mussel more acclimatized to regions of low temperatures and fluctuating salinity levels (Sarver and Foltz, 1993). Higher instances of valve closure in response to decreased salinity may contribute to the increased salinity tolerance in *M. trossulus* (Lockwood and Somero, 2011).

Oxidative Stress Response

The production of ROS occurs naturally in all cells as a byproduct of cellular energy metabolism and, within certain boundaries, are essential in maintaining certain

biological functions – acting as messengers during redox signaling in preparation for response to stressors, activating various transcription factors to facilitate cross-talk between the nucleus and the mitochondria, promoting cellular proliferation and growth, and as a form of immune defense against invasive pathogens (Halliwell, 1992; Thannickal, 2000; Finkel and Holbrook, 2000; Jo et al., 2001). Yet, regardless of where or how ROS production occurs, increases in intracellular ROS levels impact cellular processes in two different ways – either by activating certain signaling pathways that activate an anti-oxidant defense and/or by damaging various intracellular biomolecules (Finkel and Holdbrook, 2000).

Previous work in *Mytilus* and *Crassostrea virginica* (the eastern oyster) demonstrates that in addition to temperature stress and acute salinity changes, pH and hypoxia are also responsible for elevated levels of endogenous and exogenous reactive oxygen species (ROS) (Sokolova et al., 2012; Tomanek, 2011). Research shows that mollusks have a limited capacity to regulate dynamic changes in pH, and as a result periods of elevated P_{CO_2} induce oxidative stress in these bivalves (Tomanek et al., 2011). Increased abundance of proteins involved in energy metabolism also occurs as a result of heat-induced oxidative stress, with a specific up-regulation of those involved in the detoxification of ROS (e.g. thioredoxin, aldehyde dehydrogenase, and superoxide dismutase) or decreased ROS production through redox regulation (Sokolova et al., 2012; Tomanek, 2011). As a result, we find that several naturally occurring environmental stressors are responsible for the marked increase in oxidative stress experienced by marine invertebrates due to global climate change (Sokolova et al., 2012; Tomanek, 2011).

Periods of oxidative stress are often characterized by an imbalance in the endogenous homeostatic equilibrium (the normal redox state of tissues) between cellular pro-oxidants (ROS) and anti-oxidants (Bellomo et al., 1990). More specifically, this occurs when the production of pro-oxidants exceeds the organism's ability to scavenge reactive chemical radicals (Bellomo et al., 1990).

While biochemical processes in several cellular compartments, including the peroxisome and the endoplasmic reticulum, generate ROS, the majority of intracellular ROS are generated following the leakage of electrons to molecular oxygen (O_2) along the mitochondrial electron transport chain (ETC) at complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome *c* reductase) (Liu et al., 2002; Finkel and Holbrook, 2000). The movement of electrons along the ETC to oxygen, the final electron acceptor, generates over 90% of the ATP produced in cells (Finkel and Holbrook, 2000; Gunter et al., 2012). ROS accumulation, and the flux of electrons through the ETC, increases in response to the heightened demand for ATP during periods of cellular stress (Kultz, 2005; Tomanek and Zuzow, 2010; Solovaka et al., 2012). Thus, the generation of ROS might be a basic mechanism through which environmental stress diverts energy from maintenance to the defense of cellular homeostasis.

Electron leakage along the ETC, or even from steps in the TCA cycle, generates superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) (Finkel and Holbrook, 2000). The transfer of one electron to oxygen generates superoxide, a highly reactive radical, while the transfer of an additional electron to superoxide will generate hydrogen peroxide (Finkel and Holbrook, 2000; Hermes-Lima et al., 1998). Formation of superoxide radicals occurs in either the mitochondrial matrix

or the inner membrane space, from which they can diffuse into the cytosol through voltage-gated channels (Gunter et al., 2012). However, given the charge on superoxide radicals, this process is difficult, and thus mitochondrial (Mn-SOD) and cytosolic (Cu/Zn-SOD) forms of superoxide dismutase are typically responsible for catalyzing the conversion of superoxide to hydrogen peroxide – a ROS species capable of diffusing through cellular membranes triggering extensive oxidative damage (Gunter et al., 2012). Hydrogen peroxide, although not a radical itself, can accept electrons from transition metals (e.g. iron) and other donors to become a hydroxyl radical, which is the most reactive oxygen radical (Finkel and Holbrook, 2000; Hermes-Lima et al., 1998).

ROS generated as by-products of oxygen metabolism, are capable of chemically altering the structure and function of nearly all cellular constituents (e.g. DNA, proteins, lipids) and their damaging effects have been implicated as a causative factor in several human diseases (Bellomo et al., 1990; Jo et al., 2001). Thus, given that oxygen utilization generates toxic side products, organisms have developed several enzymatic and non-enzymatic antioxidant defenses to readily detoxify ROS (Gunter et al., 2012).

To reduce the risk of hydroxyl radical formation, catalase (CAT) catalyzes the conversion of hydrogen peroxide to water and molecular oxygen; while, glutathione peroxidase (GPx) is involved in the catalysis of hydrogen peroxide to water and oxidized glutathione (Gunter et al., 2012; Finkel and Holbrook, 2000). CAT activity primarily occurs in peroxisomes, while GPx activity is found in both the cytosol and mitochondria (Ben-Yoseph et al., 1996). Thus, given the mitochondrion's susceptibility to oxidative damage and that CAT is absent in the mitochondria of most eukaryotic cells, GPx plays an important role in ROS scavenging (Jo et al., 2001). Additionally, cells contain small

water-soluble antioxidants such as ascorbate (vitamin C), β -carotene and α -tocopherol (vitamin E) to detoxify ROS (McCall and Frei, 1999).

NADPH as Part of the Oxidative Stress Response

As previously mentioned, cells mitigate damage induced by ROS through the production of anti-oxidants and the activation of enzymes, like superoxide dismutase, that directly target ROS (Thannickal, 2000). The action of these anti-oxidative enzymes is facilitated by a powerful reducing agent NADPH, the availability of which determines the effectiveness of ROS scavengers and reduced environment necessary for cellular function and suppression of ROS accumulation (Singh et al., 2008).

Thus, during periods of oxidative stress, intracellular homeostasis is maintained by the tightly linked regulation of NADH and NADPH regulated pathways (Singh et al., 2007). While the former is involved in the production of ATP via oxidative phosphorylation, NADPH is mainly produced during the oxidative pentose phosphate pathway (PPP), which is responsible for alleviating the oxidative environment established by NADH-producing pathways (Singh et al., 2007).

In addition to non-enzymatic/enzymatic anti-oxidant activity, intracellular removal of H_2O_2 is achieved by the coordinated action of several metabolic pathways, including the PPP and glycolysis, which are directly coupled to ROS detoxification by GPx (Ben-Yoseph et al., 1996). Periods of oxidative stress lead to the stimulation of the PPP, which subsequently oxidizes a large proportion of glucose normally catabolized through glycolytic pathways (Ben-Yoseph et al., 1996). During this process, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase generate reducing

equivalents in the form of NADPH, which is then used by glutathione reductase to regenerate reduced glutathione (GSH) (Ben-Yoseph et al., 1996). Therefore, GPx is coupled to the PPP via glutathione reductase, as GPx activity requires GSH for detoxification of H_2O_2 (Ben-Yoseph et al., 1996).

The oxidation state of glutathione affects its transport across the mitochondrial membrane for subsequent use by GPx for radical detoxification (Jo et al., 2001). Once oxidized in the matrix by GPx, glutathione can no longer be transported to the cytosol for reconversion back to GSH (Jo et al., 2001). This highlights the importance of NADPH as a necessary reducing equivalent to facilitate glutathione reductase activity (Jo et al., 2001).

However, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are not the only enzymes capable of alleviating oxidative conditions through the production of NADPH. Alternatively, malic enzyme and NADP-dependent isocitrate dehydrogenase (NADP-IDH) also generate NADPH in eukaryotes, and are therefore capable of mediating the reduction of GSSG by glutathione reductase (Andres et al., 1979). While the mechanism for maintaining the mitochondrial NADPH pool remains to be elucidated, given the cytosolic localization of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malic enzyme, strong evidence suggests that NADP-IDH, a key enzyme in the tricarboxylic acid cycle, is the major producer of mitochondrial NADPH (Jo et al., 2001).

Protein Acetylation and Global Metabolic Changes

The production of enzymes that scavenge ROS and the down-regulation of ROS producing metabolic pathways require global regulation (Seo et al., 2004). Possible mechanisms of regulation include changes in protein abundance (at the transcriptional level) or a change in enzyme activity through posttranslational modifications (PTMs), covalent modifications on the side chains or backbones of nascent proteins (Seo et al., 2004; Walsh et al., 2005). However, increasing abundance of proteins is energetically expensive for cells, and therefore may not be an optimal means for regulating activity during periods of acute stress (Lodish et al., 2007). In addition, given that a large number of proteins denature or unfold during these periods of acute stress, expending energy to synthesize new proteins is potentially wasteful (Lodish et al., 2007). Regulation of protein activity through PTMs not only allows for a faster cellular response to acute changes in the environment, but also increases the functional diversity of the proteome (Seo et al., 2004).

PTMs such as phosphorylation, methylation and acetylation are crucial for rapid regulation of protein function with respect to changes in enzymatic activity, cellular localization, and protein-protein interactions (Seo et al., 2004). Out of over 200 identified post-translational modifications, acetylation is the most common type – with acetylated proteins found in the nucleus, cytoplasm, and mitochondria (Polevoda and Sherman, 2002; Finley and Haigis, 2012). Some proteins require acetylation for function, while others that are acetylated do not depend on this modification for activity (Polevoda and Sherman, 2002). While acetylation plays a role in transcriptional regulation through the modification of chromatin-associated proteins, the biological significance of this

modification varies as it can also affect proteins with a broad range of functions in cellular processes and metabolic transitions including transcription factors, nuclear import factors/DNA binding proteins, and cytoskeletal proteins, e.g. α -tubulin (Zhao et al., 2010; Seo et al., 2004; Polevoda and Sherman, 2001).

Protein acetylation can occur on a variety of amino acid residues, the most common being lysine (Bao and Sack 2010). Moreover, lysine acetylation is both ubiquitous and conserved in many essential enzymes involved in glycolysis and the TCA cycle and is shown to play a role in the coordination of these different metabolic pathways during periods of cellular stress (Zhao et al., 2010). For example, acetyl-CoA serves as both the acetyl donor for acetyltransferases, and is the primary entry point for the catabolism of glycolytic products in the TCA cycle (Finley and Haigis, 2012). High levels of glucose lead to the increased abundance of intracellular acetyl-CoA, and thus provide more substrate for protein acetylation (Finley and Haigis, 2012). Therefore, nutrient availability and energetic status is essential to acetyl-CoA protein acetylation of metabolic enzymes (Finley and Haigis, 2012). Similarly, deacetylation of proteins is associated with periods of limited nutrient availability (Bao and Sack, 2010).

Role of Sirtuins in Cellular Processes and Stress Response

The seven mammalian Sir2 orthologs (SIRT1-7), class III lysine deacetylases (KDAC), use NAD^+ as a co-factor to facilitate reversible acetylation of lysine residues (Canto and Auwerx, 2011; Polevoda and Sherman, 2002; Houtkooper et al., 2012). The deacetylation reaction mediated by sirtuins produces nicotinamide through the removal of an acetyl group while also generating 2' and 3'-O-acetyl-ADP-ribose (OAADPR) as a

by-product (Lawson et al., 2010). While OAADPR is hypothesized to function as a signaling molecule, nicotinamide, functions as an inhibitor of sirtuin activity through a negative feedback mechanism (Lawson et al., 2010).

Sirtuins received significant attention following their discovery and implicated role in lifespan-extension during periods of acute stress in budding yeast (Blander and Guarente, 2004). Further research shed light on Sir2's NAD^+ -dependent histone deacetylase activity and functionality in transcriptional regulation (Houtkooper et al., 2012; Blander and Guarente, 2004). Characterization of the highly conserved Sir2 domain also revealed its presence in nearly all organisms, from bacteria to higher eukaryotes (Blander and Guarente, 2004).

Mammalian sirtuins are categorized into four different classes based on results from sequence-based phylogenetic analysis: SIRT1-SIRT3 belong to class I, SIRT4 to class II, SIRT5 to class III and SIRT6 and SIRT7 to class IV (Houtkooper et al., 2012). While all of them are universally expressed and share a conserved catalytic core, the various SIRT classes vary in the specific enzymatic activity (Canto and Auwerx, 2011; Houtkooper et al., 2012). Sirtuins primary mode of action is through lysine deacetylation, however recent studies show that sirtuins also catalyze ADP-ribosylation, desuccinylation, and demalonylation (Houtkooper et al., 2012; Finley and Haigis 2012; Table 1). Moreover, SIRT4 has no detectable NAD -dependent deacetylase activity, and functions primarily as an ADP-ribosyltransferase (Huang et al., 2010).

Table 1. Sirtuin localization and function (modified from Houtkooper et al., 2012)

Sirtuin	Class	Localization	Activity
SIRT1	I	Nucleus, cytosol	Deacetylation
SIRT2	I	Cytosol	Deacetylation
SIRT3	I	Mitochondria	Deacetylation
SIRT4	II	Mitochondria	ADP-ribosylation
SIRT5	III	Mitochondria	Deacetylation, demalonylation, desuccinylation
SIRT6	IV	Nucleus	Deacetylation, ADP-ribosylation
SIRT7	IV	Nucleolus	Unknown

These nutrient and redox-stress responsive enzymes are involved in several biological processes including, but not limited to, cell cycle progression, cellular stress, genomic stability, apoptosis, senescence, life cycles, and transcriptional repression – all of which play a critical role in maintaining cell integrity, providing a link between energy homeostasis and cellular fate (Lawson et al., 2010; Houtkooper et al., 2012; Bao and Sack, 2010). Sirtuins regulate these biological processes both at the transcriptional level, and through direct modification of various metabolic enzymes (Schwer and Verdin, 2008).

Thus, the involvement of sirtuins in key cellular processes and their linkage to the pathogenesis of numerous severe diseases makes them great potential drug targets (Lawson et al., 2010).

SIRT1 – Nuclear Sirtuins: Protectors Against Oxidative Stress and DNA Damage

SIRT1, the most studied of all sirtuin isoforms, is localized to the nucleus and has implicated activity in cell proliferation, stress responses, apoptosis and cancer (Canto and Auwerx, 2011). It possesses a functional role in (1) transcriptional regulation – through the deacetylation of histones and transcription factors, and (2) metabolic regulation – as it

governs the synchronization of energy availability with metabolic activity (Huang et al., 2010; Canto and Auwerx, 2011).

For example, p53, a sequence-specific transcription factor, regulates several intracellular functions through the activation or inhibition of various gene targets (Ryan et al., 2001). Several lines of study prove p53 specificity depends on several PTMs, which target its activity towards mitochondrial biogenesis, DNA base excision repair, or apoptosis (Zhao et al., 2003). SIRT1 deacetylation of p53 is shown to suppress p53-induced apoptosis and delay the onset of senescence (Giannakou and Partridge, 2004).

Similarly, the effects of SIRT1 on the Forkhead-O-box (FOXO) family of transcription factors are comparable to that of p53 (Giannakou and Partridge, 2004; Houtkooper et al., 2012). SIRT1 is shown to interact with and deacetylate FOXO proteins, which induce the expression of several genes involved in the oxidative stress response, DNA repair, cell cycle arrest, metabolism apoptosis, and longevity (Canto and Auwerx, 2011; Giannakou and Partridge, 2004). SIRT1 deacetylation of FOXO3 leads to the induction of oxidative stress resistance genes and the enhancement of autophagy, suggesting that perhaps SIRT1 activity is prompted by periods of environmental stress (Canto and Auwerx, 2011; Houtkooper et al., 2012). A suspected mechanistic link between SIRT1 and FOXO activities can thus be inferred based on their complementary activation (Canto and Auwerx, 2011). Given that FOXO and p53 are shown to interact with one another during periods of oxidative stress, it is hypothesized that perhaps the synchronization of their activity mediates the functional role of SIRT1 in extended lifespan (Giannakou and Partridge, 2004).

SIRT1 deacetylation also regulates the activity of nuclear peroxisome proliferator-activated receptor (PPAR)- γ coactivator 1 α (PGC-1 α), to induce mitochondrial gene expression and simultaneously increase glucose metabolism (Rodgers et al., 2005; Zschoernig and Mahlknecht, 2008). Studies reveal constitutive expression of PGC-1 α upon the mutation of its lysine residues, similar to expression levels when constantly deacetylated; however, only during periods of physiological stress is expression of PGC-1 α found to be governed by SIRT1 activation (Rodgers et al., 2005). This is due to the imbalance of AMP/ATP ratios, and subsequent activation of AMP-activated protein kinase (AMPK) (Canto and Auwerx, 2011). Preemptive phosphorylation of PGC-1 α by AMPK is necessary for the successive deacetylation by SIRT1 (Canto and Auwerx, 2011). Phosphorylation of FOXO proteins by AMPK also occurs during periods of energy stress; however, whether or not this governs SIRT1 deacetylation of FOXO proteins still requires further elucidation (Canto and Auwerx, 2011). Nevertheless, the affect of these PTMs suggests that the activation of transcription factors (e.g. PGC-1 α and FOXO) might be a tightly co-regulated process mediated by sirtuins (Canto and Auwerx, 2011).

SIRT2 – Cytosolic Sirtuins: Regulators of Cell Cycle Progression and Cell Proliferation

SIRT2 is the only sirtuin primarily localized to the cytoplasm, however it can transiently shuttle into the nucleus in a cell cycle dependent manner, playing a key role in tubulin deacetylation and co-localization with microtubules (Houtkooper et al., 2012; Bao and Sack, 2010). SIRT2 is of particular importance during cell cycle checkpoint transitions and is considered a mitotic exit regulator, as it mediates chromosome

condensation and haploid cell formation (Bao and Sack, 2010; Harting and Knoll, 2010). This is achieved through both deacetylation of tubulin isoforms and histone 4 (H4K16) during the G2/M transition of mitosis (Bao and Sack, 2010). While the exact role of SIRT2 in cell cycle regulation still requires further elucidation, it is hypothesized that SIRT2 function in cell cycle regulation is stimulated by stress-induced signals (Harting and Knoll, 2010).

In addition to tubulin, SIRT2 deacetylates forkhead transcription factors, FOXO1 and FOXO3, both of which play a role in DNA repair, cell cycle progression, apoptosis, and metabolism (Bao and Sack, 2010; Harting and Knoll, 2010). The deacetylation of FOXO3a, and increased transcription of genes involved in the production of antioxidants suggests a protective function for SIRT2 against oxidative stress (Taylor et al., 2008). Moreover, its interaction with 14-3-3 β/γ is implicated in various processes regulated by the p53 family of transcription factors (Bao and Sack, 2010). Thus, SIRT2 functions not only as a deacetylase, but also as a binding partner to facilitate various cellular activities (Bao and Sack, 2010).

SIRT3, SIRT4, and SIRT5 – Mitochondrial Sirtuins: Key Regulators of Metabolism

In line with the previously established hypothesis that sirtuins serve as regulators of energy homeostasis, SIRT3, 4, and 5 are directly localized to the mitochondria, the “powerhouse” of the cell (Li and Kazgan, 2011; Gunter et al., 2012). These double membrane bound organelles consume 85-95% of the oxygen used by cells, producing the ATP necessary to carry out metabolic functions in a process known as cellular respiration (Li and Kazgan, 2011). Additionally, over 90% of intracellular ROS are generated as a

result of cellular respiration, oxidative phosphorylation, and numerous enzymatic complexes (Li and Kazagan, 2011).

Recent findings reveal that that nearly 20% of mitochondrial proteins are singularly or multiply acetylated, and therefore subject to regulation by sirtuins – making sirtuins essential for normal mitochondrial function (Finley and Haigis, 2012; Bao and Sack, 2010; Huang et al., 2010). Several studies using knockout mice reveal a dramatic increase in protein acetylation in mice lacking SIRT3, but not in those lacking SIRT4 or SIRT5, suggesting that deacetylase activity of SIRT3 is more widespread than SIRT4 or SIRT5 activity (Huang et al., 2010; Ahn et al., 2008). This, and the unique target enzymes for mitochondrial sirtuins, supports the hypothesis that PTMs, specifically deacetylation, regulate enzyme activity thereby energy metabolism and the production and scavenging of ROS (Huang et al., 2010; Ahn et al., 2008).

SIRT3, the most studied of the mitochondrial sirtuins, plays a key role in maintaining/elevating basal ATP levels by directly altering acetylation levels of complex I and complex II of the ETC (Bao and Sack, 2010; Ahn et al., 2008; Huang et al., 2010). SIRT3 deacetylation also impacts the function of various proteins involved in fatty acid oxidation, ketogenesis, oxidative phosphorylation, antioxidant defense, and amino acid metabolism (Li and Kazgan 2011). Several studies show SIRT3 deacetylates and activates acetyl-CoA synthetase 2 (AceCS2), an ATP-dependent enzyme responsible for catalyzing the conversion of free acetate to acetyl-CoA, which can then be sequestered in the TCA cycle for ATP production (Bao and Sack, 2010; Huang et al., 2010; Ahn et al., 2008). SIRT3 also targets isocitrate dehydrogenase 2 (ICDH2), a key regulator of the TCA cycle and the oxidative stress response, glutamate dehydrogenase (GDH), long-

chain acyl CoA dehydrogenase (LCAD), and 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) synthase 2 (Bao and Sack, 2010; Li and Kazgan, 2011). SIRT3 protects the cells against ROS through the activation of superoxide dismutase 2 (SOD2), a known mitochondrial antioxidant enzyme (Houtkooper et al., 2012).

Unlike SIRT3, SIRT5 is a NAD-dependent protein lysine deacetylase, demalonylase and desuccinylase (De et al., 2001; Schlicker et al., 2008). While these sirtuins have a conserved NAD⁺-binding domain, catalytic domain, and deacetylase activity, SIRT3 and SIRT5 appear to have specific targets and functions in the mitochondria (Li and Kazgan, 2011; Schlicker et al., 2008). Instead, SIRT5 deacetylase activity targets cytochrome *c*, a protein with functional roles in oxidative metabolism and apoptosis (Schlicker et al., 2008). Conversely, studies show that SIRT5 that the regulatory power of SIRT5 may lie elsewhere as its deacetylase activity is much weaker than its desuccinylase and demalonylase activity (Du et al., 2011). Desuccinylation of carbamoyl phosphate synthase 1 (CPS1), a protein involved in urea metabolism, by SIRT5 has been demonstrated *in vivo* (Du et al., 2011).

SIRT6 and SIRT7 – Nuclear Sirtuins: Metabolism and Longevity

While SIRT6 was initially described as an ADP-ribosyltransferase enzyme, recent studies show it possesses deacetylase activity, the function of which promotes proper chromatin activity in several physiologic contexts, with functional roles in the maintenance of genomic stability, repair, and longevity (Bao and Sack, 2010; Houtkooper et al., 2012). This is accomplished through the deacetylation of histone H3 lysine 9 (H3K9) and lysine 56 (H3K56), which is shown to preserve telomere integrity (Bao and

Sack, 2010). Studies also show that decreased SIRT6 deacetylase activity results in an increased susceptibility to DNA damage, and therefore, decreased genomic stability (Bao and Sack, 2010).

SIRT6's role is still unclear but recent studies suggest that it plays a role in healthspan and energy metabolism (Houtkooper et al., 2012). For example, SIRT6 transcriptional regulation is implicated in cellular resistance to hypoxia, oxidative damage and control of glucose metabolism through co-repression of HIF- α (Bao and Sack, 2010; Houtkooper et al., 2012).

Finally, SIRT7 is localized in the nucleolus and is found to activate transcription of rDNA and subsequent mitotic exit through its association with RNA polymerase I and rDNA transcription factor UBF (Bao and Sack, 2010; Houtkooper et al., 2012). While SIRT7 remains the least studied of the seven isoforms, its absence in knockout experiments is reported to have profound effects on longevity and genomic integrity (Bao and Sack, 2010; Taylor et al., 2008). Depletion of SIRT7 inhibits cell proliferation and causes cell cycle arrest, triggering apoptosis (Bao and Sack, 2010). In addition, hyperacetylation of p53 following SIRT7 depletion suggests that SIRT7 may in fact possess NAD⁺-dependent deacetylase activity (Taylor et al., 2008). This activity might be co-regulated by SIRT1 (Taylor et al., 2008; Houtkooper et al., 2012).

Proteomics

Overview

The goal of environmental proteomics is to analyze the proteome of organisms in response to periods of acute environmental stress, through both specific changes and

shifts in global protein expression patterns (Campos et al., 2012). The proteome, the protein complement of the genome, encompasses all proteins expressed by the genetic material of an organism; and at any given moment in time, it provides merely a snapshot of an ever-changing process under constant modification in response to internal and external stimuli (Rabilloud, 2000; Walsh et al., 2005; Abbott, 1999). Moreover, the proteome is influenced by a whole suite of external and internal factors, including both the subcellular location of proteins and their affiliation with highly dynamic protein-protein complexes (Lovric, 2011; Fig. 3). Thus, protein expression patterns serve as the molecular phenotype for a given genotype (Lovric, 2011).

Figure 3: Internal and external influences on the proteome – which is in constant flux (modified from Lovric, 2011).

Genomic studies are powerful for assessing the evolutionary relationship among different species, however they are limited in that there is high potential for molecular variation to occur between a gene and its corresponding protein, e.g. mRNA splicing, promotion, and expression (Lovric, 2011; Rabilloud, 2000). Thus, the limited information gleaned following the release of complete organismal DNA sequences is not due to technological limitations surrounding the synthesis of said contiguous sequences, but

rather results from the lack of knowledge obtained regarding the extent or nature of the functional competence of the expressed gene products (Rabilloud, 2000). Some studies use the post-genomic approach, transcriptomics, which identifies cellular transcript levels of mRNA for all or most of the genome (Abbott, 1999). However, there are several limitations to this methodology as well, the most prominent being that, unlike proteins, which are ultimately responsible for all cellular functions, mRNA abundance is not typically consequential for fitness and thus provides narrow insight into stress responses and their evolution (Feder and Walser, 2005). A clear disparity can be seen between the relative expression levels and degradation rates of mRNA and their corresponding proteins, making mRNA transcript levels inaccurate predictors of protein abundance (Abbott, 1999; Feder and Walser, 2005). As previously mentioned, alternative splicing and post-translational modifications increase the diversity of proteins expressed from a given transcript, as a single mRNA can give rise to >23,000 different proteins (Abbott, 1999; Feder and Walser, 2005; Lovric, 2011).

However, working with proteins poses several challenges, especially with respect to the study of less abundant proteins (Rabilloud, 2000). This shortcoming is further emphasized by the absence of an amplification tool equivalent to PCR for protein processing (Rabilloud, 2000). Therefore, while measuring protein abundance may be more difficult than assessing levels of mRNA transcripts, it provides advantages in that it allows for deeper insight into biological systems (Abbott, 1999). Given their sensitivity to intrinsic regulation, external perturbations, and fast response time, proteins are ideal biochemical indicators of metabolic stress and are thus the focus of many ecological studies (Dahlhoff, 2003; Tomanek and Zuzow, 2010).

As previously mentioned, proteomic techniques are able to bypass the principle limitations of genomics and transcriptomics to identify which proteins are involved in specific functions (Lovric, 2011). Proteomics employs a holistic perspective, which provides a comprehensive and quantitative model with predictive abilities to better characterize molecular mechanisms in more complex biological systems (Aggarwal, 2003; Tomanek and Zuzow, 2010). This allows for an understanding of the cellular stress response on multiple levels, from individual cellular components to changes across several metabolic pathways (Tomanek and Zuzow, 2010).

Like most other systems biology approaches, proteomics is discovery-based, meaning that instead of generating one testable hypothesis, the entire system is assessed as a whole and the data collected can be used to test an array of hypotheses (Aggarwal, 2003; Tomanek and Zuzow, 2010). Additionally, through comparative proteomics, closely related organisms, adapted to unique environments and stressors, can be compared to further our understanding of their physiological tolerance limits (Tomanek and Zuzow, 2010).

There are two typical approaches implemented for quantification and analysis of complex proteomic samples: top-down and bottom-up approaches (Lovric, 2011). Top-down approaches are decomposition based, as they break down a system to gain insight into its compositional sub-systems (Lovric, 2011). When put into context, whole proteins are analyzed by mass spectrometry, which generates the data necessary for characterization of those proteins (Lovric, 2011). Meanwhile, bottom-up approaches are synthesis based, piecing together systems to give rise to a grander system; e.g. peptides

generated from mass spectrometry are used to characterize proteins that have been pre-separated using 2DGE (Lovric, 2011).

Regardless of the approach used, all proteomics workflows can be broken down into three main parts: separation, quantification, and identification (Lovric, 2011). The ‘classic’ and most commonly used proteomic approach is gel-based, in which proteins are extracted from tissues, separated on gels by isoelectric point and molecular weight, digested, identified via mass spectrometry, and quantified using relevant statistical software (Berth et al., 2007; Lovric, 2011). Gel-based methods pose several advantages in that 2D gels are both easy to handle and reproduce, they can separate more than 10,000 proteins in a single gel, and the available software is well suited for efficient analysis of a large number of samples (Berth et al., 2007). Additionally, 2DGE allows for the separation of various protein isoforms with different posttranslational modifications, as protein phosphorylation adds a negative charge to one or several amino acids, glycosylation neutralizes negative charges, and acetylation suppresses positive charges on amino groups (Michel et al., 2003). Thus, this approach provides information on both changes in protein expression levels and their posttranslational modifications (Görg et al., 2004).

Other separation techniques include semi-gel-free and gel-free systems, e.g. shotgun gel-free liquid chromatography (Berth et al., 2007). These are usually employed to supplement or enhance the intrinsic limitations of gel-based methods, which include, but are not limited to: poor detection of low abundance proteins, extremely basic or acidic proteins, or poorly water-soluble proteins, e.g. membrane or nuclear proteins (Rabilloud, 2002). However, despite these limitations, the gel-based method the most

widely used bottom-up approach, and thus will be the focus of this discussion (Lovric, 2011).

Protein Separation

Protein separation can be broken down into three main parts: sample preparation (pre-fractionation), isoelectric focusing (IEF; 1st dimension separation), and 2D gel electrophoresis (2DGE; 2nd dimension separation), the most important of these being sample preparation (Lovric, 2011). While 2DGE allows for the analysis of a wide array of sample types, from whole tissue samples to isolated cell fractions, there is no one method of sample preparation that can be ubiquitously applied to all analyses, and thus each protocol must be optimized (Görg et al., 2004). An effective sample preparation approach must meet the following four criteria: (1) maximum retention of original proteins in the sample, (2) successful removal of any additional sample contaminants, e.g. lipids, nucleic acids, salts, polysaccharides, and buffers, (3) preservation of the PTMs of the proteins, and (4) the resulting sample should be compatible with successive analytical steps in the proteomic workflow (Lovric, 2011). The applied methodology must thus ensure that proteins are completely denatured, disaggregated, reduced and solubilized (Görg et al., 2004).

Prior to experimentation, all tissue samples must be flash frozen in liquid nitrogen to preserve cellular activity over the course of the experiment and prevent subsequent changes to the composition of the proteome, e.g. inactivation of proteolytic enzymes (Berth et al., 2007). Following dissection, tissues are homogenized with homogenizing tissue buffer to facilitate lysis of the cell membrane and the release proteins from their

intracellular environment (Görg et al., 2004). While mechanical force helps to accelerate membrane decomposition, neutral buffer chaotropes like urea and thiourea work to disrupt the water shell around proteins – interfering with hydrophobic interactions and breaking hydrogen bonds that naturally maintain secondary and tertiary protein structures (Görg et al., 2004; Shaw and Riederer, 2003). Some laboratories add protease inhibitors to prevent the action of proteases, however at high enough concentrations, chaotropes can function to adequately inhibit their activity in samples as well (Shaw and Riederer, 2003).

Zwitterionic detergents (surfactants), like amidosulfobetaine (ASB-14), work in combination with urea and thiourea to solubilize integral membrane proteins and break down protein-protein interactions (Görg et al., 2004; Lovric, 2011). Reducing agent dithiothreitol (DTT) also acts to cleave inter- and intra-molecular disulfide bridges and prevent their re-oxidation to maintain proteins in an unfolded state (Görg et al., 2004; Shaw and Riederer, 2003). Additionally, this buffer contains a mixture of carrier ampholytes (IPG buffer), ions that can act as an acid or base to prevent the fluctuations of the desired pH range and enhance sample solubility (Görg et al., 2004). Generally, the optimal pH range is between 4 and 7 as the majority of the proteins of interest are within this physiological range (Lodish et al., 2007; Lovric, 2011).

The resulting homogenate contains all cellular components including lipids, nucleic acids, polysaccharides, salts and other interfering substances that need to be inactivated or removed (Görg et al., 2004). Nucleic acids are negatively charged and thus interfere with focusing during first dimension separation by binding to proteins (Shaw and Riederer, 2003). Additionally, both nucleic acids and polysaccharides can interact

with carrier ampholytes to clog gel pores, giving rise to “streaky” polyacrylamide gels (Görg et al., 2004; Shaw and Riederer, 2003). Salts, which are especially common in marine samples, can also result in a multitude of focusing problems, causing electroendosmosis, the precipitation of proteins from IEF strips, and increased conductivity during IEF – prolonging charge-based separation of proteins (Görg et al., 2004; Shaw and Riederer, 2003). Hydrophobic interactions between lipids and membrane proteins can lead to protein loss due to the insolubility of protein complexes, whereas hydrophobic interactions between lipids and detergents can lead to their “consumption” unless excess detergent is present (Görg et al., 2004; Shaw and Riederer, 2003).

These contaminants can be removed via several methods, however in the case of our protocol, trichloroacetic acid (TCA)/acetone precipitation followed by a subsequent acetone wash (to remove excess TCA) was employed to purify our protein mixture (Görg et al., 2004). Unfortunately, protein loss is inevitable during this process, yet precipitation is essential prior to 2DGE (Görg et al., 2004). The resulting purified mixture is re-solubilized in a salt-free IPG rehydration buffer similar to the previously used homogenization buffer in that it includes both neutral chaotropes and ampholytes. However, this buffer additionally contains: octylphenoxypolyethoxyethanol (Nonidet P-40), a non-ionic detergent for solubilizing membrane proteins and preventing protein-protein interactions; 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), a non-denaturing zwitterionic detergent for solubilizing proteins; and dithioerythritol (DTE), reducing agent with functions similar to DTT (Görg et al., 2004; Lovric, 2011).

Given that 2DGE is sensitive to total protein, protein quantitation is fundamental to ensure that the amount of protein loaded on each gel is consistent across all samples. There are several spectrophotometric-based assays including Lowry or dye-binding assays, which rely on the protein-catalyzed reduction of Cu^{2+} to Cu^+ , or binding of Coomassie dye to protein, respectively (Mackintosh et al., 2005). However, both methods have limited sensitivity as they interfere/complex with several buffer components, including: reducing agents (DTT), chaotropes (thiourea), carrier ampholytes and detergents (CHAPS and SDS) (Mackintosh et al., 2005). The 2-D Quant Kit (GE Healthcare Bio-Sciences) was developed to circumvent previously encountered compatibility issues, as interfering compounds are discarded with the supernatant to allow for more accurate quantification of precipitated proteins (Mackintosh et al., 2005). The assay is based on the specific binding of Cu^{2+} ions to proteins suspended in a copper-containing solution. Protein concentration is inversely related to the absorbance (measured at 480nm) of unbound Cu^{2+} -colorimetric agent complexes (Mackintosh et al., 2005; 2-D Quant Kit, GE Healthcare). For analytical purposes, the amount of protein loaded onto each IPG gel strip is determined by several parameters, e.g. pH gradient, to minimize background smearing and obtain optimal resolution of the greatest number of proteins (Görg et al., 2004). In this experiment, samples are diluted to 200 μg in working IPG rehydration buffer based on results from the concentration assay.

Proteins are then separated along a pH gradient according to their pI (isoelectric point), the pH at which the protein carries no net charge (Lovric, 2011). All proteins are amphoteric molecules and thus contain charged or potentially charged amino acid side chains depending on the pH of their surroundings (Lovric, 2011; Ahn and Simpson,

2007). The charge of each protein is unique given of the inherent variation in amino acid composition and additional post-translational modifications, allowing for the separation of different proteins and charge-isoforms of the same protein (Lovric, 2011). Isoelectric focusing (IEF) takes advantage of this phenomenon, as proteins migrate to their pI when placed in an immobilized pH gradient within an electric field (Lovric, 2011; Ahn and Simpson, 2007).

At this point, the aforementioned diluted protein solution is applied to ready-made immobilized pH gradient (IPG) strips (Görg et al., 2004). First dimensions strips are available in a variety of lengths (e.g. 11 cm; 18 cm; 24 cm), and pH ranges (e.g. wide: IPG 3-12; medium: IPG 4-7; narrow: IPG 4.4-5.5), such that separation can be customized to the specifications of each experiment (Görg et al., 2004). The inverse relationship between protein retention and resolving power should be taken into consideration when selecting the pH range of the IPG gel strip (Righetti, 1983). In general, increased protein retention and separation are associated with narrower pH ranges, however protein resolution is compromised (Righetti, 1983). As previously mentioned, pH 4-7 strips were chosen for the scope of this project, as most cellular proteins fall within this range (Righetti, 1983).

Before proteins can be separated by molecular weight, IPG gels strips must be equilibrated to ensure all proteins fully bind with sodium dodecylsulfate (SDS), an anionic detergent whose hydrophilic sulfate group imparts a negative charge on peptides along the denatured protein backbone (Lovric, 2011). During the 15-minute equilibration process, samples are incubated in an equilibration buffer of Tris-HCl (pH 8.8), urea, glycerol, SDS, and a reducing agent, DTT, which acts to denature proteins by cleaving

internal disulfide bonds (Görg et al., 2004). A second equilibration in the same buffer, with DTT replaced by iodoacetamide (IAA) is required to bind covalently with free DTT and resultant cysteine residues so they cannot reform disulfide bonds (Görg et al., 2004). This helps to maintain proteins in their denatured state (Görg et al., 2004).

Once proteins are charged and equilibrated, they are separated by their molecular weight on a sodium-dodecyl-sulfate polyacrylamide gel (SDS-PAGE), which is a matrix composed of acrylamide and “cross-linking” bisacrylamide monomers (Lovric, 2011; Görg et al., 2004). Protein migration is dictated by the size of the pores within this “mesh,” which can be controlled by adjusting the percentages of acrylamide and bisacrylamide used when casting gels (Lovric, 2011). IPG strips are placed on top of a pre-cast polyacrylamide gel within an electric current, causing the negatively charged proteins to migrate through the gel matrix towards the positive cathode (Lovric, 2011). As proteins travel vertically down the polyacrylamide gel, smaller molecular weight proteins traverse through the matrix more quickly than larger proteins, resulting in smaller proteins near the bottom of the gel and larger proteins closer to the top (Lovric, 2011).

Following 2nd dimension separation, gels must be stained for visualization and analysis of proteins within the gel (Berth et al., 2007). Various staining methods are available for protein detection, and thus the specific characteristics of each stain must be considered prior to selection, because not all stains are compatible with each identification process (Görg et al., 2004). Protein stains are either fluorescent (e.g. SYPRO Ruby, LavaPurple) or colorimetric (e.g. Colloidal Coomassie Blue, Silver Stain) (Ball and Karuso, 2007). Colloidal Coomassie Blue (CBB) is one of the more commonly

used staining methods, as it is easy to use, inexpensive, has a wide dynamic range, and is compatible with most protein detection methods, including mass spectrometry (Görg et al., 2004). Yet, its weaker ability to resolve low abundance proteins (10 ng of protein/spot) means that no more than a few hundred spots can be detected on each gel (Görg et al., 2004; Ball and Karuso, 2007). Silver staining methods are much more sensitive (0.1 ng protein/spot) than CBB stains, but given their complexity they are not as reproducible and have a lower dynamic range (Görg et al., 2004). Additionally, silver stains are found to be incompatible with certain methods of detection, as they interfere with peptide ionization during mass spectrometry (Görg et al., 2004).

Limitations of the colorimetric stains have led to the development of fluorescent stains, with sensitivities comparable to that of silver stains (1-2 ng protein/spot), highly dynamic ranges to increase protein quantification on gels, and compatibility with mass spectrometry (Ball and Karuso, 2007; Görg et al., 2004). The major drawback to these stains however is their cost – not only are the stains themselves expensive, but they require expensive compatible equipment, e.g. scanners and UV trans-illuminating spot pickers (Ball and Karuso, 2007). No matter which staining method is employed, once gels are stained they need to be digitized, using the appropriate scanner or CCD-camera-based systems for subsequent analysis (Lovric, 2011; Berth et al., 2007). The quality of the raw data significantly impacts image analysis, as background noise and artifacts on gels may reduce the accuracy of spot detection and quantification; therefore, proper calibration of scanning devices is imperative prior to image acquisition (Berth et al., 2007).

Protein Quantification

After successful separation of proteins, gel images are analyzed for differential expression between control and experimental samples using one of the several available gel image analysis software packages, e.g. Delta2D (DECODON, Greifswald, Germany), PDQuest (BioRad, Hercules, CA), and Melanie III (Genebio, Geneva, Switzerland) (Berth et al., 2007). There are several advantages to Delta2D, the system used in this project, in that unlike other software packages, it successfully removes gel image distortions and allows for gel comparison prior to spot boundary detection through the use of congruent false color images (Berth et al., 2007). However, regardless of the program used, protein quantification follows the same basic steps (Lovric, 2011).

First, gel images are loaded into the software program and grouped together by treatment (Berth et al., 2007). Given that the location of a protein on a 2D-gel fluctuates slightly between gels, variation in spot positioning is removed via a process known as “warping” (Berth et al., 2007; Fig. 4). The lack of congruency can be attributed to a variety of factors including, but not limited to, variations in the pH or temperature of the running buffer, gel polymerization, or current leakage during electrophoresis (Berth et al., 2007). This accentuates the need for precision during the protein separation portion of the workflow, as elimination or minimization of these differences improves warping accuracy and successive statistical analyses (Berth et al., 2007).

Figure 4: 2DGE-based proteomics analysis in Delta2D (modified from Berth et al., 2007).

When warping, gels are compared pixel for pixel based on obvious spot patterns from two gels displayed in different colors (Lovric, 2011). More explicitly, these gels are manually warped to one another with calibrated match vectors that connect corresponding points, aligning protein spots on a single master gel with comparable spots on every other gel (Görg et al., 2004; Lovric, 2011). This not only allows for the analysis of analogous spot patterns, but also highlights obvious differences between treatment groups as image geometry is transformed through warping to produce a nearly exact overlay (Berth et al., 2007). Given the manual nature of this process, spot matches must be made carefully and accurately, with excessive revision, prior to spot detection (Görg et al., 2004).

Once spot positions are matched on each gel, all gels within each group are superimposed to generate a proteome map (fusion image), which is a single “consensus spot pattern” representative of how proteins are distributed in the experiment (Berth et al., 2007). This composite gel image is compared against each of the original images to align match vectors for the generation of spot boundaries (Berth et al., 2007). These spot boundaries are derived by a process called “spot detection,” in which the software uses Gaussian models/algorithms to automatically determine spot boundaries and intensity (Berth et al., 2007; Lovric, 2011). Automatically detected spots are then manually edited ensure all boundaries are representative of true spots prior to statistical analysis of differential protein expression (Berth et al., 2007). These boundaries are subsequently transferred to all original gel images using match vectors from the wrapping process (Berth et al., 2007). After background subtraction, raw spot volumes (determined by both the area and pixel density of each spot) are normalized against the total spot volume

of all proteins in the same gel and on other gel images, to quantify the relative amount of protein in each spot (Berth et al., 2007). This process aims to eliminate inherent differences between gel images often due to: variance in protein loading, image exposure times, staining efficiency, and other inconsistencies accrued during image acquisition (Berth et al., 2007).

Protein expression profiles can be generated based on normalized spot volumes to showcase statistically significant changes in protein abundance for each spot across treatment groups (Berth et al., 2007). Currently, analytical software packages provide a suite of basic univariate statistical tools so that each experiment can be appropriately analyzed based on the experimental design and number of treatment groups (Berth et al., 2007). Additionally, most also support the export of data in tabular form so more advanced methods can be employed (Berth et al., 2007).

In the simplest case, the standard t-test is used for experiments comparing two samples, e.g. treatment vs. control, where the null hypothesis is that there is no difference in protein expression level between the treatment and control groups. The rejection of the null hypothesis is indicative of differentially expressed patterns of protein abundance between the two groups (Berth et al., 2007; Rencher and Christensen, 2012). For more complex experiments with multiple treatments, one- or two-way ANOVAs are implemented (Delta2D, DECODON, Greifswald, Germany; Rencher and Christensen, 2012). While the aforementioned tests are relatively robust to the violation of their requirement of normally distributed errors and equality of variances, permutation-based analyses (e.g. randomization-based ANOVAs) are often used to protect against departures from these requirements (Ludbrook and Dudley, 1998; Anderson and Braak,

2003). Thus, in proteomic studies it is often preferable to utilize this alternative approach over the traditional t- or F-tests when there are several independent treatment groups (Ludbrook and Dudley, 1998). It is important to note however, that automated permutation-based tests are for univariate analyses allowing for the study of only a single protein at a time and are thus not appropriate for multivariate analyses where a collection of proteins are simultaneously compared. In these cases a different approach needs to be implemented.

In the instance of this experiment, we want to test a null hypothesis that the six treatments have the same effect on average protein abundance in each sample. However, this situation poses a multiple hypothesis problem when analyzed using traditional univariate (including permutation) methods, in that for each expression profile generated, a separate test of significance is run (Berth et al., 2007). This is especially problematic when analyzing large proteomic data sets, as they generally involve the simultaneous comparison of several hundred spots (Berth et al., 2007). Moreover, the chance of making a Type I statistical error (incorrectly rejecting the null hypothesis and thereby accepting a false positive identification by chance alone) is directly related to the number of unique proteins being compared (Ludbrook, 1998). Generally speaking, this risk is maintained at a level determined by the investigator (based on the level of significance, e.g. p-value, alpha), however as the number of tests increases, the probability of making a type I error is inflated beyond the originally established parameters (Ludbrook, 1998). Thus, alpha, a measure of the false positive rate (FPR), is often lowered to minimize the discovery of false positives and thereby increase the likelihood that the proteins identified

as significant are in fact significant (Berth et al., 2007; Ludbrook, 1998; Storey and Tibshirani, 2003).

However, overcorrection of this threshold often proves problematic, as protecting against the detection any one false positive is often too stringent in larger experiments, leading to several missed findings (Storey and Tibshirani, 2003). On the contrary, even with a small alpha, significant changes in protein expression across treatments will still be falsely discovered (Berth et al., 2007; Ludbrook, 1998). To better characterize this, a distinction must first be made between the FPR and the false discovery rate (FDR) (Berth et al., 2007).

The FPR, (represented by alpha for a single comparison) is the probability of the presence of truly null comparisons that are falsely deemed significant among the collection of multiple comparisons (Storey and Tibshirani, 2003). Conversely, the FDR, represented by the q-value, is the percentage of all tests deemed significant that are in fact truly null, or in this context, the proportion of proteins deemed significant that are in fact false leads (Storey and Tibshirani, 2003). In the context of the experiment on hand, the established FPR is the *probability* of identifying one or more null spots as significant predictors of protein abundance changes when there really are none; while the FDR is the *proportion* of all spots deemed significant between all pairwise comparisons of spots when there really are none.

Therefore, in order to create an analysis that is neither too lenient, and incorrect, or too limited, and therefore not biologically relevant, several studies implement the q-value (Storey and Tibshirani, 2003). However, it may be too restrictive in proteomics studies.

As previously mentioned, many univariate permutation-based tests are not always appropriate for multivariate analyses. A more conservative multivariate approach can be utilized to account for the FPR and to characterize the handful of proteins most responsible for changes in abundance. Given that in some experiments there are fewer observations (gels) than variables (spots), and thus not enough data to look at all spots simultaneously, the spots contributing to most of the variability in the data set can be selected based on criteria determined *a priori*.

Principal component analyses (PCA) can be employed to assess the importance of specific proteins whose abundances changed significantly following treatment (Berth et al., 2007). PCA, a method of multivariate analysis, serves to reduce the dimensionality in large complex datasets by creating linear combinations of the variables that best preserve the primary sources of variability within the cloud of data (Rencher and Christensen, 2012).

In context with this experiment, the analysis considers each gel image as a data vector whose values are determined by the spot intensities at each location on that particular gel (Berth et al., 2007). The associated eigenvalues and eigen vectors from the principal components analysis quantify the contribution of each protein in the separation of samples as they provide information about where most of the variability lies (Rencher and Christensen, 2012; Berth et al., 2007). The coefficients of the eigenvector with the highest eigenvalue provide information about the spot locations (or combinations of locations) that are associated with the greatest variability across gels (Rencher and Christensen, 2012).

There are several approaches one can take to determine how many principal components to keep. In one such instance, a fixed proportion of the total variance is retained (Rencher and Christensen, 2012). Principal components that explain less than 80% of variability are excluded (retain λ_i explaining $> 80\%$). A subset of variables can then be determined by selecting as many variables as the number of retained principal components (Rencher and Christensen, 2012). To select variables (i.e., spots), for each retained principal component, the spot corresponding to the maximum of the absolute values of the eigenvector coefficients in the first principal component is selected (Rencher and Christensen, 2012). This process can then be repeated for each subsequent principal component, unless the spot corresponding to the maximum coefficient has already been selected, in which case, the variable corresponding to the next maximum can be chosen (Rencher and Christensen, 2012). Other methods include: excluding components that express less than the average amount of variability, using a scree plot to identify a natural break in the pattern of variation, or using a hypothesis test (likelihood ratio test) to exclude insignificant eigenvalues (however, this tends to be a very conservative estimate) (Rencher and Christensen, 2012). Any of these methods will generate a subset of spots to be used for subsequent statistical analyses.

For spots previously determined by PCA, a MANOVA (multivariate ANOVA containing multiple dependent variables) is run to test for differences in the average density of spots between treatment groups (Rencher and Christensen, 2012). Unlike ANOVA, the MANOVA takes into account multiple independent and dependent variables within the same model, allowing for greater power and reduced type I error (Rencher and Christensen, 2012). A MANOVA identifies linear combinations of the

dependent variables that best discriminate among the groups in the particular model (Rencher and Christensen, 2012).

Lastly, a discriminant analysis can be used to determine which spots best discriminate between groups (Rencher and Christensen, 2012). Discriminant plots help interpret the nature of the variability across factors using lengths of factor vectors (Rencher and Christensen, 2012). The magnitude and direction of a given factor vector tells us about the weight the factor has in that linear combination (Rencher and Christensen, 2012). If all variables are measured on the same scale, discriminant coefficients, as opposed to standardized coefficients (used for non-commensurate data), can be used to rank the variables in order of which spots explain most of the variability between treatment groups (Rencher and Christensen, 2012). A step-wise discriminant analysis can also be used to pick the variables that most strongly discriminate between groups (Rencher and Christensen, 2012). While these results are strongly biased, they account for possible correlations, which aren't accounted for in Delta2D's univariate methods.

While this method allows for the identification of proteins that are most significantly distinguishing treatment groups, it doesn't take into account that the proteins identified as most significant may not in fact be the causal proteins. Rather, they may only be highly correlated with the expression of a causal protein. An additional approach, variable clustering, can be implemented to further improve data interpretation. More specifically, the variable clustering algorithm begins with all variables (spots) in a single cluster, and proceeds by splitting and assigning variables to new clusters (according to the principal components analysis of the clusters) until no new splits or assignments are

possible (the second largest eigenvalue for the principal components is less than one) (Variable Clustering Procedure – VARCLUS; JMP Pro 11.1.1. Cary, North Carolina). Therefore, a large set of variables can be replaced by individual clusters and tested en masse improving type I error protection with little loss of information or predictive power. Proteins within significant clusters can be further analyzed.

For each cluster, a MANOVA (multivariate ANOVA containing multiple dependent variables) is run to test for differences in the average density of spots between treatment groups. This is once again followed by a discriminant analysis to determine which spots best discriminate between groups.

Protein Identification

The final step of the proteomic workflow is to identify significant proteins of interest and interpret their role in the cellular response to different treatments (Görg et al., 2004). Mass spectrometry (MS) is the most widely used approach to accomplish this in proteomic analyses, as an array of protein identification methods can be carried out by different classes of machines, e.g. matrix-assisted laser desorption/ionization (MALDI) tandem time of flight (TOF/TOF) mass spectrometer, or electrospray ionization (ESI) high pressure liquid chromatography (HPLC) (Aebersold and Mann, 2003). MS methods pose several advantages as they have the capacity to detect PTMs on various protein isoforms and allow for the high throughput detection of proteins in the low femtomole range and even below (Görg et al., 2004; Suckau et al., 2003). While not all methods will be discussed in depth in this review, we will focus on MALDI TOF/TOF MS, which is

the preferred method of protein identification following 2DGE proteomic methods (Lovric, 2011).

Briefly, protein identification via mass spectrometry can be broken down into three steps. First, protein spots of interest are manually excised from gels using a spot cutter and subsequently digested using proteases that cut at predetermined sites along the amino acid backbone to produce a unique set of predictable peptide fragments (Abersold and Mann, 2003). The most commonly used enzyme is trypsin, which cleaves proteins on the C-terminal of lysine and arginine (Lovric, 2011).

Secondly, using the mass spectrometer, the experimental mass and abundance of each protein is determined and matched to a database of calculated peptide masses to generate peptide mass fingerprints (PMFs) for each peptide in the sample mixture (Aebersold and Mann, 2003). To confirm this match, further fragmentation through a process known as tandem mass spectrometry (MS/MS) produces peptide fragment fingerprints (PFFs) (Tomanek, 2011). These provide additional information regarding the amino acid composition of each peptide (Tomanek, 2011).

Expressed sequence tag (EST) libraries are commonly used to search uninterpreted PMFs and PFFs against known nucleic acid sequences (Choudhary et al., 2001). “In general, the reading frame for translation is unknown, and the nucleic acid sequence must be translated in all six frames before searching. This generates a large quantity of effectively random sequence, within which some degree of matching to the experimental data will occur by chance” (Choudhary et al., 2001). However, MS/MS data is found to contain enough information to successfully discriminate a positive match from a match due to random chance alone (Choudhardy et al., 2001).

PMFs and PFFs are combined to increase the chances of positive identifications, and searched against genomic databases, e.g. EST libraries, where experimental data is compared to theoretical enzymatic digestions (Tomanek, 2011; Lovric, 2011). Positive peptide matches are contingent on several factors, which include, but are not limited to the purity of the peptide sample, the type of database search conducted (e.g. search parameters, error rate), the presence of theoretically possible sequences in the database, and PTMs/variable peptide modifications (Lovric, 2011).

In general, each mass spectrometer is comprised of an ion source, a mass analyzer, and a detector (Aebersold and Mann, 2003; Suckau et al., 2003; Fig. 5). ESI and MALDI are two ionization methods most commonly used to generate gas-phase ions from nonvolatile analytes (Aebersold and Goodlett, 2001). Both ESI and MALDI are referred to as “soft ionization” techniques given that analyte fragmentation during the ionization process is minimal, thus allowing for the successful transfer of intact ionized peptides into the gas-phase (Aebersold and Goodlett, 2001).

Figure 5: MALDI-TOF-TOF mass spectrometer schematic (modified from Aebersold and Mann 2003 and Suckau et al., 2003).

ESI is commonly paired with top-down proteomics approaches, in which analysis of complex protein samples yields a multitude of isotopic peaks and charge states (Lovric, 2011). These ions are then further fragmented, and the resulting fragment masses are compared to expected masses from known sequences to effectively localize mutations and PTMs within a given sequence (Lovric, 2011). One of the primary differences between ESI and MADLI is that ESI ionizes peptides in solution, and is therefore often coupled with liquid based chromatography, e.g. HPLC (Aebersold and Mann, 2003).

Meanwhile, MALDI ionizes peptides out of solid media, making more suitable for analyses that utilize the bottom-up approach (Aebersold and Mann, 2003). The most reliable and efficient method for preparing media on the MALDI target plate is the dried droplet technique (Lovric, 2011). A small volume of digested protein solution is embedded in a dry, crystalline matrix (e.g. α -hydroxycyano cinnamic acid) and gently mixed by pipetting before plating on a target plate (Lovric, 2011). The target plate is then placed in the mass spectrometer, where laser pulses sublimate and ionize the samples out of the matrix (Aebersold and Mann 2003). This leads to the formation of positively charged peptides, which are accelerated by voltage plates toward the mass analyzer (Aebersold and Mann 2003).

The mass analyzer then measures the mass-to-charge ratio (m/z) of each ionized peptide in the sample (Aebersold and Mann 2003). The four types of mass analyzers currently preferred in proteomic-based research include: the ion trap, the linear time-of-flight (TOF), the Fourier transform ion cyclotron (FT-MS), and the quadrupole (Aebersold and Mann 2003). MALDI is typically coupled with TOF analyzers, which are based on the simple principle that if two ions carrying the same charge were accelerated

by the same electric field, they would be accelerated at different speeds based on their mass (Lovric, 2011). Without going into the mathematics behind this, basic physics tells us the lighter ion will not only be accelerated faster and to a higher speed, but will reach the target before the heavier ion (Lovric, 2011). Once the ions reach their maximum speed they will drift until they reach the target; therefore, the mass of each ion can be determined based on the amount of time spent in the ‘flight tube’ (Lovric, 2011).

The MALDI-TOF/TOF mass spectrometer includes two co-linearly arranged TOF analyzers separated by a collision cell (Suckau et al., 2003). The first TOF analyzer initially separates high abundance precursor ions of one m/z ratio, which are then fragmented in the collision cell by high-energy collisions (Leung and Pitts 2008). These fragment ions then enter the second TOF analyzer, which are separated to determine the amino acid sequences of the precursor ions (Leung and Pitts 2008). This configuration allows for better sensitivity and greatly increases the accuracy mass measurements for PMFs – as the likelihood that two proteins will share the same overall molecular weight is much greater than the likelihood that they share all trypsin fragments (Aerbersold and Mann, 2003).

From the mass analyzer, ionized peptides move to the detector, which determines the abundance of ions at each individual m/z value (Aebersold and Mann 2003). The detector will pick up multiple peptide fragments to display a unique pattern of numerous peaks with different molecular weights (Lovric, 2011). The PMF for each sample is produced from the resultant pattern of m/z ratios and typically presented in graphical form, with the m/z range along the x-axis and intensity along the y-axis (Lovric, 2011). Peak amplitude is directly correlated with protein expression levels, as high abundance

peptides appear as taller peaks within the PMF, while conversely, low abundance peptides are displayed as shorter peaks (Lovric, 2011). The molecular weights of these experimental peaks can then be compared with a database (MASCOT) of theoretical proteins from sequenced organisms all cleaved *in silico* into trypsin fragments (Görg et al., 2004; Tomanek, 2011). Therefore, PMFs allow researchers to identify a given protein in a sample with relative confidence, especially if the study organism genome is sequenced (Görg et al., 2004). As previously mentioned, MS/MS can be used for further fragmentation of high abundance peptides, e.g. generation of PFFs (Tomanek, 2011). The PFF and the PMF of a given protein can then be combined in a single search containing both MS and MS/MS data (Tomanek, 2011).

The search engine used in this experiment, MASCOT, utilizes a molecular weight search engine (MOWSE) algorithm to score protein hits against different databases depending on experimental organisms (Perkins et al., 1999). In this experiment, PMFs and tandem mass spectra were searched against two databases: (1) an expressed sequence tag (EST) library containing thousands of different gene sequences for *M. californianus* and *M. galloprovincialis*, and (2) Swiss-Prot (last update: June 2009) with 17,360 molluscan protein sequences (Tomanek and Zuzow, 2010).

The MASCOT scores assigned to each protein matched in the sequence databases represent the probability that the match is a chance event (Perkins et al., 1999). A protein is considered to be a significant match if the MOWSE score is above the significance threshold ($p\text{-value} < 0.05$) or two or more peptide fragments are matched (Perkins et al., 1999). Therefore, “high” scores suggest there is a good probability that the hit is indicative of a correctly identified protein (Perkins et al., 1999). For the MASCOT search,

PMFs can be additionally searched against NCBI BLAST (basic local alignment search tool) databases to determine protein identification.

After identification of positive peptide matches, Delta2D hierarchical clustering can be used to generate heat maps, which group proteins into clusters based on similarities in their expression profiles, thereby connecting proteins with associated degrees of variation observed in each group (Berth et al., 2007). By joining or “clustering” similarly expressed proteins, we can identify both outliers and proteins that show similar changes in their abundance in response to the same treatment. This sort of information provides insight into which proteins share functions in related metabolic responses and how they interact in various pathways (Berth et al., 2007). Protein identifications can additionally be linked to their loading values within the components of a PCA, allowing further elucidation of functional relationships between proteins from complex data sets.

Additional Analytical Methods: Western Blot

While the high resolving power of 2DGE is a sufficient means for protein purification and analysis, it is sometimes necessary to quantify the activity of a particular protein of interest across treatments, e.g. sirtuins (Towbin et al., 1979). Western blotting was first described by Towbin, et.al in 1979 and has since become one of the most commonly used methods in molecular biology, recognized for its ability to detect the presence, relative abundance, relative molecular mass, posttranslational modifications, and protein interactions of individual proteins in complex protein mixtures (MacPhee, 2010).

Western blotting, also known as immunoblotting, is to proteins what Northern blotting is to RNA and Southern blotting is to DNA (Kurien and Scofield, 2006; Sambrook and Russell, 2001). In all three techniques, components are separated via gel electrophoresis and then probed with reagents specific to a specific sequence of amino acids (Western blotting) or nucleotides (Southern/Northern blotting) (Sambrook and Russell, 2001). In general, western blotting involve three basic steps: (1) separation of proteins by molecular weight, (2) transfer of proteins from a SDS polyacrylamide gel to an absorbent membrane, and (3) detection of the protein of interest using a proper primary and secondary enzyme labeled antibody (probe) for visualization (Mahmood and Yang, 2012). The nature of these probes is what really distinguishes Western from Southern and Northern blotting (Sambrook and Russell, 2001).

Antibodies (immunoglobulin) are available in a variety of classes, affinities, and idiotypes; however, as far as molecular biology is concerned, the most important property is their specificity to the antigenic epitopes displayed by target proteins (Sambrook and Russell, 2001). Thus, while simple equations can be used to predict the hybridization rate and specificity for a given nucleic acid probe, antibodies bind to target proteins based on preferential recognition of native or denatured epitope conformations (Sambrook and Russell, 2001). Consequently, not all antibodies are appropriate for any given assay, and therefore must be selected based on experimental conditions.

While both monoclonal and polyclonal antibodies can be utilized for Western blot analysis, each possesses their own advantages and disadvantages, making them useful for different applications (MacPhee, 2010). Polyclonal antibodies are produced in response to a foreign antigen through the activation of multiple B-cells, all of which target a

different epitope on that particular antigen (Singh et al., 2014). Therefore, a variety of antibodies are produced with different specificities and epitope affinities (Singh et al., 2014). Conversely, monoclonal antibodies produced by a single B-cell are specific to one epitope (Singh et al., 2014).

While polyclonal antibodies have the propensity to non-specifically bind to proteins unrelated to the protein of interest, they pose several advantages (MacPhee, 2010). For example, a stronger signal is produced following polyclonal incubation and blot development, due to the fact that they are capable of recognizing multiple epitopes on any one antigen (Singh et al., 2014; MacPhee, 2010). This results in a more robust detection than that typically seen following probing with monoclonal antibodies (MacPhee, 2010). Nevertheless, monoclonal antibodies are far more sensitive to changes in antigen concentration, thereby reducing the background noise and cross-reactivity with non-specific proteins (MacPhee, 2010). However, not all monoclonal antibodies are appropriate for use as probes, especially during instances where the specific epitope site on the protein of interest is denatured during gel electrophoresis (Sambrook and Russell, 2001; MacPhee, 2010). Therefore, background research to determine optimal assay conditions is imperative to improve chances of blotting success.

Before proteins can be run on a polyacrylamide gel, it is important to consider the quality and compatibility of the protein lysis buffer, to ensure proteins of interest are both extracted and effectively solubilized, particularly through the use of a compatible detergent (MacPhee, 2010). For more detailed information on the use of different detergents see MacPhee, 2010. The resulting homogenate is then precipitated and quantified (as previously discussed) in order to determine the appropriate volume of

sample to be diluted into the sample buffer, e.g. Leammli buffer (Mahmood and Yang, 2012). Since membranes have a limited capacity to bind proteins, protein quantification prior to polyacrylamide gel electrophoresis is essential (MacPhee, 2010).

Leammli buffer contains several components necessary to facilitate the directional migration of proteins in the polyacrylamide gel including: glycerol, which helps samples sink into wells; Tris base, which provides the appropriate pH; and sodium dodecyl sulphate (SDS) and β -mercaptoethanol (BME), which both act to denature proteins by cleaving internal disulfide bonds (Görg et al., 2004; Mahmood and Yang, 2012). Additionally, denatured proteins bind with SDS and as a result become negatively charged (MacPhee, 2010).

Previously, an overview of 2DGE articulated the basic principles behind the use of electrophoresis to separate complex mixtures of proteins based on both their isoelectric point and molecular weight. However, for the purposes of the western blot, denatured proteins are separated based on size only through two different types of agarose gel: a higher, slightly acidic (pH 6.8) stacking gel and a lower, slightly basic (pH 8.8) resolving gel (Mahmood and Yang, 2012). Due to the lower acrylamide concentration, the stacking gel is very porous, which allows proteins to form sharply defined bands (Mahmood and Yang, 2012). These bands are then “resolved” in the resolving gel, whose smaller pores (due to the increased acrylamide content) serve to separate proteins based on size (Mahmood and Yang, 2012).

After separating the protein mixture, an exact replica of the gel can be made onto an absorbent membrane (Mahmood and Yang, 2012; MacPhee, 2010). Several factors affect the efficiency of this transfer, including: the molecular mass of the proteins being

transferred, the membrane used, and the nature of the gel (MacPhee, 2010; Kurien and Scofield, 2006). In general, thinner gels containing smaller proteins result in a more complete transfer, as larger proteins blot poorly and thinner gels handle poorly (Kurien and Scofield, 2006). Additionally, while there are a number of membranes to choose from – nitrocellulose, polyvinylidene difluoride (PVDF), activated paper, or activated nylon – each comes with its own caveats (Kurien and Scofield, 2006). Yet, despite that proteins are not covalently bound, and the membrane is brittle and dry unless submerged, nitrocellulose membranes persist as the best compromise under most circumstances (Kurien and Scofield, 2006).

Protein transfer from polyacrylamide gels to nitrocellulose membranes can be achieved in three different ways: (1) simple diffusion, (2) vacuum-assisted solvent flow, and (3) electrophoretic elution (Kurien and Scofield, 2006). For the purpose of this discussion we will focus on the parameters necessary for electrophoretic elution, however a detailed description of previous methods can be found in Kurien and Scofield, 2006. This methodology is particularly advantageous given its speed and completeness of transfer compared to other blotting approaches (Kurien and Scofield, 20016).

Electrophoretic elution can be carried out in semi-dry or wet conditions, however wet conditions are preferred as to avoid gel desiccation and the successful transfer of larger proteins (Mahmood and Yang, 2012; MacPhee, 2010). Proteins are transferred from the gel onto the membrane using an electric field created by two vertical stainless steel-platinum electrodes, oriented perpendicular to the surface of the gel (Mahmood and Yang, 2012; Kurien and Scofield, 2006). In this procedure, the membrane is placed between the gel surface and the positive electrode, with a fiber pad (sponge) and piece of

filter paper sandwiched on either end to protect both the gel and blotting membrane (Mahmood and Yang, 2012). To ensure the transfer of a clear image, the gel and membrane must be in very close contact with one another, avoiding air bubbles in between the transfer sandwich (Mahmood and Yang, 2012; MacPhee, 2010). Additionally, it is very important that the membrane is placed between the gel surface and the positive electrode to facilitate the migration of negatively charged proteins from the gel to the membrane (Mahmood and Yang, 201).

Often, researchers want to check the homogeneity and transfer efficiency across the membrane to confirm that comparable protein was loaded in each lane of the gel (MacPhee, 2010). Direct detection of proteins is achieved through the use of organic dyes (Ponceau S), fluorescent labels (SYPRO Ruby), various silver staining methods, colloidal particles (gold, silver, copper, iron, or India ink), or metal chelate stains (ferrozine-ferrous complex and ferrocyanide-ferric complex) (Kurien and Scofield, 2006; MacPhee, 2010). Because dyes may interfere with antibody binding and detection, reversible staining, e.g. Ponceau S, is advantageous to prevent the use of a contaminated Western blot prior to subsequent immunodetection analysis (MacPhee, 2010).

Once blots are prepared, membranes are blocked with a protein buffer mixture to improve the sensitivity of the assay by preventing non-specific binding of antibodies to the membrane, thereby reducing background interference and by promoting the re-naturation of antigenic sites (Mahmood and Yang, 2012; MacPhee, 2010). This mixture usually consists of 5% non-fat milk powder in Tris buffered saline containing 0.1% Tween-20 (TBST) or 5% BSA in a similar buffer (MacPhee, 2010).

However, while in the past nonfat dried milk was preferred for blocking solutions, as it is both affordable and widely available, milk proteins are not compatible with every method of detection, thus emphasizing the need to optimize buffer composition based on the antibody datasheets (Mahmood and Yang, 2012; MacPhee, 2010). For example, casein, a phosphoprotein and biotin, is found to interfere with results from assays utilizing biotin or anti-phosphoprotein antibodies (Mahmood and Yang, 2012). In such instances implementation of Triton X-100, BSA, or Nonidet P-40 (NP40) for eliminating the binding of unwanted proteins is also proven effective (Kurien and Scofield, 2006). Additionally, the use of acidic buffers instead of milk following protein transfer is shown to increase sensitivity for non-denaturing blots (Kurien and Scofield, 2006). Therefore, no single blocking buffer can be universally applied to every experiment as each antibody-antigen pair has unique characteristics (Sambrook and Russell, 2001).

Next blocked membranes are then exposed to a series of incubations with different immunochemical reagents and wash steps (Sambrook and Rusesell, 2001). Membranes are first probed with unlabeled primary antibodies specific to the protein of interest (Sambrook and Russell, 2001). Since the primary antibody is generally needed in greater amounts than the secondary antibody, primary antibody is often incubated with BSA for reuse in the event of failed or poor blot results (Mahmood and Yang, 2012).

As previously mentioned, the greatest contributing factor to Western blot success is the quality and specificity of the primary antibody (Sambrook and Russell, 2001). Therefore, antibodies that lack specificity will generate a weak signal or multiple, often non-specific, bands on a blot (Gomes, 2009). Antibody concentration is unique to each assay, and therefore must be previously determined based on manufacturing information

and preliminary experimentation (Mahmood and Yang, 2012). Because of this, antibody concentration is subsequently diluted to optimal concentration in TBST or phosphate buffered saline (PBS) wash buffers (Mahmood and Yang, 2012).

Intermittent washing between each incubation period is necessary to remove unbound reagents/antibody and minimize the background noise, thereby increasing the signal to noise ratio (Mahmood and Yang, 2012). Excessive washing can cause a decreased signal due to the elution of the antibody and/or antigen from the blot, while insufficient washing will result in high background (Mahmood and Yang, 2012). Background optimization is of particular importance for chemiluminescent detection systems, where sensitivity is determined by the efficiency of background suppression (Sambrook and Russell, 2001).

Target antigens are ultimately indirectly detected by one of several secondary antibodies (e.g. radioactive: ^{125}I -labeled protein A or G antibodies; enzyme-conjugated: horseradish- (HRP) or alkaline phosphatase-coupled (AP) antibodies), the choice of which is ultimately dependent on the species of the animal in which the primary antibody was raised (Sambrook and Russell, 2001; Kurien and Scofield, 2006). For example, if the primary antibody is a rabbit polyclonal antibody, then the secondary antibody must be an anti-rabbit IgG secondary antibody obtained from a non-rabbit host, e.g. goat. While bands produced by radioactive antibodies are easy to quantitate, these antibodies are expensive and have short shelf lives due to radioactive decay (Kurien and Scofield, 2006). Therefore, due to their high sensitivity and low cost, enzyme-linked antibodies are more commonly utilized for Western blots (Kurien and Scofield, 2006).

Just as there are several different tags that can be linked to secondary antibodies, there are also a variety of methods used to detect the resulting signal produced following substrate addition, e.g. autoradiography, enzymatic production of a colored precipitate, or enhanced chemiluminescence (ECL) (Sambrook and Russell, 2001; Mahmood and Yang, 2012). Chemiluminescent methods, the most popular method for detection in Western blot analysis and the approach implemented in this particular experiment, generally involve the use of only a few chemical components to generate a detectable signal (Sambrook and Russell, 2001; Kurien and Scofield, 2006; MacPhee, 2010). Following secondary antibody incubation, 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), the most widely used substrate, is added and subsequently oxidized by HRP in the presence of hydrogen peroxide (Kurien and Scofield, 2006; MacPhee, 2010). This in turn produces an excited state product, which decays via a light-emitting pathway (Kurien and Scofield, 2006). The use of para-substituted phenolic compounds can enhance light emission up to 1,000-fold by replacing luminol as the substrate (Kurien and Scofield, 2006; MacPhee, 2010). Several manufacturers also offer ECL-based Western blot detection kits to preserve the signal duration (MacPhee, 2010).

Blot images are then visualized by exposure to film designed for ECL or through the use of digital imaging equipment containing CCD cameras (MacPhee, 2010). The latter is much more sensitive due to its resolving power and broad dynamic detection range, as the signal produced is inconsistent across the concentration range of samples (MacPhee, 2010; Kurien and Scofield, 2006). Therefore, it is important to note that data obtained from Western blots provides only a relative comparison of protein levels, and is thus considered only semi-quantitative (Mahmood and Yang, 2012). Blots images are

then loaded into corresponding software programs, e.g. Total Lab TL120 v2006f, for subsequent statistical analyses.

While this technique is widely used and accepted, several problems can arise throughout the procedure that can generally be grouped into five classes: (1) unexpected bands, (2) lack of bands, (3) faint bands (weak signal), (4) high background on the blot, and (5) patchy or uneven spots on the blot (Mahmood and Yang, 2012). See Mahmood and Yang, 2012 and Gomes 2009 for a comprehensive review of Western blot troubleshooting.

Hypotheses and Objectives

The purpose of this study is to determine the impact of periods of acute oxidative stress on the distribution ranges of two species of blue mussel, *M. galloprovincialis* and *M. trossulus*. Furthermore, we seek to elucidate the role sirtuins play in this response. As previously mentioned, while under acute heat stress, related *Mytilus* congeners undergo a shift in redox potential from NADH-fueled respiratory pathways (catabolic) to pathways producing NADPH (anabolic) as a way to decrease the production of ROS and provide reducing equivalents to detoxify ROS (Tomanek and Zuzow, 2010). We hypothesize that sirtuins (a family of NAD-dependent deacetylases) are involved in the regulation of this metabolic transition.

Therefore, we predict this transition will result in altered acetylation patterns of novel proteins related to the production of reducing equivalents (e.g. NADPH) supplying the cell with reactants to neutralize free radicals. This will provide a mechanism for coping with the typically highly variable abiotic environment of the intertidal zone which

is much more stressful than the subtidal environment that is just a few feet away.

Furthermore, an overall greater ability to scavenge ROS or an energy metabolism that produces fewer ROS during stress, could provide a physiological advantage that enables an organism to perform better under more stressful conditions, as are predicted to occur due to climate change. This can translate into a competitive advantage and an extension of the distribution range.

II. MANUSCRIPT

Introduction

Intertidal organisms experience a physically variable environment as a result of the circatidal rhythm, causing periods of immersion and emersion that differ greatly in food availability, osmolarity, oxygen partial pressure and temperature (Palmer, 1973; Newell, 1979). Tidal oscillations induce environmentally extreme conditions in intertidal organisms along a vertical gradient – with a positive correlation between increased oxidative stress and increased exposure to air or height within the intertidal zone (Letendre et al., 2009; Petes et al., 2008). This is due in part because oxidative stress, indicated by the increase in abundance of anti-oxidant enzymes, is a co-stressor of temperature, osmotic, hypoxic and pH stress in intertidal mussels (Tomanek, 2012). The acute shift of the intracellular redox balance toward oxidation, known as oxidative stress, is due to increased production or insufficient scavenging of reactive oxygen species (ROS).

While the production of ROS occurs naturally in all cells through normal cell activity as a byproduct of energy metabolism, protein maturation in the endoplasmic reticulum, and peroxisomal activity, the damaging effects of ROS, e.g. oxidative DNA damage, lipid peroxidation and protein carbonylation, are generally controlled by non-enzymatic and enzymatic ROS scavenging systems (Murphy, 2009; Murphy, 2012; Halliwell, 1992; Finkel et al., 2009; Lesser, 2006). The activity of these ROS-scavenging systems is facilitated by the reducing agent NADPH, the availability of which determines the effectiveness of ROS scavengers and reduced environment necessary for cellular function and suppression of ROS accumulation (Pollak et al., 2007). Thus, both the

production of enzymes that remove ROS and the down-regulation of ROS producing metabolic pathways require regulation. Regulation of protein activity through posttranslational modifications (PTMs; e.g. phosphorylation, methylation, and acetylation) not only allows for a faster cellular response to acute changes in the environment, but also increases the functional diversity of the proteome, the protein complement of the genome (Seo et al., 2004).

Lysine acetylation is both ubiquitous and conserved in many essential metabolic enzymes found in glycolysis and the TCA cycle (Zhao et al., 2010). The reversible acetylation of lysine residues is facilitated by NAD-dependent deacetylases, known as sirtuins. These enzymes are involved in several biological processes including, but not limited to, cell cycle progression, apoptosis, senescence, life cycles, and transcriptional repression – all of which play a critical role in maintaining cell integrity, providing a connection between energy homeostasis and intracellular fate (Houtkooper et al., 2012)(Lawson et al., 2010; Polevoda et al., 2002).

Previous work in mollusks demonstrates that temperature stress, acute salinity changes, pH, and hypoxia are all responsible for elevated levels of endogenous and exogenous reactive ROS (Sokolova et al., 2012; Tomanek, 2011; Tomanek 2012). Moreover, research shows that these organisms have a limited capacity to regulate these dynamic changes, and as a result, fluctuations in the above-mentioned stressors induce oxidative stress in marine bivalves, like the blue mussel, *Mytilus* (Tomanek et al., 2011).

In addition, while under acute heat stress, related *Mytilus* congeners undergo a shift in redox potential from NADH-fueled respiratory pathways (catabolic) to pathways

producing NADPH (anabolic) as a way to decrease the production of ROS and provide reducing equivalents to detoxify ROS (Tomanek and Zuzow, 2010).

Given their sensitivity to intrinsic regulation, external perturbations, and fast response time, proteins are ideal biochemical indicators of metabolic stress (Dahlhoff, 2003; Tomanek and Zuzow, 2010). Proteomics is a systems biology approach that allows for the simultaneous characterization of protein abundance changes and identification of which proteins are involved in specific functions in response to a variety of environmental stressors (Lovric, 2011; Tomanek, 2011). Additionally, through comparative proteomics, closely related organisms, adapted to unique environments and stressors, can be compared to further our understanding of their physiological tolerance limits (Tomanek, 2014).

In this study we chose to examine the closely related blue mussel congeners, *Mytilus galloprovincialis* and *Mytilus trossulus*. Blue mussels belonging to the genus *Mytilus* are sessile marine invertebrates that can thrive on both natural substrates, such as the rocky intertidal, and artificial substrates, like pier pilings and ship hulls. Natural and anthropogenic migrations have given rise to the warm-adapted Mediterranean *M. galloprovincialis* successfully invading and displacing the native cold-adapted congener, *M. trossulus* along the coast of California as far north as San Francisco Bay (Lallias et al., 2009; Tomanek and Zuzow, 2010; Braby and Somero, 2006).

This study used two-dimensional proteomics to analyze the responses of the two blue mussels, *M. galloprovincialis* and *M. trossulus*, to acute oxidative stress in the presence of sirtuin inhibitors in order to assess the species-specific role of sirtuins. We hypothesize that sirtuins (a family of NAD-dependent deacetylases) are involved in the regulation of

the metabolic transition to NADP producing pathways. Therefore, we predict this transition will result in altered acetylation patterns of novel proteins related to the production of reducing equivalents (e.g. NADPH), supplying the cell with reactants to neutralize free radicals. Comparisons of the proteomes within and between each species during periods of (1) chemically induced oxidative stress, (2) sirtuin inhibition, and (3) simultaneous periods of oxidative stress and sirtuin inhibition were made following two-dimensional gel electrophoresis (2DGE) and tandem mass spectrometry (MS/MS) protein identification. These identifications provide us with insights into specific differences in the oxidative stress response of each species, perhaps the underlying mechanism governing species biogeographic range distribution and tolerance limits.

Results and Discussion

Animal Mortality

Mussels of both species were acclimated to a natural tidal cycle for two weeks under natural conditions, suspended in a basket underneath a pier that shaded the animals. With the beginning of a natural high tide, we started to expose whole mussels to all six treatments for 8 h, approximately the length of a high or low tide during a semi-circatidal rhythm. Following treatment, whole mussels recovered under control conditions (with constant aeration) for ~24 h to allow mussels to translate proteins in response to treatments. Each treatment initially included 6 individuals. While no individuals of *M. galloprovincialis* died during any of the treatments, five individuals of *M. trossulus* died over the course of the experiment: one each during exposure to NAM, MND and suramin and menadione or nicotinamide and menadione (two individuals) simultaneously (SUR/MND or NAM/MND), reducing the number of individuals analyzed from 36 to 31 for *M. trossulus*. Thus, four of these individuals were exposed to an inhibitor, suggesting that *M. trossulus* might be more sensitive to the effect of sirtuin inhibition.

Variable Clustering and MANOVA Analysis

Composite images (proteome maps), generated based on all gel images for both *M. galloprovincialis* and *M. trossulus*, represent the average of the normalized pixel volume of all proteins detected (Figs 1A and 1B, respectively). In *M. galloprovincialis*, of the 316 spots detected, 131 (or 41%) were identified by tandem mass spectrometry regardless of significance (Table S1). To minimize the data set and obtain a more

conservative statistical analysis (reduce the chance of making a type I error), we reduced the complete set of proteins to 34 protein clusters based on the amount of variability explained by each protein (VARCLUS analysis). Twenty of the thirty-four (or 59%) clusters were significantly different between treatments (MANOVA, p -value ≤ 0.05). These clusters contained 57 proteins (Fig. 2A).

(a)

(b)

Figure 1: Proteome map generated from all 2D gel images of gill tissue after exposure of whole animals to an 8 h treatment (nicotinamide, nicotinamide and menadione, suramin, suramin and menadione, or menadione) and a control (SW only) followed by a 24.5 h recovery (SW only), and through separation of proteins by isoelectric point (horizontal axis) and mass (vertical axis). Each represents a composite gel image of (a) all thirty-six gel images (N=6 per treatment), depicting 316 protein spots from gill tissue of *M. galloprovincialis*; and (b) all thirty-one gels (N=4-6 per treatment), depicting 293 protein spots from gill tissue of *M. trossulus*. The proteome map represents average pixel volumes for each protein spot. Numbered spots were those that showed changes in abundance in response to treatments (Variable Clustering and MANOVA) and were identified using tandem mass spectrometry (for protein identifications see Table S1 and S2).

In *M. trossulus*, of the 293 spots detected, 93 (or 32%) were identified by mass spectrometry (Table S2). The complete set of identified proteins was replaced by fifty-eight clusters (VARCLUS analysis). Subsequent MANOVA tests found twenty-eight of the fifty-eight clusters to differ between treatments. Significant clusters contained 49 proteins (Fig. 2B).

(a)

(b)

Figure 2: Hierarchical clustering using Pearson's correlation of proteins from (a) *M. galloprovincialis* and (b) *M. trossulus* that changed significantly with oxidative stress and sirtuin inhibition and were identified with tandem mass spectrometry (Variable Clustering and MANOVA). Blue coloring represents a lower than average protein abundance (standardized volume), whereas yellow represents greater than average protein abundance. The columns show individual mussels, which cluster according to treatment (N=6 per treatment: *M. galloprovincialis*; N=4-6 per treatment: *M. trossulus*). The rows represent the standardized protein abundances, which are identified to the right. Treatment groups are abbreviated as follows: Control (CTL), Nicotinamide (NAM), Nicotinamide + Menadione (NAM/MND), Suramin (SUR), Suramin + Menadione (SUR/MND), Menadione (MND).

Western Blot Analysis of SIRT3 and SIRT5

Western blotting was used to quantify the abundance of SIRT3 and SIRT5 in response to treatments (Figs S2A and S2B). We were not successful to detect SIRT1, which is marked for proteasomal degradation by oxidants/aldehydes (Arunachalam et al., 2010; Caito et al., 2010). Human SIRT3 and SIRT5 (MW = 32.7 kDa and 32.3 kDa, respectively) differed in molecular mass from putative *Mytilus* SIRT3 and SIRT5 bands by several kDa, possibly due to interspecific differences to the human standards. For example, a NCBI Blast search showed that the molecular mass of SIRT5 in the marine invertebrate *Aplysia californica* is 38.8 kDa. However, SIRT3 has not been identified in any mollusk (or invertebrate) to use for comparison and our results are putative as long as there is not a species-specific antibody or at least a comparison with other closely related invertebrate taxa confirming the uniqueness of the assigned band as SIRT3.

Both SIRT3 and SIRT5 showed differences in average abundance between species and treatment groups (p-value < 0.01 or lower for all comparisons; General Linear Model). Tukey post-hoc analyses showed that SIRT3 levels were higher in *M. galloprovincialis* (p-value $\leq$ 0.05), while levels of SIRT5 were generally higher in *M. trossulus* across all treatments (p-value $\leq$ 0.05; Fig. S1A). Not considering species separately, levels of SIRT3 significantly decreased in response to menadione (MND) and simultaneous exposure to SUR/MND (Fig. S1B). Species-specific pairwise comparisons showed that SIRT3 levels were higher in NAM/MND than in SUR/MND treatment in *M. galloprovincialis* (Fig. 3A). However, while levels were also higher in NAM/MND than in the MND treatment, those differences were marginally insignificant (Tukey post-hoc analysis, P-value $\leq$ 0.05). Similarly, SIRT3 levels were also higher in the NAM/MND in

comparison to the SUR/MND and MND treatments in *M. trossulus*. Thus, while MIOS decreased SIRT3 levels, sirtuin inhibition during NAM/MND increased levels, in contrast to SUR/MND, to levels similar to the control. These results suggest that MIOS decreased SIRT3 levels, clearly indicating that SIRT3 levels are sensitive to an increase in the cellular ROS load and that NAM but not SUR inhibited the response that lowered SIRT3 levels during MIOS.

Conversely, levels of SIRT5 significantly increased following exposure to SUR/MND in *M. galloprovincialis* (Fig. 3B). SIRT5 levels were also higher in response to MND, in comparison to all other treatments, with the exception of SUR/MND, during which SIRT5 levels increased further in *M. galloprovincialis*. There were no differences for *M. trossulus*.

(b)

Figure 3: A General Linear Model was run for all bands to determine differences in average (a) SIRT3 and (b) SIRT5 calibrated volumes between species and treatment groups. Treatment groups are abbreviated as follows: Control (CTL), Nicotinamide (NAM), Nicotinamide + Menadione (NAM/MND), Suramin (SUR), Suramin + Menadione (SUR/MND), Menadione (MND).

For the comparison of the proteomic responses of both *Mytilus* congeners to menadione induced oxidative stress (MIOS), we clustered proteins that changed significantly by functional categories: including protein homeostasis, oxidative stress, energy metabolism, cytoskeletal dynamics, signal transduction, immune defense, and heavy metal binding. Discriminant analyses (DA), which provide 95%-confidence intervals around samples of one treatment, and abundance patterns of these significant and functionally related proteins are used to compare the responses between congeners. A potential caveat of this comparison is that the functional categories include different proteins, potentially representing different cellular processes within a category, e.g. T-complex protein and small HSP are included in the protein homeostasis category in *M.*

galloprovincialis but are absent from the *M. trossulus* data set. Another trade-off of using only significant proteins for the DA is that homologous proteins changing in only one congener are only included in its DA. While the DAs compared all treatments to each other, we will focus our discussion on three comparisons: the one between CTL and MND to evaluate the effect of oxidative stress without inhibitors, between MND and SUR/MND to assess the effect of suramin (SIRT1, 2 and 5) and between MND and NAM/MND to assess the effect of nicotinamide (SIRT1, 2 and 3). Similar effects of the inhibitors might indicate a role for SIRT1 and SIRT2, while different effects (NAM/MND versus SUR/MND compared to each other or relative to MND) were interpreted as indicating SIRT3 and SIRT5 specific effects, respectively.

Protein Homeostasis

Protein homeostasis includes the correct folding of newly translated proteins, the refolding of unfolded proteins subsequent to stress and protein degradation of denatured proteins (Morimoto et al., 2012). Specifically, proteins that we identified as being involved in protein homeostasis play a role as chaperones of the actin and tubulin-based cytoskeleton (sHSPs and TCP-1, respectively) and chaperones with anti-oxidant function (translationally controlled tumor protein), in protein maturation, including glycosylation, in the endoplasmic reticulum (glucose-regulated proteins 78 and 94, and calreticulin), proteolysis (proteasome), and protease inhibition (cystatin).

The separation of treatment groups through the DA of proteins involved in protein homeostasis showed greatly differing patterns between the two congeners (Fig. 4A and 4B). In *M. galloprovincialis*, CTL and MND overlapped in 95%-confidence intervals

(CIs) and both overlapped with SUR/MND. Only NAM/MND differed from MND, indicating that SIRT3 has a greater effect on protein homeostasis during oxidative stress than SIRT5 in *M. galloprovincialis*. In contrast, in *M. trossulus*, CTL and MND differed, and both NAM/MND and SUR/MND differed from MND and each other, indicating that SIRT3 and SIRT5 affect protein homeostasis during oxidative stress. Thus, in *M. galloprovincialis*, MND alone does not seem to affect protein homeostasis, possibly because of other effective cellular responses, e.g. anti-oxidant proteins, and SIRT3 mediated responses.

(a)

Figure 4: Discriminant Analysis on all significant spots involved in protein homeostasis for (a) *M. galloprovincialis* and (b) *M. trossulus*. The contribution of spots in differentiating the treatment groups can be visualized in the above plots.

Among the proteins representing protein homeostasis, glucose-regulated protein 78 (GRP78), calreticulin and endoplasmin (the HSP90 homolog GRP94) are involved in protein maturation in the endoplasmic reticulum (ER) and were represented by isoforms in both species (endoplasmin being the exception). More specifically, these ER chaperones aid in the synthesis, folding, and assembly of secretory and membrane proteins (Feige and Hendershot, 2011; Appenzeller-Herzog, 2011).

In both congeners, levels of calreticulin, an ER chaperone that binds to glycosylated proteins that are misfolded, slightly decreased during MND relative to the control, but not significantly (Fig. 5A and 5B). However, while calreticulin was similar between MND and SUR/MND, levels were higher in comparison to both during NAM/MND in *M. galloprovincialis*. While MND also reduced levels of calreticulin relative to the CTL in *M. trossulus* (but not significantly so), in contrast to *M.*

galloprovincialis, MND differed from SUR/MND but not NAM/MND. Thus both SIRT3 and SIRT5 play an important putative role in regulating the abundance of calreticulin during oxidative stress in *M. galloprovincialis* and *M. trossulus*, respectively.

(a)

(b)

Figure 5: Expression profile graphs showing the relative expression of proteins involved in protein homeostasis in (a) *M. galloprovincialis* and (b) *M. trossulus*. Spot volumes

were obtained by normalizing against the volume of all proteins, and means $\pm$ 1 SEM are shown (N=6 per treatment: *M. galloprovincialis*; N=4-6 per treatment: *M. trossulus*). Treatments with significant differences in expression level are marked with different letters. Treatment groups are abbreviated as follows: Control (C), Nicotinamide (N), Nicotinamide + Menadione (N/M), Suramin (S), Suramin + Menadione (S/M), Menadione (M).

GRP78 aids in the proper folding of secretory and membrane proteins (Melnick et al., 1994). Levels of two GRP78 isoforms remained unchanged during CTL, MND, NAM/MND and SUR/MND in *M. galloprovincialis* but one isoforms (spot 70) showed differences between NAM/MND and SUR/MND. In *M. trossulus*, two GRP78 isoforms did not change between the CTL and MND, but one (spot 9) was higher during SUR/MND in comparison to both MND and NAM/MND. Both isoforms showed opposite changes in abundances with SUR/MND relative to the MND group, possibly indicating two isoforms differing in a post-translational modification, which is dependent on the activity of SIRT5, e.g. succinylation.

Endoplasmin (or glucose-regulated protein 94), a HSP90 homolog localized in the ER, works in concert with GRP78, and did not differ between CTL, MND, NAM/MND and SUR/MND in *M. trossulus* (Fig. 5B). In addition, simultaneous exposure to SUR/MND further increased its abundance in *M. trossulus*. While not significant, patterns of abundance changes clustered with those of GRP78 and calreticulin. Thus, all three ER chaperones seem to be affected by suramin only, and thus, by extension, SIRT5 in *M. trossulus*. The opposite patterns, an effect of NAM or SIRT3 was observed for calreticulin and possibly one of the GRP78 isoforms in *M. galloprovincialis*. Thus, ER chaperones abundance changes are putatively affected by SIRT3 and SIRT5 in *M.*

galloprovincialis and *M. trossulus*, respectively. A recent study showed that sirtuins interacts with several HSP70s and GRP78 (Law et al., 2009).

Translationally controlled tumor protein (TCTP), a heat-stable antioxidant protein, is one of the most abundant proteins expressed in normal cells (Gnanaekar et al., 2009; Nagano-Ito et al., 2009). More specifically, in addition to acting as a molecular chaperone to protect against thermal damage, research using retroviral-mediated expression cloning has demonstrated TCTP's ability to protect cells from H₂O₂-induced toxicity and cell death (Gnanaekar et al., 2009; Nagano-Ito et al., 2009). In *M. trossulus* two isoforms of TCTP, were shown to remain relatively constant in abundance across treatment groups, with the exception of a significant decrease during periods of simultaneous exposure to NAM/MND for one and an increase for the other isoform. (Fig. 5B). This pattern of opposite shifts in abundance with sirtuin inhibition, in this case NAM, suggest that these isoforms represent two different PTMs, most likely affected by SIRT3.

Both congeners changed the abundance of two proteasome type β isoforms (Fig. 5A and 5B). Neither one of those isoforms changed with MND in *M. galloprovincialis*, but one (spot 46) showed higher levels during NAM/MND in comparison to both MND and SUR/MND, suggesting a role for SIRT3. One isoform (spot 58) showed a similar pattern in *M. trossulus*. The other isoform only showed a significant effect of NAM under non-stressful conditions. Thus, MND only affects proteasome type β in *M. trossulus* but NAM/MND (but not SUR/MND) leads to higher levels relative to MND. While this proteasome subunits seems to be affected by SIRT3 in both congeners, the abundance of this subunit seems to be more sensitive to oxidative stress in *M. trossulus*.

A reduction in proteasome activity during oxidative stress could prolong the lifespan of oxidative stress proteins as it has been shown in human endothelial cells exposed to low levels of proteasome inhibition (Bieler et al., 2009). It is possible that this is a last resort response while protein synthesis is inhibited due to stressful conditions.

M. galloprovincialis also showed changes in molecular chaperones that are involved in the folding of tubulin and actin (T-complex protein 1; TCP1) and the stabilization of the cytoskeleton (small HSPs; Sternlicht et al., 1993; Brackley and Grantham, 2009). TCP1 decreased with oxidative stress (MND), but sirtuin inhibition had no effect. In contrast, small HSP 24.1 differed between NAM/MND and both MND and SUR/MND (all were similar to CTL), suggesting that SIRT3 was involved in the response of this cytoskeletal chaperone to oxidative stress in *M. galloprovincialis*.

Oxidative Stress

In *M. galloprovincialis*, the proteins directly involved in responding to oxidative stress belong to the peroxiredoxin-thioredoxin (Prx-Trx) ROS-scavenging system and depend on NADPH as a reducing equivalent to maintain thioredoxin (Trx) in a reduced state via the action of thioredoxin reductase (Murphy, 2012). A third protein, cystathionine γ -lyase (CGL), is part of the pathway synthesizing cysteine, which is a rate-limiting step towards the antioxidant glutathione (GSH). GSH plays a role similar to Trx, as it is used by GSH peroxidase to scavenge hydrogen and organic peroxides (Murphy, 2012).

In *M. galloprovincialis*, only the MND group was separated from all other treatments, all of which overlapped with the CTL (Fig. 6). Interestingly, both

NAM/MND and SUR/MND differed from MND but not the control group or from each other. Thus, since both sirtuin inhibitors affected the response to MND in similar ways, it is possible that SIRT1 and SIRT2 might affect the abundance changes of these three anti-oxidant proteins response in *M. galloprovincialis*.

Figure 6: Discriminant Analysis on all significant spots involved in ROS scavenging and redox regulation (oxidative stress) for *M. galloprovincialis*. The contribution of spots in differentiating the treatment groups can be visualized in the above plot.

In contrast, in *M. trossulus* we did not identify a single anti-oxidant protein, even including some we identified previously, e.g. DyP-type peroxidase, superoxide dismutase (SOD) and Trx, and which responded to temperature and hyposalinity stress (Fields et al., 2012; Tomanek and Zuzow, 2010; Tomanek et al., 2012).

The results from the DA are reflected in the changes in protein abundance (Fig. 7). Both, CGL and Prx5 showed an unexpected lower abundance under MND relative to the CTRL, and both increased their abundance in response to the simultaneous treatment of sirtuin inhibition and oxidative stress, suggesting that one of or both SIRT1 and

SIRT2, which are inhibited by both inhibitors, are affecting these changes. Trx levels were different between NAM/MND and SUR/MND, possibly indicating a role for one of the sirtuins SIRT3 or SIRT5. It is surprising that CGL and Prx5 cluster together and that their abundance changes differ from Trx, especially since Prx5 is reduced by Trx and not GSH (Murphy, 2012). However, there is a precedent for a role of GSH as a reductant for oxidized peroxiredoxin. Despite this, we have no explanation for why their abundance decreased with oxidative stress, except for the possibility that these two represent isoforms that are less active under oxidative stress and that we did not identify the active form, which supposedly increased during MND, as scenario that has been shown for Prx (Rabilloud et al., 2002; Wagner et al., 2002).

Figure 7: Expression profile graphs showing the relative expression of proteins involved in ROS scavenging and redox regulation (oxidative stress) in *M. galloprovincialis*. Spot volumes were obtained by normalizing against the volume of all proteins, and means $\pm$ 1 SEM are shown (N=6 for all groups). Treatments with significant differences in expression level are marked with different letters. Treatment groups are abbreviated as follows: Control (C), Nicotinamide (N), Nicotinamide + Menadione (N/M), Suramin (S), Suramin + Menadione (S/M), Menadione (M).

Both, the Trx-Prx and glutathione ROS-scavenging system depend on NADPH as the reducing equivalent for Trx and GSH (Murphy, 2012). It is therefore noticeable that one (spot 24) of the two isoforms of NADP-dependent isocitrate dehydrogenase (NADP-ICDH) in *M. galloprovincialis* showed higher abundance under SUR/MND than NAM/MND (Fig. 7), similar to Trx. The other isoform in *M. galloprovincialis* and the only one identified in *M. trossulus* showed similar abundance changes between SUR and SUR/MND. These results might suggest that SIRT5 is regulating both, Trx and NADP-ICDH. The importance of NADP-ICDH was shown when decreased expression led to elevated levels of ROS, whereas the reverse was the case for cells that overproduce NADP-ICDH (Jo et al., 2001; Lee et al., 2002).

The six isoforms that make up the Prx family can be distinguished by intracellular location, structure and function (Radyuk et al., 2010). More specifically, mitochondria contain two Prx isoforms, Prdx3 and Prdx5 (Mailloux et al., 2013). While Prx3 activity is directly linked to the Trx system, Prx5 functions also in the antioxidant defense against mtDNA damage and cell survival (Radyuk et al., 2010; Banmeyer et al., 2005; Kroptov et al., 2006; Nguyen-nhu and Knoop 2003; Yuan et al., 2004; Knoop et al., 2011). Reduced glutathione, one of the major cellular anti-oxidants and scavengers of ROS in the minimal stress proteome, is a redox-active tripeptide (consisting of glutamate, glycine, and cysteine) whose relatively high concentration compared to oxidized glutathione (GSSG) is known to help maintain homeostatic conditions necessary for disulfide-bond formation in the ER (Appenzeller-Herzog, 2011). The synthesis of GSH is highly dependent on the availability of its rate-limiting precursor, cysteine, the majority of which is derived from methionine through the transsulfuration pathway (Kandil et al.,

2010; Diwakar and Ravindranath, 2007; Garcia-Garcia et al., 2012). CGL, the final (rate-limiting) enzyme in the transsulfuration pathway, is responsible for catalyzing the conversion of L-cystathionine into L-cysteine, α -ketobutyrate, and hydrogen sulfide (Diwakar and Ravindranath, 2007). Several studies show that perturbations in the transsulfuration pathway decrease levels of cellular GSH, suggesting an important link between functional levels of CGL and GSH (Diwakar and Ravindranath, 2007; Meister, 1995; Mosharov et al., 2000). Moreover, recent work showed that not only does hydrogen sulfide, a byproduct of cysteine synthesis, directly inhibit ROS/oxidative stress, but also has implicated roles in increasing the abundance of SIRT1 (Zhang et al., 2013). Therefore, in addition to its role in GSH regulation, CGL also functions to regulate intracellular levels of SIRT1 – a class of sirtuins directly affected by NAM and SUR. In *M. galloprovincialis* levels of CGL remain relatively constant following sirtuin inhibition, however they decreased during periods of MIOS (Fig. 7).

Energy Metabolism

The production of ROS is closely linked to energy metabolism (Murphy, 2009). More specifically, we identified several proteins that play roles in maintaining intracellular redox balance (NADP-ICDH), energy production (cytochrome c reductase, phosphoenolpyruvate carboxykinase, malate and dihydrolipoyl dehydrogenase), and amino acid metabolism (methylmalonate-semialdehyde dehydrogenase, 3-hydroxyisobutyrate dehydrogenase).

A comparison of the DA of metabolic proteins showed that both SUR/MND and NAM/MND differed from MND (all were separated from CTL), suggesting that SIRT1,

2, 3 or 5 affect metabolism in during oxidative stress in both species. However, under non-stressful conditions SUR (but not NAM) differed from CTL in *M. galloprovincialis*, while NAM (but not SUR) differed from the CTL in *M. trossulus*, suggesting that SIRT5 has a greater effect in the former and SIRT3 a greater effect in the latter species under homeostatic conditions (Fig. 8A and 8B).

(a)

Energy Metabolism

Protein ID

- (3) UDP-N-acetylhexosamine pyrophosphorylase
- (4) Amine oxidase
- (15) 4-hydroxybutyrate CoA transferase
- (19) NADP-dependent isocitrate dehydrogenase
- (24) NADP-dependent isocitrate dehydrogenase
- (39) O-methyltransferase mdmC
- (59) Dihydropteridine reductase
- (60) V-type proton ATPase
- (61) V-type proton ATPase
- (90) Methylmalonate-semialdehyde dehydrogenase
- (131) Phosphoglycerate kinase 1
- (284) Cytochrome c reductase

(b)

Energy Metabolism

Protein ID

- (10) Phosphoenolpyruvate carboxykinase (cyto.)
- (17) Dihydrolipoyl dehydrogenase (mito.)
- (22) 4-hydroxybutyrate CoA-transferase
- (26) Ornithine aminotransferase
- (35) UDP-glucose 4-epimerase
- (48) 3-hydroxyisobutyrate dehydrogenase (mito.)
- (63) Cytochrome c reductase
- (166) NADP-dependent isocitrate dehydrogenase
- (226) Malate dehydrogenase (cyto.)

Figure 8: Discriminant Analysis on all significant spots involved in energy metabolism for (a) *M. galloprovincialis* and (b) *M. trossulus*. The contribution of spots in differentiating the treatment groups can be visualized in the above plots.

Metabolic Proteins

Since the state of energy metabolism is greatly affected by intracellular pH (Hochachka and Somero, 2002), we were interested in the putative role of vacuolar-type H^+ -ATPase (v-ATPase), which function is to pump protons into the lumen of organelles such as lysosomes, endosomes, and synaptic vesicles, hydrolysing ATP in the process (Hirata et al., 2003; Hinton et al., 2007). Several studies show yeast lacking v-ATPase accumulate elevated levels of ROS and thus have increased susceptibility to oxidative protein damage (Kane, 2007; Milgrom et al., 2007). Similarly, another study revealed v-ATPase's sensitivity to fluctuations in intracellular H_2O_2 concentration (Wang and Floor, 1998).

While one (spot 60) of the two v-ATPase isoforms in *M. galloprovincialis* showed higher levels in response to NAM/MND relative to SUR/MND and SUR, with levels during MND treatment in-between, the other decreased during both periods of MND and SUR/MND relative to both CTL and NAM/MND (spot 61) (Fig. 9A). Thus, while spot 60 might not be affected by either one of the sirtuin inhibitors, spot 61 showed an increase with NAM/MND (but not SUR/MND) relative to MND alone, suggesting that SIRT 3 modified its abundance.

(a)

(b)

Figure 9: Expression profile graphs showing the relative expression of proteins involved in energy metabolism (including proteins involved in inorganic ion transport, amino acid, and fatty acid metabolism) in (a) *M. galloprovincialis* and (b) *M. trossulus*. Spot volumes were obtained by normalizing against the volume of all proteins, and means $\pm$ 1 SEM are shown (N=6 per treatment: *M. galloprovincialis*; N=4-6 per treatment: *M. trossulus*). Treatments with significant differences in expression level are marked with different letters. Treatment groups are abbreviated as follows: Control (C), Nicotinamide (N), Nicotinamide + Menadione (N/M), Suramin (S), Suramin + Menadione (S/M), Menadione (M).

Cytochrome c reductase, a key component of the electron transport chain (ETC; Complex III) and source of ROS (Liu et al., 2002; Finkel and Holbrook, 2000; Gunter et al., 2012; Droese and Brandt, 2008) showed no change during MND or sirtuin inhibitors/MND (Fig. 9A and 9B). The one isoform in *M. galloprovincialis* showed a difference between NAM and SUR under non-stressful conditions.

While the electron transport chain (ETC) contributes to ROS production, the NADH/NAD⁺ ratio, which is controlled in large part by the activity of dehydrogenases of the citric acid cycle, also contributes to mitochondrial ROS production (Tahara et al., 2007). In *M. trossulus*, we identified an isoform of dihydrolipoyl-dehydrogenase

(DHLDH), the E3 component of α -ketoglutarate dehydrogenase (α -KGDH), a key component of the Krebs cycle, and of pyruvate dehydrogenase, which is the primary link between glycolysis and Krebs cycle (Martin et al., 2005; Tretter and Adam Vizi, 2004). As DHLDH has been identified as a potential source of ROS during periods of oxidative stress in the mitochondria, an anti-oxidative strategy requires lowering the abundance of DHLDH in order to minimize ROS production (Martin et al., 2005; Tretter and Adam Vizi, 2000; Tretter and Adam Vizi, 2004; Tahara et al., 2007). Thus, the decrease of DHLDH can be seen as an anti-oxidative response to MND. Sirtuin inhibitors did not modify this response further.

Three proteins representing a branching point of anaerobic metabolism were identified in *M. trossulus*. While phosphoenolpyruvate carboxykinase (PEPCK) converts PEP to oxaloacetate, malate dehydrogenase further converts oxaloacetate to malate, which feeds into the citrate acid cycle (Michal and Schomburg, 2012). PEPCK was different between SUR and SUR/MND (Fig. 9B). During glycolysis, pyruvate kinase converts PEP to pyruvate. Bivalves employ a condensation reaction in which an amino acid, arginine, condenses with pyruvate to form iminoacids, a reaction catalyzed by octopine dehydrogenase (ODH; Müller et al. 2012). ODH abundance was lower during SUR/MND in comparison to both MND and NAM/MND, suggesting a role for SIRT5.

The decrease in levels of key metabolic enzymes linking glycolysis to the Krebs cycle (DHLDH – and thus PDH) suggest the down-regulation of NADH production, providing support for the hypothesis that perhaps *M. trossulus* is experiencing a shift in redox potential from NADH-fueled respiratory pathways to pathways producing NADPH. This is further supported by the slight increase in NADP-ICDH in *M. trossulus*

– which acts to facilitate the production of NADPH as a reducing equivalent for either the glutathione or the thioredoxin-peroxiredoxin pathway. These metabolic changes are shown to occur in several studies in response to the acetylation status of metabolic enzymes, a PTM affected by the activity of sirtuins (Choudhary et al., 2009, Wang et al., 2010; Zhao et al., 2010). Similar findings were observed by Tomanek and Zuzow, 2010 – providing further support for this hypothesis.

Cytoskeleton

The actin cytoskeleton requires constant reorganization and regulation in order to facilitate the coordinated action of several cellular functions (Hall, 1998). Cytoskeletal dynamics not only govern cellular structure and motility, but also are involved in cell division, endo- and exocytosis, cell polarity and signal transduction (Valderrama et al., 2001). Cytoskeletal proteins that we identified play a role in actin-treadmilling (F-actin capping protein, profilin), actin filament stabilization (fascin) and severing (severin, which shares an actin severing domain with gelsolin; coactosin is homologous to actin depolymerization factor), ciliary structure (dynein heavy chain, radial spoke head proteins, tektins), and extracellular matrix (collagen).

The separation of treatment groups through the DA of proteins involved in cytoskeletal modifications and regulation showed several similar patterns between the two congeners (Fig. 10A and 10B). In both congeners, NAM/MND, SUR/MND, and MND are separated from each other (and from the CTL, with the exception of SUR/MND in *M. trossulus*), suggesting that SIRT3 and SIRT5 affect the abundance of cytoskeletal proteins during oxidative stress.

Figure 10: Discriminant Analysis on all significant spots involved in cytoskeletal dynamics for (a) *M. galloprovincialis* and (b) *M. trossulus*. The contribution of spots in differentiating the treatment groups can be visualized in the above plots.

Actin-Binding Proteins

Controlled actin polymerization, “tread milling,” expands the cell membrane, drives the formation of cellular protrusions, and facilitates intracellular movement (Le Clainche and Carlier, 2008; Adams, 2004). F-actin capping proteins bind and thereby cap the barbed end of actin filaments, thereby increasing the availability of monomeric actin while preventing/terminating actin filament growth (Le Clainche and Carlier, 2008). In *M. galloprovincialis*, one F-actin capping protein isoform (spot 41) increased in response to MND (Fig. 11A). The same isoform decreased in both NAM/MND and SUR/MND relative to MND, suggesting an influence of sirtuins, possibly SIRT1 and SIRT2. A second isoform (spot 31) showed a difference between NAM/MND (but not SUR/MND) and MND, further supporting a role for sirtuins in modifying the activity of F-actin capping protein. Paralleling these changes, in *M. trossulus*, abundances of F-actin capping protein are lower in NAM/MND than in both, MND and SUR/MND. This suggests that abundances of these isoforms are dependent on the activity of sirtuins, possibly SIRT3 only, in both congeners.

(a)

(b)

Figure 11: Expression profile graphs showing the relative expression of proteins involved in cytoskeletal dynamics in (a) *M. galloprovincialis* and (b) *M. trossulus*. Spot volumes were obtained by normalizing against the volume of all proteins, and means $\pm$ 1 SEM are shown (N=6 per treatment: *M. galloprovincialis*; N=4-6 per treatment: *M. trossulus*). Treatments with significant differences in expression level are marked with different letters. Treatment groups are abbreviated as follows: Control (C), Nicotinamide (N), Nicotinamide + Menadione (N/M), Suramin (S), Suramin + Menadione (S/M), Menadione (M).

While F-actin capping protein halts actin polymerization, profilin binds globular (G actin) and uses ATP activity to promote nucleation and growth of barbed G-actin (globular actin) filaments (Suetsugu et al., 1999). Not only does profilin induce actin assembly, but also it is involved in actin-based motility and cytoskeletal regulation through the activation of membrane-bound signaling molecules (Suetsugu et al., 1999). In *M. trossulus*, profilin abundance significantly decreased in response to MND. Furthermore, profilin levels were lower during NAM/MND, but higher during SUR/MND than in MND (Fig. 11B), possibly suggesting opposite effects by SIRT3 and SIRT5. Thus, the fluctuations in profilin abundance support the hypothesis that oxidative stress ceases actin filament growth in *M. trossulus* and that sirtuins affect this response. These results coincide with conclusions drawn from other studies, as previous work shows a decrease in actin “tread milling” and cell membrane expansion in *M. trossulus* following periods of acute stress (Tomanek, 2012; Tomanek and Zuzow, 2010).

Dynamic Microtubule Network

Not only do actin-based cytoskeletal elements need constant reorganization, but dynamic remodeling of the microtubule cytoskeleton is also essential for the maintenance of several cellular processes including cell division, cilia and flagella motility, intracellular transport, and differentiation (Gardner et al., 2011; Yang et al., 2006). Microtubules are dimers of α and β subunits that are constantly transitioning between tightly regulated periods of polymerization and depolymerization (Gardner et al., 2011). During periods of oxidative stress, we see an increase in α tubulin 3 and β tubulin isoforms in *M. trossulus*. A previous study found that menadione-induced thiol oxidation decreases or inhibits the

assembly of microtubules through the oxidation of sulfhydryl groups on tubulin dimers (Bellomo et al., 1990). Thus, we hypothesize that the increased abundance of α tubulin 3 chain and β tubulin is potentially due to decreased polymerization in response to oxidative *in vivo* conditions (in vitro conditions for protein separation are generating a reducing environment).

Ciliary axonemes are composed of two central microtubules surrounded by nine microtubule doublets, which are interconnected by nexin links, radial spokes, and dynein arms – all of which were identified in one or both congeners (Yang et al., 2006). Combined, these components not only provide support and structure, but also allow for bending and flexibility (Yang et al., 2006). Tektins are a group of highly specialized microtubule stabilizing proteins that aid in the assembly of ciliary and flagellar axonemes (Amos, 2008). A decrease in tektin-4 abundance in *M. galloprovincialis* during MND leads us to believe that mussels arrests the assembly of new ciliary elements during oxidative stress.

Dyneins, multi-complex molecular motor proteins composed of heavy and light chains, can be classified as either axonemal or cytoplasmic (Asai and Koonce, 2001). The heavy chain component of each dynein contains both an ATPase and microtubule motor domain, driving movement through ATP-dependent reactions. Cytoplasmic dynein facilitates the retrograde transport of intracellular cargo (e.g. vesicles made by the endoplasmic reticulum, endosomes, and lysosomes), using chemical energy derived from ATP, while axonemal dynein governs the beating of cilia and flagella (Asai and Koonce, 2001). As previously mentioned, ciliary movement (e.g. beating) is of particular importance in *Mytilus* for nutrient acquisition across the gill epithelium (Moore, 1971).

Dynein plays a key role in this process as a loss in coordination of beating or the complete cessation of beating decreases the organism's ability to effectively sequester nutrients by filtration (Moore, 1971). While abundances of dynein differed between NAM/MND and SUR/MND in *M. galloprovincialis*, in *M. trossulus* abundances were higher during NAM/MND than both MND and SUR/MND (Fig. 11A and 11B). Overall these results indicate that SIRT3 regulates the activity of dynein heavy chain 10 (axonemal) during oxidative stress.

Additionally, we see regulation of dynein control through the radial spoke head proteins (RSPs) – proteins that transmit signals to the dynein arms (Yang et al., 2006). Previous studies showed that cilia or flagella lacking radial spoke head proteins (RSPs) cease function or show signs of abnormal motility (Yang et al., 2006). Similarly, errors in ciliary orientation are associated with physiological dysfunction, and thus proper orientation of cilia relative to tissue axes is just as important as motility itself (Marshall and Kintner, 2008). One of two different RSPs showed higher abundances during NAM/MND than both MND and SUR/MND in *M. trossulus* (Fig. 11B). The two isoforms, RSP1 and RSP9, are encoded by separate genes and are regulated independently (Gingras et al., 1998). Based on our results, sirtuins seem to affect the function of dynein through PTMs..

Extracellular Matrix Proteins

Collagen proteins are not only involved in structuring the extracellular matrix, but also play an integral role in byssal thread production by foot tissue – an important component for intertidal adhesion (Harrington and Waite, 2007). In *M. galloprovincialis*, we

identified several different collagen proteins (short-chain collagen C4, collagen α -6(VI) chain, and collagen α -5(VI) chain). At least one (spot 71) of two collagen VI chain isoforms showed higher abundance during NAM/MND when compared to MND and SUR/MND (Fig. 11A). It is therefore possible that SIRT3 plays a regulatory role in the abundance of collagen VI chain isoforms. Short chain collagen C4 abundance differed between NAM/MND or SUR/MND in *M. galloprovincialis*, opposite the pattern of collagen VI. In *M. trossulus*, a short chain collagen isoform had lower levels following both NAM/MND and SUR/MND than the CTL. Thus, sirtuins seem to modify collagen isoforms, at least in *M. galloprovincialis*, in response to oxidative stress.

Intracellular Signaling

Aspects of cell structure, motility and adhesion as well as vesicle transport are in part controlled by the small G-protein superfamily, including Ras, Rho, Arf, Sar1, and Ran families (Sasaki and Takai 1998; Burridge and Wennerberg, 2004; Marks et al., 2009). More specifically, members of the Rho family facilitate signaling between membrane receptors and the actin cytoskeleton (Hall 1998). Specifically, proteins that we identified as being involved in signal transduction play a role in Rho GTPase activity (G-protein: type- β and Rho GDP-dissociation inhibitor 1), nuclear particles (major vault proteins), apoptotic cascades (tripartite motif containing protein 2 – TRIM2), vesicular transport (Rab GDP dissociation inhibitor 2, and Rab2), cell cycle progression, stress signal amplification, and actin-binding (14-3-3 protein γ , Cdc42, and mitogen-activated protein kinase 1).

The separation of treatment groups through the DA of proteins involved in signal transduction showed distinct patterns between the two congeners (Fig. 12A and 12B). In *M. galloprovincialis*, the DA overlaid all treatments except MND. This suggests that abundance patterns of signaling proteins were affected by oxidative stress, and that sirtuin inhibition (NAM or SUR) reversed this response, similar to the pattern for oxidative proteins. Therefore, we suspect that at least one of the sirtuins inhibited by NAM and SUR, e.g. SIRT1, 2, 3 or 5, has an affect on the abundance of proteins involved in signal transduction in this congener.

(a)

Figure 12: Discriminant Analysis on all significant spots involved in signal transduction for (a) *M. galloprovincialis* and (b) *M. troossulus*. The contribution of spots in differentiating the treatment groups can be visualized in the above plots.

In *M. troossulus*, MND overlaps with CTL and NAM/MND with MND (Fig. 12B).

So there is no response to MND. Only SUR/MND is separate from all the aforementioned treatments. This implies that in *M. troossulus* proteins involved in signaling are more affected by sirtuin inhibition during oxidative stress than oxidative stress alone. Thus, it is possible that SIRT5 plays an important regulatory role in signal transduction in *M. troossulus*.

G-proteins relay signals to downstream effector proteins when in a GTP-bound activate state (Sasaki and Takai 1998). Downstream, Ras-related proteins serve as “molecular switches,” regulating several components of intracellular actin dynamics (Sasaki and Takai 1998). When bound to GTP, these small G-proteins are in their activated state (Sasaki and Takai 1998). Rho-GDP dissociation inhibitors (Rho-GDI)

sequester Rho to the cytosol, and thus are key regulators of down-stream Ras/Rho-mediated processes (Hall 1998; Sasaki and Takai 1998).

In both congeners, we identified isoforms of Rho GDI1 (Fig. 13A and 13B). In *M. galloprovincialis* a difference in abundance was observed between organisms exposed to NAM/MND (but not SUR/MND) and MND. Conversely, in *M. trossulus*, Rho GDI differed between SUR/MND and all other treatments. Therefore, results suggest that sirtuins Rho GDI1 during oxidative stress. More specifically, SIRT3 possibly plays a role in affecting Rho-GDI in *M. galloprovincialis*, whereas SIRT5 seems to affect Rho-GDI in *M. trossulus*. In both cases, the inhibitor treatment increased the abundance of Rho-GDI (supposedly the de-acetylated isoform).

(a)

(b)

Figure 13: Expression profile graphs showing the relative expression of proteins involved in signal transduction (including proteins involved in vesicular and nuclear transport) in (a) *M. galloprovincialis* and (b) *M. trossulus*. Spot volumes were obtained by normalizing against the volume of all proteins, and means $\pm$ 1 SEM are shown (N=6 per treatment: *M. galloprovincialis*; N=4-6 per treatment: *M. trossulus*). Treatments with significant differences in expression level are marked with different letters. Treatment groups are abbreviated as follows: Control (C), Nicotinamide (N), Nicotinamide + Menadione (N/M), Suramin (S), Suramin + Menadione (S/M), Menadione (M).

Cdc42, another member of the Rho family, prevents binding of capping proteins to allow for nucleation (Huang et al., 1999; Marks et al., 2009). Cdc42 not only stimulates the formation of actin-rich cell membrane protrusions, but also coordinates cell cycle progression, stress fiber formation, motility, intracellular vesicle trafficking, and survival through the activation of stress signaling pathways, e.g. c-Jun NH₂-terminal kinase (JNK) pathway and the p38 mitogen-activated protein kinase (MAPK) cascade (Hall 1998; Cowan and Storey, 2003; Marks et al., 2009). While the opposing expression patterns of both cdc42 and F-actin capping protein in *M. galloprovincialis* seems contradictory (Fig. 13A and 13B), perhaps the decreased expression of cdc42 during periods of oxidative stress results to offset the effects of F-actin capping protein cytoskeletal regulation. The significant decrease in cdc42 during MND could also be a cellular defense mechanism implemented to decrease rates of mitosis until more favorable extracellular conditions are available.

As mentioned above, various signaling molecules can enhance intracellular communication. For example, MAPKs are signal-transducing enzymes that are involved in several cellular processes including proliferation and survival (Chang and Karin, 2001). The MAPK signaling cascade controls cellular responses to physical and chemical stresses by connecting cell-surface receptors to intracellular regulatory proteins through the subsequent phosphorylation of multiple cytoplasmic MAPKs (Seger and Krebs, 1995; Chang and Karin 2001). MAPKs amplify any given signal by several orders of magnitude, making them powerful global regulators of cell function (Seger and Krebs, 1995). Although while there was an increase in the abundance, although not significant, of MAPK1 between NAM/MND versus MND in *M. galloprovincialis*, suggesting an

effect of SIRT 1, 2 and 3, the significantly higher abundance during SUR/MND relative to MND in *M. trossulus* suggested a role for SIRT5, and therefore an effect dissimilar between the congeners, but similar to the interspecific differences for Rho-GDI.

The protein 14-3-3 γ is highly conserved and plays a critical role in a variety of intracellular processes including, but not limited to, signal transduction, cell cycle progression, cell cycle arrest, and stress responses (Pearce and Humphrey, 2001; Jin et al., 2008). More specifically, to elicit specific responses, these proteins alter the sub-cellular location, activity, PTMs, and molecular interaction of their substrates (Jin et al., 2008). In *M. trossulus* we identified an isoform of 14-3-3 γ that increased in abundance following NAM in comparison to MND and SUR/MND. While insignificant, an increase was also observed following simultaneous exposure to NAM/MND. Taken together, results suggest 14-3-3 γ levels are coupled to sirtuin activity, supporting the findings of an earlier study, which showed that 14-3-3 γ may act as a positive regulator of SIRT2 (Jin et al., 2008).

Signaling and Apoptosis

Bcl2 proteins are a family of proteins that control mitochondria membrane permeability; thereby making them key regulators of mitochondria-mediated cell death (Marks et al., 2009). Bim, a BH3-only member of the Bcl-2 family, stimulates apoptotic cascades in response to periods of ER stress (Puthalakath et al., 2007; Thompson et al., 2011). When the global attenuation of protein synthesis and/or the increased abundance of molecular chaperones can no longer process high volumes of unfolded proteins caused by periods of cellular stress, Bim is dephosphorylated – and thereby activated and not degraded

(Puthalakath et al., 2007). To avoid triggering the signal-controlled apoptotic cascade, recent proteomic findings showed that tripartite motif containing protein 2 (TRIM2; a ubiquitin-protein ligase E3) ubiquitinates p42/p44 MAPK phosphorylated Bim (Thompson et al., 2011). Therefore, we hypothesize that TRIM2 acts as a pro-survival protein during periods of oxidative stress in *Mytilus*.

In *M. galloprovincialis* we saw an increase in TRIM2 following exposure of NAM in comparison to the CTL, but otherwise there were no differences (Fig. 13A). In *M. trossulus* we saw a decrease in response to SUR/MND in comparison to the CTL (Fig. 13B), indicating that sirtuin inhibition or/and MND treatment decreased TRIM2. MND alone lowered TRIM2 abundance, but not significantly, suggesting that SIRT inhibition by SUR indeed affected, possibly lowered, TRIM2 abundance. Since there was no difference between NAM/MND and MND (or CTL), it is possible that SIRT5 affects TRIM2 in *M. trossulus*.

Nuclear Transport

Both congeners showed a conspicuous row of proteins (~100kDa), which were identified as the major vault protein (MVP). While MVPs are pervasive components of vault particles, their exact function has still yet to be elucidated (Suprenant et al., 2007; Berger et al., 2009). They are implicated in affecting stress-induced signaling cascades, including the MAPK pathway, and thus may be mediators of the oxidative stress response (Berger et al., 2009). Isoforms of MVP showed interspecific differences in how they responded (Fig. 13A and 13B). In *M. galloprovincialis* one MVP (spot 65) decreased during MND relative to the SUR treatment. Another one (spot 66) decreased during

SUR/MND versus CTL, NAM/MND and SUR but not MND. In *M. trossulus* levels decreased during SUR/MND relative to all other treatments, including MND, similar to the MVP (spot 66) in *M. galloprovincialis*. It is therefore likely that SIRT5 affects the abundance of MVP isoforms during MIOS in both congeners, may be more so in *M. trossulus*.

Vesicular Transport

In *M. galloprovincialis* we identified two small G-proteins that play a role in the vesicular transport from the ER to the Golgi: Rab-GDP dissociation inhibitor 2 (Rab-GDI2) and Rab2 (Marks et al., 2009). Like Rho-GDI1, Rab-GDI2 regulates the intracellular location of Rab, thereby controlling its activity (Marks et al., 2009). While the exact role for Rab proteins in vesicular trafficking has yet to be elucidated, their common mode of action involves the recruitment of tethering proteins for contact between the vesicle and target membrane (Marks et al., 2009). While Rab-2 showed higher abundances during NAM/MND in comparison to both MND and SUR/MND, Rab-GDI2 showed lower levels during NAM/MND in comparison to MND (but not SUR/MND). It thus seems possible that SIRT3 plays a role in modifying the docking process of vesicles through opposing effects on Rab2 and Rab-GDI2 in *M. galloprovincialis* during oxidative stress.

Immune Defense

The complement system, part of the innate and adaptive host defense against invasive pathogens, is activated through one of three pathways (Gestal et al., 2010). Proteins that

we identified as being involved in the immune response, C1q-domain containing proteins, are target recognition proteins of the classical complement pathway, capable of binding to multiple antigen-antibody complexes for pathway activation (Gestal et al., 2010; Carland and Gerwick, 2010). While invertebrates are known to be equipped with innate immune systems, there has been little evidence of specific adaptive immunity, despite that the two systems are intricately linked (Gestal et al., 2010). However, recent studies published regarding the presence of a complement system in bivalves (e.g. mussels and scallops) reveal that C1q protects mollusks against both bacterial and parasitic infections – suggesting not only a pro-survival role for this gene, but also providing evidence for a more evolved immune system in these marine invertebrates than previously identified (Gestal et al., 2010; Zhang et al., 2008). Fibrinogen is part of the complement cascade and the pore-forming apextrin might be involved in the downstream effects of the complement-activation cascade (Romero et al. 2011; Estévez-Calvar et al. 2011).

In *M. galloprovincialis*, the DA of immune-related proteins separated MND from all three CTL, NAM/MND and SUR/MND, which overlapped (Fig. 14). Thus, SIRT inhibitors reset the cellular response to CTL, possibly indicating that SIRT1 and 2 are affecting the complement cascade during oxidative stress. Only one complement protein changed in *M. trossulus*, and thus a DA was not performed.

Figure 14: Discriminant Analysis on all significant spots involved in immune defense for *M. galloprovincialis*. The contribution of spots in differentiating the treatment groups can be visualized in the above plots.

In *M. galloprovincialis*, only one of the complement containing proteins (MgC1q44) showed lower levels during NAM/MND and SUR/MND in comparison to MND (Fig. 15A). In *M. trossulus*, the one isoform of MgC1q6 remained at relatively constant levels, except for a significant decrease following NAM/MND (Fig. 15B). While both sirtuin inhibitors affected the MgC1q44 isoform in *M. galloprovincialis*, only nicotinamide, and therefore SIRT3, affected the MgC1q6 isoform in *M. trossulus* during oxidative stress.

Figure 15: Expression profile graphs showing the relative expression of proteins involved in immune defense in (a) *M. galloprovincialis* and (b) *M. trossulus*. Spot volumes were obtained by normalizing against the volume of all proteins, and means $\pm$ 1 SEM are shown (N=6 per treatment: *M. galloprovincialis*; N=4-6 per treatment: *M. trossulus*). Treatments with significant differences in expression level are marked with different letters. Treatment groups are abbreviated as follows: Control (C), Nicotinamide

(N), Nicotinamide + Menadione (N/M), Suramin (S), Suramin + Menadione (S/M), Menadione (M), Suramin (SUR), Suramin + Menadione (SUR/MND), Menadione (MND).

Heavy Metal Binding Proteins

Accumulation of heavy metals (e.g. iron, copper, cadmium etc.) generates superoxide anions, peroxides, and hydroxyl radicals by interacting with protein-bound metals (Flora et al., 2008). The long-term effects of metal exposure not only include impairment of signaling pathways, like those involving small G-proteins and MAPK, but can also lead to apoptosis as a result of periods of metal-induced oxidative stress (Flora et al., 2008). Thus, the bioaccumulation of these metals in an organism's tissues is dependent on biochemical processes of metal metabolism, detoxification, and storage occurring within cells. In *M. trossulus* we identified four heavy metal binding proteins (HMBP). Previous findings show several proteins capable of binding and transporting both essential and non-essential heavy metals in marine invertebrates, e.g. histidine rich glycoproteins (HRG), extracellular fluid proteins, and metallothioneins (Amiard et al., 2006; Devoid et al., 2007; Yin et al., 2005). However, we failed to explicitly identify these protein types in our study.

The DA separated the NAM/MND group from all other groups, which overlapped with the CTL group. However, the MND and SUR/MND treatments were also separated from each other (Fig. 16). Since the exposure to MND and both NAM/MND and SUR/MND differed, something that is reflected for each of the four isoforms as well (Fig. 17), we hypothesize that sirtuins had an effect on the abundance of HMBPs during oxidative stress in *M. trossulus* (Fig. 17).

Figure 16: Discriminant Analysis on all significant spots involved in heavy metal binding for *M. trossulus*. The contribution of spots in differentiating the treatment groups can be visualized in the above plots.

Figure 17: Expression profile graphs showing the relative expression of proteins involved in heavy metal binding in *M. trossulus*. Spot volumes were obtained by normalizing against the volume of all proteins, and means $\pm$ 1 SEM are shown (N=4-6 per treatment). Treatments with significant differences in expression level are marked with different letters. Treatment groups are abbreviated as follows: Control (C), Nicotinamide (N), Nicotinamide + Menadione (N/M), Suramin (S), Suramin + Menadione (S/M), Menadione (M).

SIRT3 and SIRT5 Levels

To evaluate the role of constitutive levels of sirtuins in explaining the responses to sirtuin inhibition during MND, we quantified SIRT3 and SIRT5 levels, using heterologous antibodies (Fig. 3A and 3B). Relative to the CTL, MND lowered SIRT3 levels only in *M. trossulus*. NAM/MND increased SIRT3 in comparison to SUR/MND in *M. galloprovincialis*. However, while levels were also higher in NAM/MND than in the MND treatment, differences were not significant ($P\text{-value} \leq 0.05$). Similarly, SIRT3 levels were also higher in the NAM/MND in comparison to the SUR/MND and MND treatments in *M. trossulus*. Thus, while MND decreased SIRT3 levels, NAM/MND (but not SUR/MND) increased levels to those of the CTL. These results suggest that MND decreased SIRT3 levels (significantly in *M. trossulus* only), indicating that SIRT3 levels are sensitive to an increase in ROS production and that NAM, but not SUR inhibited the response that lowered SIRT3 levels, confirming the specificity of NAM for affecting SIRT3.

SIRT5 levels were higher in response to MND, in comparison to all other treatments, with the exception of SUR/MND, during which SIRT5 levels increased further in *M. galloprovincialis* only. There were no differences for *M. trossulus*.

Conclusions

By using DAs as a tool to explore the role of certain cellular processes, we can discern some of the broader interspecific differences and summarize the response to oxidative stress. First, menadione caused a change in the abundance of three anti-oxidant proteins, i.e. CGL, Prx5 and Trx, in *M. galloprovincialis*, but not in *M. trossulus*,

separating MND from all other treatments. This separation also suggested that sirtuin inhibition inhibited the response to oxidative stress, placing an important role on sirtuins in responding to oxidative stress in *M. galloprovincialis*. Somewhat confusingly though, two of these proteins showed lowered abundances with MND, possibly because they represent isoforms whose PTMs changed towards a more active form which we did not identify.

Second, the DA of several signaling proteins involved in the cellular stress response separated the MND from the CTL in *M. galloprovincialis* but not in *M. trossulus*, further supporting that the former species activated a proteomic response to oxidative stress that was missing or muted in *M. trossulus*. This pattern extends to the signaling role of small G-proteins in vesicular transport (Rab-GDI2).

Third, the DA for protein homeostasis separated MND from CTL in *M. trossulus* but not *M. galloprovincialis*. Thus, we hypothesize that the changes in anti-oxidant and stress signaling proteins in *M. galloprovincialis* possibly prevented a challenge to proteostasis. DAs of metabolic and cytoskeletal proteins separated MND and CTL in both species. It is unclear, if those changes were preventing (e.g. switch to higher NADPH production) or representing ROS-induced damage (cytoskeleton).

Similar interspecific differences are seen for the effect of NAM/MND in comparison to MND on anti-oxidant, stress signaling and proteostasis proteins, with *M. galloprovincialis* but not *M. trossulus* displaying significant changes. This pattern might expand to both metabolic and signaling proteins and would be attributed to the role of SIRT3.

Intracellular Signaling

While sirtuin inhibition did not appear to play a role during normal homeostatic conditions, the DA revealed that both SUR and NAM had an effect on the MIOS response in *M. galloprovincialis*. Based on this and expression profiles of corresponding proteins, we suspect that at least one of the three isoforms inhibited by SUR or NAM (SIRT1, 3, or 5) has an effect on the abundance of proteins involved in signal transduction in this congener. Moreover, we hypothesize a role for sirtuins in the expression of TRIM2 during periods of MIOS, whose increased abundance could lead to selective advantage in this congener during periods of acute stress.

Meanwhile, in *M. trossulus* proteins involved in signal transduction are more affected by inhibition of SIRT5 than organismal stress state. In addition to results from our current study, findings in previous studies support our hypothesis that several proteins involved in signal transduction directly interact with sirtuins (MAPK and 14-3-3 protein γ).

A broader pattern of interspecific differences is missing for the effect of suramin (SUR/MND versus MND) during oxidative stress. However, comparisons of DAs of functional groups (cytoskeleton, energy metabolism, proteostasis and signaling) between CTL and SUR showed an opposite and complementary pattern between the congeners. Thus, it is possible that SIRT5 plays a similar role under stressful but a species-specific role under non-stressful conditions in both *Mytilus* congeners.

While the DAs provide a broader perspective, even within a functional group in which DAs suggest a common effect, e.g. of SIRT3 on cytoskeletal proteins, species-specific pattern indicate possible differences between the species.

Cytoskeleton

Both *M. galloprovincialis* and *M. trossulus* rely on sirtuins to regulate cytoskeletal dynamics and extracellular matrix proteins. Based on results from the DA and abundance changes – we hypothesize that while SIRT3 regulates cytoskeletal protein expression under normal homeostatic conditions in *M. galloprovincialis*, cytoskeletal dynamics in *M. trossulus* are tightly regulated by both organismal stress state and sirtuin mediated responses. More specifically, based on changes in the abundance of tektin-4 and dynein heavy chain 10, *M. galloprovincialis* seems to halt the assembly of new ciliary elements, possibly to conserve energy for maintain homeostasis of other cellular processes. In *M. trossulus*, based on changes in actin binding proteins (F-actin capping protein and profilin) and extracellular matrix proteins, oxidative stress might temporarily arrest actin filament growth.

Overall, the two *Mytilus* congeners showed the greatest differences in changes of protein abundance for oxidative stress proteins (including NADP-dependent isocitrate dehydrogenase) and the common hemolymph protein heavy metal binding protein (*M. trossulus* only). Both congeners showed similar effects in response to simultaneous sirtuin inhibition and MIOS for proteins involved in protein degradation (proteasome), cytoskeletal modifications (actin and tubulin), proteins regulating actin filament growth

(F-actin capping protein), amino acid metabolism and stress signaling (G-proteins, small G-proteins and MAPK). Results indicate that protein acetylation plays an important role in the oxidative stress response of *M. galloprovincialis*. More specifically this suggests that sirtuins play an important role in regulating the oxidative stress response in *M. galloprovincialis* and thus contribute to the greater stress resistance of this species. Furthermore, the changes in the abundance of several molecular chaperones suggest a greater effect of sirtuins in regulating the cellular response to heat stress, which could in part explain why this species is more heat-tolerant than the native *M. trossulus*.

Implications of Global Climate Change

The invasion of non-native species has the potential to shift the local community dynamics established in a currently thriving ecosystem (Schneider, 2008). Moreover, identification of the particular mechanisms and attributes that successful invasive organisms use to infiltrate a new region can help explain the higher fitness observed compared to native species occupying a similar niche (Schneider, 2008). While, biotic interactions may play an important role in future physiological tolerance limits, shifts in abiotic factors play a more prominent role in observed changes (Somero, 2010). Fluctuations in temperature, oxygen availability, salinity, and pH can alter both the rates of physiological processes, and the integrity of cellular components in organisms (Somero 2010; Tomanek and Zuzow, 2010). These concepts are of particular importance considering that global climate change may challenge an organism's ability to acclimate to variable environmental conditions and survive rapid climatic shifts (Tomanek and Zuzow 2010).

Until recently, changes in the chemical composition of both terrestrial and marine ecosystems have occurred over geological time spans, allowing for the natural adaptation and evolution of species (Roessig et al., 2004). However, recent anthropogenic activity has increased atmospheric greenhouse gases, including carbon dioxide (CO₂), methane (CH₄), and nitrous oxide (N₂O), in large enough quantities to cause profound shifts in global radiation retention, ocean and atmospheric temperatures, sea levels, precipitation events, wind patterns, and polar ice melt (Roessig et al., 2004; Rhein and Rintoul 2013; Doney et al., 2012).

Currently, the average global surface temperature is increasing – with the highest recorded temperatures ever observed occurring in the most recent decades (Mieszkowska et al., 2006; Rhein and Rintoul 2013). Predictive models anticipate acceleration in the current warming trend for both terrestrial and marine habitats, increased ocean acidification and an expansion of low-oxygen zones, with potentially wide-ranging ecological consequences and, as a result, shifts in species' biogeographic ranges (Mieszkowska et al., 2006; Jones et al., 2010; Doney et al., 2012). Additionally, a warmer atmosphere holds more humidity and causes more intense precipitation events, which leads to hyposaline stress in estuaries and coastal zones (Doney et al., 2012; Fig. X).

As previously mentioned, most intertidal organisms experience a fluctuating environment as a result of circatidal rhythms, which are dynamic and continuous changes in tidal levels characterized by periods of immersion and emersion (Palmer, 1973). Tidal oscillation induces environmental stress in intertidal organisms along a vertical gradient – with a positive correlation between increased levels of stress and increased exposure to air in mussels (Letendre et al., 2009). While intertidal organisms are adapted to these

fluctuating conditions, periods of prolonged emersion coinciding with extreme UV-radiation, temperatures, osmotic conditions (pH shifts) and prolonged hypoxia can be stressful, altering both the rates of physiological processes and the integrity of cellular components in organisms (Finke et al., 2007; Somero 2010; Tomanek and Zuzow, 2010).

Climate change will affect all of these factors simultaneously and it is thus important to evaluate how species that are closely related, but differ in their competitiveness and invasiveness along environmental gradients, respond to these factors. Similarly, while biotic interactions play an important role in successful invasions, the physiological processes that set tolerance limits to abiotic factors have become a key component in defining and predicting the invasiveness of a species (Somero, 2010; Lockwood and Somero 2011). Therefore, due to its influence on these periods of environmental stress and inherent biological processes, the ubiquitous effects of climate change will exacerbate the impact of invasive species on native species' biodiversity and distribution.

The invasion of non-native species has the potential to alter the local community dynamics of an established marine ecosystem (Schneider, 2008). Literally hundreds of marine species invaded the Pacific coast over the last few decades and some of these species have become keystone species in their novel environment (Sorte et al., 2010). However, the particular mechanisms and attributes that successful invasive organisms use to infiltrate a new region are largely unknown but could help explain the higher fitness observed compared to native species occupying a similar niche (Schneider, 2008). Thus, the physiological response of both native and invasive species to abiotic stress provides

insight into the weak elements of the cellular processes that are activated to resist and rebound from perturbations by the environment.

Previous studies on heart rates, transcriptomics and proteomics all indicate that *M. galloprovincialis* is more heat-tolerant than *M. trossulus* (Braby and Somero 2006; Lockwood and Somero 2011; Schneider 2008; Tomanek and Zuzow 2010). While, *M. trossulus* is a euryhaline, cold-adapted mussel more acclimatized to regions of low temperatures and fluctuating in salinity levels (Sarver and Foltz, 1993). Thus, given the anticipated trends in global climate change, *M. trossulus* will not likely persist in the warmer regions of southern California without continuous reintroduction by ships (Suchanek et al., 1997). Shifting currents are unlikely to disperse larvae enough to reestablish populations of *M. trossulus* in these regions and therefore, further contraction of *M. trossulus* is anticipated in response to current global warming trends. As one of the most abundant intertidal invertebrates, these mussels play a fundamental role in establishing and maintaining local ecological dynamics (Braby and Somero, 2006). Given that anticipated climate change will shift the local ecological homeostasis and community dynamics of mussel populations, changes in both biotic and abiotic factors will have far reaching effects on a many currently thriving ecosystems.

Materials and Methods

Animal Collection and Acclimation

Mytilus galloprovincialis specimens were collected from Santa Barbara, CA, USA (34°24'15"N, 119°41'30"W) and *M. trossulus* specimens were collected from Newport Harbor, OR, USA (44°38'25"N, 124°03'10"W). Upon arrival, animals were suspended in mesh bags from the Cal Poly pier for two weeks where they were acclimated to a circa-tidal regime of 6 h periods of immersion and emersion. One day prior to experimentation, animals were transported to the Cal Poly campus and contained in a re-circulating seawater tank at a temperature of 13°C.

Experimental Design

Following acclimation, whole organisms were placed in differing concentrations of menadione, suramin and nicotinamide. The quinone derivative, menadione (2-methyl-1,4-napthoquinone, vitamin K3; 95% menadione sodium bisulfite; M5750 Sigma Aldrich) is a cytotoxic agent known to cause macromolecular damage, disruption of calcium homeostasis, thiol depletion, and elevated levels of lipid peroxidation (Chiou and Tzeng, 2000). It was thereby chosen as the pro-oxidant to induce ROS proliferation *in vitro* through redox cycling at multiple cellular sites with a concurrent decrease in NADPH (Lor et al., 2010). More specifically, the one-electron reduction of menadione results in the formation of unstable semiquinone radicals whose reaction with oxygen is what is known to produce superoxide and hydroxyl radicals (Chiou and Tzeng, 2000; Tonquill and Tarhan, 2014; Chiou et al., 1999). This reaction is paired with a concurrent

decrease in NADPH levels, which are necessary to restore antioxidant defenses (Tongul and Tarhan, 2014).

The proteomic response to sirtuin inhibition was analyzed through the use of suramin (Enzo Life Sciences) and nicotinamide (Enzo Life Sciences), which effectively block the C-pocket of the NAD⁺-binding site on sirtuins, inhibiting deacetylation (Lawson et al., 2010). Additionally, suramin inhibits sirtuin activity by binding into the both the B- and C-pockets of the NAD⁺ binding site as well as to the substrate-binding site rendering it a highly effective exogenous inhibitor of SIRT1, SIRT2, and SIRT5 (Schuetz et al, 2007; Cimen et al., 2010; Milne and Denu 2008; Peck et al. 2010). Nicotinamide, an endogenous physiological inhibitor of SIRT1, SIRT2 and SIRT3, is the by-product of the deacetylation reaction (Chen 2010; Schuetz et al., 2007; Milne and Denu 2008; Peck et al. 2010).

Prior to exposure, organisms were randomly separated into six treatment groups, with six individuals in each group (Table 1). Whole organisms were exposed to these compounds in filtered seawater for 8 h to simulate the oxidative stress associated with a long low tide, followed by a 24.5 h recovery period in seawater (SW) under constant aeration to allow for the translation of proteins. Gill tissues were dissected and flash-frozen in liquid nitrogen and stored at -80°C at the end of the recovery period.

Table 1. Experimental Outline

Group	Treatment	Abbreviation	Group Size (n)
1	Pure SW (Control)	CTL	6
2	25 mM Nicotinamide	NAM	6
3	25 mM Nicotinamide 1 mM Menadione	NAM/MND	6
4	100 μ M Suramin	SUR	6
5	100 μ M Suramin 1 mM Menadione	SUR/MND	6
6	1 mM Menadione	MND	6

*Concentrations determined from relevant literature and pilot experimentation using *M. californianus* (McDonagh and Sheehan, 2008; Tedesco et al., 2010; Trapp et al., 2007; Sanders et al., 2007).

Homogenization

Samples were weighed and homogenized in 4 volumes homogenization buffer (7M urea, 2M thiourea, 40mM Tris-HCl, 1%, amidosulfobetaine-14, 0.5% IPG buffer, 40mM dithiothreitol) with ground-glass homogenizers. Centrifugation at 13.200 g for 30 minutes at 20°C (room temperature) was done to obtain a supernatant. Each aliquot was washed in 4 volumes 10% trichloroacetic (TCA)/acetone to precipitate the proteins overnight at -20°C.

Samples were subsequently centrifuged at 4°C for 15 minutes at 13.200 g. The supernatant was decanted and each pellet was subsequently washed with 100% acetone to remove any remaining TCA. After centrifugation the acetone was immediately decanted and the pellet was dissolved in rehydration buffer (7M urea, 2 M thiourea, 2% cholamidopropyl-dimethylammonio-propanesulfonic acid, 2% nonyl phenoxypolyethoxyethanol 40, 0.002% bromophenol blue, 0.5% IPG buffer, 100 mM dithio-erythritol). The resulting rehydrated protein was pooled and protein concentration was determined using a 2D Quant kit (GE HealthCare) following the manufacturer's instructions.

Gel Electrophoresis

Proteins (400ug) were separated based on their isoelectric point along 11 cm BioRad ReadyStrips IPG strip (pH 4-7) and then passively rehydrated for 5 h, followed by 12 h of active rehydration (50V), using an isoelectric focusing cell (BioRad).

IPG strips were incubated for two 15 minute intervals in SDS equilibration buffer (6 M urea, 50 mM Tris-HCl, pH 8.8, 30% glycerol, 2% SDS, 0.002% bromphenol blue) – first with 65mM dithiothreitol to reduce all disulfide bonds and, second, with 135mM iodoacetamide to cap resulting cysteine residues and prevent protein re-aggregation. IPG strips were placed on top of 11.8% SDS-acrylamide gels and run at 200V for 55 minutes (at 10°C). Gels were stained with colloidal Coomassie Blue overnight and destained by repeated washings with Milli-Q water for 48 h. Destained gels were scanned on an Epson 1680 expression scanner.

Gel Image Analysis

Digitized two-dimensional gel images were quantitatively analyzed with 2D gel image analysis software, Delta2D (version 3.6, Decodon, Greifswald, Germany). Briefly, gel images were first wrapped to each other and a proteome map (a fusion image), a composite gel image representing all spots, was generated. The proteome map was used to generate spot boundaries, which were subsequently transferred to all gel images using the match vectors from the wrapping process. After background subtraction spot volumes were normalized against the total spot volume of all proteins in the gel image.

Trypsin Digestion

Proteins spots were excised from gels using a spot cutter (Beecher Instruments, Inc. Manual Tissue Array Punches MP15 – 1.5mm diameter). Spots of interest were placed in a destaining buffer (25mM ammonium bicarbonate (NH_4HCO_3)/50% acetonitrile) for 45 min to remove a residual colloidal Coomassie Blue stain, dehydrated with 100% acetonitrile for 10 min and digested with trypsin solution (40mM NH_4CO_3 , 10% acetonitrile, 4.3 $\mu\text{g}/\mu\text{L}$ trypsin) overnight at 37°C. Spots were then washed with analyte solution (0.1%TFA/acetonitrile, 2:1 ratio) for 1 h to elute any remaining peptides from the gel matrix. Gel plugs were discarded and the analyte solution was pooled with the trypsin solution and concentrated in the SpeedVac (Eppendorf) at 45°C for 50 min. Matrix solution (0.2mg/mL α -hydroxycyano cinnamic acid in acetonitrile, 0.1% trifluoroacetic acid (TFA)/acetonitrile) was added directly to the samples prior to plating on a 600 μm Anchorchip target plate (Bruker Daltonics). Each spot was washed 3 times with wash buffer (0.1% TFA, 10mM ammonium phosphate) and recrystallized with a buffer containing ethanol, acetone, 0.1% TFA, 6:3:1 ratio).

Mass Spectrometry

A matrix-assisted laser desorption ionization time of flight (MALDI-ToF-ToF) mass spectrometer (Ultraflex II; Bruker Daltonics Inc., Billerica MA, USA) was used to generate peptide mass fingerprints (PMFs) of significant proteins. FlexAnalysis (version 3.0; Bruker Daltonics Inc.) was used to detect peptide peaks (SNAP algorithm with a signal-to-noise-ratio of 6 for MS and 1.5 for MS/MS). Monoisotopic masses of significant peptides were determined after trypsin digestion using PMF peak intensities.

Proteins were identified using the Mascot (version 2.2; Matrix Science Inc., Boston, MA, USA) Expressed Sequence Tag (EST) library for *Mytilus* in combination with PMFs and tandem mass spectra. A score obtained from the EST library is representative of the correlation between the experimental data and the database entry of the corresponding peptide match. NCBI Blast search engine was then employed to compare the sequence homology of identified proteins to sequence data entries. Analysis of peptide spectra followed procedures from prior investigations (Tomanek and Zuzow, 2010).

Statistical Analysis

A multivariate approach was utilized to characterize the handful of proteins whose changes in abundance were most responsible for distinguishing treatment groups. Normalized spot volumes were exported from Delta 2D into JMP Pro 11.1.1 (SAS) for statistical analyses. In order to analyze the data set using a MANOVA, spot volumes were cubed-root transformed to correct for lack of distributional normality. Given that there were fewer observations than variables, and thus not enough data to look at all spots simultaneously, the spots contributing to most of the variability in the data set were selected based on criteria determined *a priori*.

Specifically, PCA on covariance were first used to identify the primary sources of variability in the data for each species. Principal components that explained less than 80% of variability were excluded (retain λ_i explaining $> 80\%$). A subset of variables was then determined by selecting as many variables as the number of retained principal components. To select variables, for each retained principal component, the spot

corresponding to the maximum of the absolute values of the eigenvector coefficients in the first principal component was selected. This process was repeated for each subsequent principal component, unless the spot corresponding to the maximum coefficient was already selected. In this case, the variable corresponding to the next maximum was chosen. This generated a subset of spots to be used for statistical analyses.

For spots previously determined by PCA, a MANOVA was run to test for differences in the average density of spots, between treatment groups. Lastly, a DA was used to determine which spots best discriminated between groups. Since all variables were measured on the same scale, discriminant coefficients, as opposed to standardized coefficients, were used to rank the variables in order of which spots explained most of the variability between treatment groups. A step-wise DA was then used to pick the variables that most strongly discriminate between groups. While these results are strongly biased, they account for possible correlations, which aren't accounted for in Delta2D's univariate methods.

While this method allows for the identification of proteins that most significantly distinguish treatment groups, it doesn't take into account that the proteins identified as most significant may not in fact be the causal proteins. Rather, they may only be highly correlated with the expression of a causal protein. An additional approach, variable clustering, was implemented to account for this correlation. More specifically, the variable clustering algorithm split and assigned variables to new clusters according to the principal component analysis of the clusters (Variable Clustering Procedure – VARCLUS). This replaced the large set of variables with individual clusters to be tested en masse.

For each cluster, a MANOVA (multivariate ANOVA containing multiple dependent variables) was run to test for differences in the average density of spots between treatment groups. This was once again followed by a DA to determine which spots best discriminate between groups.

After identification of positive peptide matches, Delta2D hierarchical clustering, using a Pearson correlation metric was employed to connect proteins with associated degrees of variation observed in each group. Only identified proteins were used for hierarchical clustering. *Post-hoc* testing to compare treatments was conducted using Tukey's analysis ($P < 0.05$), using Minitab (version 15; Minitab Inc., State College, Pa, USA).

Western Blot Analysis

Western blots were used to quantify the endogenous levels of SIRT3 and SIRT5 in the each gill sample. Protein (5 μ g) and SIRT3 and SIRT5 standards (5 ng) dissolved in Laemmli sample buffer (BioRad) were incubated at 100°C for 3 minutes and electrophoresed using a 4% SDS polyacrylamide stacking gel on top of 11.8% SDS polyacrylamide gels. Polyacrylamide gel electrophoresis (PAGE) was run at 200 V for 60 min, until the dye front reached the bottom of each gel. Separate gels containing 10 sample wells and three sirtuin standards were used for each sirtuin isoform (SIRT3 and SIRT5).

After PAGE, proteins were transferred by a western blotting to a 12 cm, 0.2 μ m nitrocellulose membrane (Criterion Blotter; Bio-Rad). The transfer was conducted at 100

V for 30 min with a Towbin transfer buffer (200 mM glycine/25mM Tris pH 8.8, 192 mM methanol, 0.1% SDS).

After transfer, nitrocellulose membranes were blocked in 5% bovine serum albumin (BSA) in TBST (Tris buffered Saline + 0.6% Tween-20; pH 7.6) for 1 h on a shaker, followed by four five-minute washes in TBST. Subsequently blots were incubated for 12 h at 4°C with primary antibody (anti-SIRT3 rabbit, polyclonal, IgG; 1:2500; Enzo) in 2.5% BSA in TBST and 0.02%thimerosal. Following incubation, the washing procedures described above were repeated. Blots were then incubated for 1 h at 20°C (room temperature) with a polyclonal goat anti-rabbit IgG antibody conjugated with horseradish peroxidase; 1:5000) in 2.5% BSA in TBST. Blots were rinsed again as described above.

Blots were imaged on a Typhoon Imager (GE Healthcare) after the membrane was overlaid with a solution of enhanced chemiluminescent (ECL) reagent (Pierce ECL 2 western blotting substrate) according to the manufacturer's instructions for 5 min. The band intensity (ECL signal) of each lane represented the amount of sirtuin present in 5µg of each sample relative to the standard. Band intensity was detected according to the manufacturer's instructions, using PMT 430 (fluorescence) under 200 microns. Total Lab TL120 v2006f software was used to determine relative levels of SIRT3 and SIRT5.

Western Blot Statistical Analysis

Band volumes for each sample were calibrated and compared to the known concentration of sample loaded (5µg). We used human SIRT3 and SIRT5 standards to account for possible variation of incubation conditions between western blots. A

MANOVA was run for all bands to protect from making a type 1 statistical error. Next, provided the MANOVA results are significant, full factorial general linear models for both SIRT3 and SIRT5 were run, followed by Tukey post hoc comparisons ($P \leq 0.05$), which were used to assess differences in average sirtuin abundance between treatment groups.

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Table S1. Protein identifications (using MS/MS) of all spots in *M. galloprovincialis* acute oxidative stress experiment.

No.	Protein ID	MW (kDa) Estimated	pI Estimated	MW (kDa) Predicted	pI Predicted	GenBank ID	Mascot Score	Peptide Matches	Sequence Coverage(%)	Functional Category
1	E3 ubiquitin-protein ligase	88.00	6.80	85.09	8.51	gi 405950771	124	5	4	Protein degradation
2	Selenium-binding protein	71.00	6.14	53.32	6.11	gi 405971621	72	3	4	Oxidative stress
3	UDP-N-acetylhexosamine pyrophosphorylase	74.00	6.51	57.23	5.46	gi 405971443	97	3	13	Energy metabolism
4	Amine oxidase	72.00	6.65	54.45	5.95	gi 228902236	140	3	5	Energy metabolism
5	Tubulin α -1 chain	67.00	5.29	53.84	4.93	gi 307204973	471	9	15	Cytoskeleton
6	β -tubulin	64.00	5.14	49.96	4.73	gi 198424255	471	10	17	Cytoskeleton
7	T-complex 1	70.00	6.62	60.31	5.96	gi 71895883	213	7	10	Molecular chaperone
9	Aldehyde dehydrogenase (mito.)	69.00	5.91	56.93	6.18	gi 405965075	201	6	9	Energy metabolism
10	Tubulin α -1C chain	69.00	6.18	50.08	4.94	gi 405965638	60	2	13	Cytoskeleton
11	Fascin	66.00	6.38	55.57	6.21	gi 405961655	68	2	3	Cytoskeleton
12	Tektin-4	61.00	6.05	52.50	5.53	gi 405967050	237	8	12	Cytoskeleton
14	Rab GDP dissociation inhibitor 2	58.00	6.58	50.55	5.60	gi 37382224	271	7	7	Vesicular transport
15	4-hydroxybutyrate CoA-transferase	58.00	6.62	51.43	6.99	gi 405966123	175	5	10	Energy metabolism
16	Mitochondrial-processing peptidase, type- β	59.00	5.95	53.39	5.84	gi 268784705	245	7	15	Protein degradation
19	NADP-dependent isocitrate dehydrogenase	56.00	6.67	50.48	6.80	gi 385288543	87	3	2	Energy metabolism
20	4-hydroxyphenylpyruvate dioxygenase	53.00	6.68	211.45	6.63	gi 405968443	128	2	19	Amino acid metabolism
23	Actin	55.00	6.51	41.82	5.11	gi 3907622	67	3	9	Cytoskeleton
24	NADP-dependent isocitrate dehydrogenase	54.00	6.66	50.84	6.80	gi 385288543	194	7	7	Energy metabolism
25	Cystathionine γ -lyase	50.00	6.90	46.90	6.57	gi 40596445	199	6	6	Oxidative stress
26	Mitogen-activated protein kinase	51.00	6.63	40.37	6.02	gi 118343753	281	7	21	Signal transduction
27	40S ribosomal protein SA	49.00	4.84	33.28	4.78	gi 405976088	249	4	11	Cytoskeleton
28	C1q domain containing protein (MgC1q6)	41.00	5.21	26.34	5.25	gi 325504311	151	3	8	Immune defense
29	C1q domain containing protein (MgC1q6)	42.00	5.11	26.34	5.25	gi 325504311	154	4	8	Immune defense
30	C1q domain containing protein (MgC1q6)	40.00	5.31	26.34	5.25	gi 325504312	224	4	32	Immune defense
31	F-actin-capping protein, type- α	40.00	6.60	32.29	5.77	gi 405951960	65	2	3	Cytoskeleton
32	Guanine nucleotide-binding protein, type- β	38.00	5.77	37.31	5.62	gi 405963261	88	3	2	Signal transduction
33	Guanine nucleotide-binding protein, type- β	37.00	5.98	37.31	5.62	gi 405963261	213	5	4	Signal transduction
34	Tripartite motif containing protein 2	37.00	5.74	115.36	6.36	gi 405972994	62	2	7	Signal transduction
35	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	35.00	6.39	39.51	6.29	gi 405975453	124	4	5	Signal transduction
36	Fibrinogen	35.00	6.42	30.22	5.98	gi 312270829	73	3	10	Immune defense
37	Small HSP 24.1	33.00	6.13	28.52	6.11	gi 347545633	22	5	4	Molecular chaperone
39	O-methyltransferase mdmC	30.00	5.16	35.94	8.34	gi 405953767	131	5	9	Energy metabolism
40	Proteasome, type- α	29.00	5.23	27.98	5.26	gi 405971985	69	3	9	Protein degradation
41	F-actin-capping protein, type- β	29.00	5.38	30.72	5.70	gi 41053959	125	6	8	Cytoskeleton
43	Apexrin	26.00	5.54	24.34	5.42	gi 339785142	105	5	20	Immune defense
46	Proteasome, type- β	21.00	5.92	77.55	6.10	gi 405969077	46	2	7	Protein degradation
47	Rho GDP-dissociation inhibitor 1	20.00	5.28	23.56	5.15	gi 405966436	66	4	4	Signal transduction
50	Proteasome, type- β	16.00	6.66	23.42	5.09	gi 405951012	46	2	6	Protein degradation
51	Ras-related protein, Rab-2	15.00	6.72	15.33	7.21	gi 405969069	44	5	9	Vesicular transport
52	C1q domain containing protein (MgC1q44)	15.00	6.38	23.59	7.66	gi 325504387	64	4	17	Immune defense
55	Peroxiredoxin-5 (mito.)	8.00	6.76	16.57	5.66	gi 405974897	149	6	10	Oxidative stress
57	Dynein heavy chain 10 (axonemal)	3.00	6.75	185.12	6.65	gi 405975546	67	2	4	Energy metabolism
58	Malate dehydrogenase	39.00	6.92	36.25	8.44	gi 8746611	207	5	10	Energy metabolism
59	Dihydropteridine reductase	28.00	6.81	24.86	6.60	gi 405946263	129	5	6	Energy metabolism
60	V-type proton ATPase	83.00	5.81	70.81	5.20	gi 405950221	124	6	4	Energy metabolism
61	V-type proton ATPase	82.00	5.69	70.81	5.20	gi 405950221	28	1	1	Energy metabolism
62	Short-chain collagen C4	47.00	5.70	24.69	8.26	gi 405960952	210	4	15	Cytoskeleton
64	G protein, type- β	39.00	5.70	34.43	5.83	gi 51872141	58	2	5	Signal transduction
65	Major vault protein	100.00	6.02	95.52	5.58	gi 405974681	433	10	10	Nuclear transport
66	Major vault protein	101.00	6.21	95.52	5.58	gi 405974681	40	6	6	Nuclear transport
67	78 kDa glucose-regulated protein	98.00	5.12	72.99	5.02	gi 405968607	261	7	6	Molecular chaperone
70	78 kDa glucose-regulated protein	86.00	5.12	72.99	5.02	gi 405968607	201	6	6	Molecular chaperone
71	Collagen α -5(VI) chain	79.00	5.21	206.03	5.81	gi 405953446	50	3	19	Cytoskeleton
72	Collagen α -5(VI) chain	79.00	5.16	247.94	5.94	gi 405975735	156	5	5	Cytoskeleton
74	HSP70	82.00	5.91	65.32	5.61	gi 405950524	81	3	3	Molecular chaperone
75	Thioredoxin	79.00	5.02	44.67	4.84	gi 405970435	93	3	15	Oxidative stress
76	Calreticulin	62.00	4.61	48.15	4.53	gi 148717307	268	7	7	Molecular chaperone
77	Protein-density lipoprotein receptor-related protein 1	31.00	4.49	39.20	5.32	gi 405960325	116	5	14	Fatty acid metabolism
78	Cdc42	14.00	6.94	21.29	6.16	gi 333449473	116	4	5	Signal transduction
80	EF-hand domain-containing protein 1	85.00	6.85	74.34	6.23	gi 405964721	27	1	3	Inorganic ion transport
82	Major vault protein	82.00	6.01	96.25	5.58	gi 405974681	355	9	10	Nuclear transport
86	α -tubulin	72.00	6.10	43.94	4.85	gi 21867221	186	3	14	Cytoskeleton
90	Methylmalonate-semialdehyde dehydrogenase	67.00	5.88	57.20	6.48	gi 405970294	189	4	9	Energy metabolism
91	Methylmalonate-semialdehyde dehydrogenase	68.00	6.01	57.20	6.48	gi 405970294	89	8	7	Energy metabolism
95	Tubulin α -1C chain	70.00	5.79	50.06	4.94	gi 405965638	86	4	10	Cytoskeleton
98	Aldehyde dehydrogenase (mito.)	66.00	6.83	60.31	5.18	gi 405965075	60	2	6	Energy metabolism
107	Retinal dehydrogenase 1	64.00	6.33	53.12	5.74	gi 405965075	73	3	4	Energy metabolism
108	Enolase	62.00	6.26	47.70	5.99	gi 332025761	287	7	7	Energy metabolism
112	ATP synthase type- β	63.00	4.97	46.25	4.95	gi 405963011	163	5	12	Energy metabolism
115	Actin	60.00	5.83	41.74	5.30	gi 405964579	118	3	6	Cytoskeleton
117	Severin	59.00	6.00	43.17	5.39	gi 405954086	95	2	7	Cytoskeleton
118	Severin	59.00	6.47	43.17	5.39	gi 405954086	237	5	6	Cytoskeleton
120	Ornithine aminotransferase (mito.)	57.00	6.42	7.95	47.42	gi 405951937	187	6	6	Amino acid metabolism
122	Actin	59.00	6.09	41.70	5.29	gi 86610891	65	3	11	Cytoskeleton
125	Actin	57.00	6.06	41.74	5.30	gi 405964579	303	6	13	Cytoskeleton
126	Severin	58.00	6.29	43.17	5.39	gi 405954086	102	4	5	Cytoskeleton
127	Pyruvate dehydrogenase E1 component, type- α (mito.)	57.00	6.25	42.28	5.32	gi 52485584	56	2	5	Energy metabolism
128	Actin-2	55.00	5.37	41.77	5.30	gi 405973339	46	3	7	Cytoskeleton
129	Actin (cytoplasmic)	55.00	5.82	41.72	5.30	gi 405964580	115	2	5	Cytoskeleton
131	Phosphoglycerate kinase 1	56.00	6.74	43.00	7.59	gi 405963233	79	2	5	Energy metabolism
133	Medium-chain specific acyl-CoA dehydrogenase (mito.)	55.00	6.86	29.79	6.79	gi 405962014	64	2	6	Energy metabolism
134	C1q domain-containing protein	55.00	6.61	18.74	8.39	gi 301598996	178	3	16	Immune defense
135	Actin-2	55.00	6.22	41.72	5.30	gi 405973339	108	4	11	Cytoskeleton
137	Actin	55.00	5.94	41.70	5.29	gi 86610891	204	4	10	Cytoskeleton
138	Actin-2	55.00	6.11	41.77	5.30	gi 405973339	137	3	7	Cytoskeleton
139	Actin-2	55.00	6.15	41.77	5.30	gi 405973339	192	5	11	Cytoskeleton
140	Actin	55.00	6.07	41.70	5.29	gi 86610891	255	5	11	Cytoskeleton
142	Actin	54.00	6.02	41.74	5.30	gi 405964579	318	6	13	Cytoskeleton
147	Malate dehydrogenase	51.00	6.03	10.60	5.33	gi 405960257	332	7	7	Energy metabolism
155	Guanine nucleotide-binding protein, type- β	46.00	5.95	37.31	5.62	gi 405963261	211	4	3	Signal transduction
158	Short-chain collagen C4	43.00	5.82	24.69	8.26	gi 405960952	184	4	9	Cytoskeleton
160	Malate dehydrogenase	42.00	6.75	36.08	8.20	gi 253709246	60	2	3	Energy metabolism
161	3-hydroxyanthranilate 3,4-dioxygenase	43.00	6.89	34.11	5.44	gi 405955617	65	2	6	Amino acid metabolism
165	Inorganic pyrophosphatase	42.00	5.37	48.31	5.75	gi 405962800	44	3	2	Energy metabolism
166	Trefoil factor	41.00	6.26	29.42	4.36	gi 74474939	59	1	11	Secretory protein
171	Short-chain collagen C4	40.00	5.66	24.69	8.26	gi 405960952	67	2	5	Cytoskeleton
173	Fumarylacetoacetate hydrolase domain-containing protein 2	40.00	5.80	22.67	5.27	gi 405955543	105	3	7	Energy metabolism
177	Small HSP 24.1	39.00	5.85	28.52	5.61	gi 347545633	262	4	11	Molecular chaperone
179	S-formylglutathione hydrolase	37.00	6.74	31.16	6.18	gi 405971478	71	2	4	Amino acid metabolism
180	Small HSP 24.1	38.00	5.69	28.52	5.61	gi 347545633	55	2	8	Molecular chaperone
183	Apexrin	36.00	6.32	24.39	8.42	gi 339785142	140	3	13	Immune defense
184	Apexrin	37.00	5.42	24.39	8.42	gi 339785142	56	3	15	Immune defense
187	Fumarylacetoacetate hydrolase domain-containing protein 2	36.00	6.09	22.67	5.27	gi 405955543	57	3	10	Energy metabolism
191	Apexrin	34.00	6.32	24.39	8.42	gi 339785142	48	4	13	Immune defense
202	Triosephosphate isomerase	32.00	6.51	27.22	7.57	gi 333449422	229	6	9	Energy metabolism
207	Apexrin	33.00	6.34	24.39	8.42	gi 339785142	256	4	15	Immune defense
208	6-phosphogluconolactonase	31.00	6.06	26.67	5.47	gi 405954693	44	2	12	Energy metabolism
215	Collagen α -5(VI) chain	28.00	5.26	247.94	5.94	gi 405975735	139	2	4	Cytoskeleton
217	HSP71	28.00	6.28	71.28	5.28	gi 76780612	43	2	4	Molecular chaperone
223	Rab-7a	21.00	6.10	23.05	5.39	gi 405954307	100	3	3	Vesicular transport
234	SAR1b	21.00	6.79	24.88	7.10	gi 405964143	76	2	2	Vesicular transport
237	HSP71	19.00	6.85	32.17	6.76	gi 76780612	74	3	4	Molecular chaperone
252	Cystatin B	13.00	5.29	10.57	5.50	gi 297341138	72	2	12	Protease inhibitor
259	Heterogeneous nuclear ribonucleoprotein 27C	8.00	5.09	58.88	8.32	gi 405977312	66	2	12</	

Table S2. Protein identifications (using MS/MS) of all spots in *M. trossulus* acute oxidative stress experiment.

No.	Protein ID	MW (kDa) Estimated	pI Estimated	MW (kDa) Predicted	pI Predicted	GenBank ID	Mascot Score	Peptide Matches	Sequence Coverage(%)	Functional Category
1	Major vault protein	92.00	5.95	96.25	5.58	gi 405974681	404	10	10	Signal transduction
3	Peptidyl-prolyl cis-trans isomerase B	37.00	6.88	23.73	5.97	gi 405963687	248	8	27	Molecular chaperone
5	Cystatin B	13.00	5.29	10.57	5.50	gi 297341138	131	3	19	Molecular chaperone
7	Endoplasmic	92.00	5.10	12.53	4.88	gi 405973525	187	6	5	Molecular chaperone
9	78 kDa glucose-regulated protein	82.00	5.13	72.99	5.02	gi 405968607	245	5	26	Molecular chaperone
10	Phosphoenolpyruvate carboxykinase (cyto.)	80.00	6.60	73.92	6.73	gi 405961262	69	3	2	Energy metabolism
11	Tripartite motif-containing protein 2	81.00	6.68	83.44	6.66	gi 405962519	66	3	2	Signal transduction
13	Intermediate filament protein (cyto.)	78.00	5.74	69.25	5.38	gi 405950795	144	7	9	Cytoskeleton
16	Tubulin α -3 chain	68.00	5.64	50.81	4.98	gi 405963072	96	2	13	Cytoskeleton
17	Dihydropyridyl dehydrogenase (mito.)	70.00	6.63	53.65	7.60	gi 387015534	212	6	8	Energy metabolism
18	β -tubulin	66.00	5.02	49.96	4.74	gi 45598623	345	8	13	Cytoskeleton
19	β -tubulin	67.00	4.91	49.96	4.74	gi 45598623	455	9	18	Cytoskeleton
20	α -aminoacidic semialdehyde dehydrogenase	68.00	6.54	55.18	5.85	gi 405978696	154	5	9	Amino acid metabolism
21	α -aminoacidic semialdehyde dehydrogenase	68.00	6.65	55.18	5.85	gi 405978696	262	6	10	Amino acid metabolism
22	4-hydroxybutyrate CoA-transferase	63.00	6.65	50.73	8.33	gi 389613607	78	4	7	Energy metabolism
23	ATP synthase, type- β	63.00	5.03	46.23	4.95	gi 46909261	630	11	25	Energy metabolism
25	Tektin-1	62.00	5.66	48.30	6.12	gi 405975636	100	4	8	Cytoskeleton
26	Ornithine aminotransferase	61.00	6.38	52.12	7.96	gi 157109474	201	6	6	Energy metabolism
27	Calreticulin	62.00	4.57	48.16	4.53	gi 148717307	273	7	10	Molecular chaperone
28	Actin	58.00	5.47	41.74	5.30	gi 405964579	340	7	16	Cytoskeleton
29	Radial spoke head 1	58.00	4.63	32.14	4.34	gi 296531372	281	6	20	Cytoskeleton
30	Mitogen-activated protein kinase 1	58.00	6.60	42.14	5.91	gi 332030495	205	6	17	Signal transduction
32	D-octopine dehydrogenase	57.00	6.56	44.31	6.02	gi 399912965	173	3	15	Amino acid metabolism
33	40S ribosomal protein SA	56.00	4.83	33.28	4.78	gi 405976088	236	5	11	Cytoskeleton
35	UDP-glucose 4-epimerase	54.00	6.57	37.33	6.72	gi 405968861	84	2	9	Energy metabolism
39	Ser/thr-protein phosphatase 1, type- β	52.00	6.71	37.81	5.84	gi 524893878	59	3	9	Signal transduction
41	C1q domain containing protein (MgC1q6)	47.00	5.19	26.34	5.25	gi 325504311	63	3	15	Immune defense
42	Heavy metal binding protein	49.00	6.19	26.86	5.09	gi 38635428	172	3	11	Oxidative stress
43	Radial spoke head 9	46.00	5.42	30.99	5.20	gi 405959092	277	7	19	Cytoskeleton
44	Heavy metal binding protein	49.00	6.39	26.86	5.09	gi 38635428	139	3	11	Oxidative stress
45	F-actin-capping protein, type- α	47.00	5.08	32.29	5.77	gi 405951960	81	3	6	Cytoskeleton
46	Heavy metal binding protein	45.00	5.54	26.86	5.09	gi 38635428	132	3	11	Oxidative stress
47	Proteasome, type- α	49.00	6.79	27.73	6.07	gi 405951408	197	5	19	Protein degradation
48	3-hydroxyisobutyrate dehydrogenase (mito.)	44.00	5.64	42.08	8.00	gi 405957947	137	5	7	Energy metabolism
49	Heavy metal binding protein	45.00	5.68	26.86	5.09	gi 38635428	173	3	11	Oxidative stress
50	Heavy metal binding protein	45.00	5.15	26.86	5.09	gi 38635428	153	3	11	Oxidative stress
52	Carbonic anhydrase	39.00	6.48	36.51	8.70	gi 405966618	138	3	9	Inorganic ion transport
54	Short-chain collagen C4	38.00	5.34	24.69	8.26	gi 405960952	66	2	3	Cytoskeleton
55	Proteasome, type- α	38.00	5.30	27.98	5.26	gi 405971985	81	5	12	Protein degradation
58	Proteasome, type- β	32.00	5.84	77.55	6.10	gi 405969077	145	5	38	Protein degradation
59	Rho GDP-dissociation inhibitor 1	32.00	5.38	23.56	5.15	gi 405966436	58	3	17	Signal transduction
62	Ras-related protein	29.00	5.45	22.79	5.55	gi 126697336	57	3	2	Signal transduction
63	Cytochrome c reductase	29.00	5.82	28.63	7.65	gi 147904657	71	3	10	Energy metabolism
65	Ubiquitin-conjugating enzyme	26.00	6.81	16.29	7.72	gi 405966174	78	3	3	Protein degradation
66	Translationally controlled tumor protein	24.00	4.91	19.58	4.76	gi 359359687	74	2	6	Molecular chaperone
68	Translationally controlled tumor protein	23.00	4.93	19.58	4.76	gi 359359687	72	2	6	Molecular chaperone
70	Dynein heavy chain 10 (axonemal)	19.00	5.02	585.11	5.65	gi 405976546	70	2	3	Cytoskeleton
71	Actin-depolymerizing factor 6	17.00	6.64	15.68	5.83	gi 297341132	109	2	13	Cytoskeleton
72	Coactosin	18.00	5.59	15.84	5.25	gi 405972070	161	4	16	Cytoskeleton
73	Profilin	11.00	6.81	15.27	6.10	gi 47551153	88	4	9	Cytoskeleton
77	Heterogeneous nuclear ribonucleoprotein 27C	4.00	5.35	58.88	8.32	gi 405977312	101	3	10	Protein synthesis
78	Calcyphosin	23.00	5.76	30.55	5.46	gi 405977572	102	5	10	Signal transduction
85	Tubulin α -1C chain	81.00	6.01	50.08	4.94	gi 405965638	144	4	17	Cytoskeleton
86	Tubulin α -1C chain	74.00	6.03	50.08	4.94	gi 405965638	77	2	10	Cytoskeleton
96	Tektin-2	66.00	5.86	47.72	5.71	gi 405950079	113	5	23	Cytoskeleton
99	Tubulin α -1C chain	74.00	6.08	50.11	4.94	gi 405965637	70	2	8	Cytoskeleton
100	Tubulin α -1C chain	71.00	6.07	50.08	4.94	gi 405965638	197	4	16	Cytoskeleton
114	78 kDa glucose-regulated protein	92.00	5.14	72.99	5.02	gi 405968607	73	4	21	Molecular chaperone
119	Actin	80.00	5.59	41.70	5.30	gi 189473617	232	5	19	Cytoskeleton
131	UDP-N-acetylhexosamine pyrophosphorylase	74.00	6.51	57.23	5.46	gi 405971443	186	3	18	Energy metabolism
144	Glutathione S-transferase GSTp1	71.00	5.43	24	5.95	gi 22094809	71	2	13	Oxidative stress
147	Tubulin α -1C chain	66.00	5.37	50.08	4.94	gi 405965638	236	6	25	Cytoskeleton
152	Tubulin α -1C chain	71.00	6.42	50.08	4.94	gi 405965638	108	3	12	Cytoskeleton
154	T-complex 1	70.00	6.48	25.49	5.59	gi 405959601	78	2	10	Molecular chaperone
155	Calreticulin	65.00	4.95	48.12	4.53	gi 148717307	76	2	10	Molecular chaperone
165	α -aminoacidic semialdehyde dehydrogenase	63.00	6.53	55.18	5.85	gi 405978696	116	2	13	Amino acid metabolism
166	NADP-dependent isocitrate dehydrogenase	60.00	6.71	50.48	6.80	gi 385268543	131	3	16	Energy metabolism
170	Severin	62.00	6.07	43.17	5.39	gi 405954086	60	2	11	Cytoskeleton
178	Actin	61.00	6.22	41.70	5.30	gi 189473617	232	5	19	Cytoskeleton
189	HSP40	57.00	6.11	153.37	5.05	gi 405965494	77	3	11	Molecular chaperone
190	Actin	55.00	5.42	41.70	5.30	gi 189473617	114	3	13	Cytoskeleton
195	Actin	53.00	5.75	41.70	5.30	gi 189473617	92	3	10	Cytoskeleton
196	Malate dehydrogenase (cyto.)	50.00	6.45	36.40	6.02	gi 73656337	141	3	8	Energy metabolism
197	C1q domain containing protein (MgC1q6)	47.00	5.29	26.34	5.25	gi 325504311	77	2	13	Immune defense
199	40S ribosomal protein SA	51.00	4.95	33.28	4.78	gi 405976088	105	5	37	Cytoskeleton
200	Actin	50.00	5.95	41.73	5.30	gi 189473617	84	2	9	Cytoskeleton
203	Guanine nucleotide-binding protein, type- β	48.00	5.81	37.31	5.62	gi 405963261	116	3	3	Signal transduction
204	Chloride intracellular channel exc-4	47.00	5.99	32.89	5.29	gi 405965094	134	3	3	Inorganic ion transport
205	Malate dehydrogenase	47.00	6.70	36.25	8.44	gi 6746611	227	3	20	Energy metabolism
209	β -tubulin	46.00	5.70	48.16	4.72	gi 405965590	168	6	25	Cytoskeleton
213	Tubulin α -1C chain	47.00	6.19	50.08	4.94	gi 405965638	123	3	12	Cytoskeleton
216	Elongation factor 1- Δ	46.00	4.83	17.13	4.81	gi 405960011	84	3	23	Protein synthesis
223	Tropomyosin	43.00	4.87	32.75	4.62	gi 6647862	109	3	16	Cytoskeleton
226	Malate dehydrogenase (cyto.)	41.00	6.42	36.42	6.15	gi 73656362	55	3	8	Energy metabolism
237	Proteasome, type- α	38.00	6.60	29.86	6.47	gi 524871178	133	3	17	Protein degradation
245	Prohibitin	37.00	5.66	29.80	5.62	gi 524879616	258	5	21	DNA synthesis
253	Cathepsin L	36.00	4.58	39.97	5.39	gi 405971603	103	3	17	Protein degradation
256	Apexin	34.00	6.29	24.39	8.42	gi 339785142	90	2	17	Molecular chaperone
260	14-3-3 protein γ	34.00	4.68	28.46	4.66	gi 405950430	90	5	23	Signal transduction
265	Signal sequence receptor, type β	32.00	6.01	20.85	6.96	gi 40643038	103	2	11	Signal transduction
285	40S ribosomal protein S12	18.00	5.96	14.90	5.79	gi 405977575	98	4	30	Cytoskeleton
292	Heterogeneous nuclear ribonucleoprotein 27C	10.00	15.20	58.88	8.32	gi 405977312	85	2	12	Protein synthesis
293	Heterogeneous nuclear ribonucleoprotein 27C	7.00	4.99	58.88	8.32	gi 405977312	68	2	12	Protein synthesis

Table S3. Overview of Tukey pair-wise comparisons in *M. galloprovincialis*.

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
	SIRT3	Sirtuin					✓		
	SIRT5	Sirtuin	✓				✓	✓	✓

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
20	4-hydroxyphenylpyruvate dioxygenase	Amino acid metabolism							

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
9	β-tubulin	Cytoskeleton						✓	
11	Fascin	Cytoskeleton							
12	Tektin-4	Cytoskeleton	✓	✓					
23	Actin	Cytoskeleton	✓						
27	40S ribosomal protein SA	Cytoskeleton		✓		✓			
31	F-actin-capping protein, type-α	Cytoskeleton						✓	
41	F-actin-capping protein, type-β	Cytoskeleton	✓					✓	✓
57	Dynein heavy chain 10 (axonemal)	Cytoskeleton							
62	Short-chain collagen C4	Cytoskeleton						✓	
71	Collagen α-5(VI) chain	Cytoskeleton		✓		✓		✓	
72	Collagen α-5(VI) chain	Cytoskeleton				✓			
125	Actin	Cytoskeleton							✓
126	Severin	Cytoskeleton						✓	
129	Actin	Cytoskeleton						✓	
135	Actin	Cytoskeleton						✓	
280	Actin	Cytoskeleton							
Discriminant Analysis			✓	✓		✓	✓	✓	✓

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
3	UDP-N-acetylhexosamine pyrophosphorylase	Energy metabolism			✓		✓		
4	Amine oxidase	Energy metabolism							
15	4-hydroxybutyrate CoA transferase	Energy metabolism	✓					✓	✓
19	NADP-dependent isocitrate dehydrogenase	Energy metabolism					✓		✓
24	NADP-dependent isocitrate dehydrogenase	Energy metabolism				✓			✓
39	O-methyltransferase mdmC	Energy metabolism						✓	
59	Dihydropteridine reductase	Energy metabolism	✓					✓	
60	V-type proton ATPase	Energy metabolism							
61	V-type proton ATPase	Energy metabolism	✓				✓	✓	
90	Methylmalonate-semialdehyde dehydrogenase	Energy metabolism							
131	Phosphoglycerate kinase 1	Energy metabolism			✓		✓		
284	Cytochrome c reductase	Energy metabolism							
Discriminant Analysis			✓		✓	✓	✓	✓	✓

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
77	Protein density lipoprotein receptor-related protein 1	Fatty acid metabolism						✓	

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
28	C1q domain containing protein (MgC1q6)	Immune defense				✓			
29	C1q domain containing protein (MgC1q6)	Immune defense							
30	C1q domain containing protein (MgC1q6)	Immune defense			✓				
36	Fibrinogen	Immune defense	✓						
43	Apexrin	Immune defense	✓						
52	C1q domain containing protein (MgC1q44)	Immune defense						✓	✓
183	Apexrin	Immune defense							
Discriminant Analysis			✓	✓	✓	✓	✓	✓	✓

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
7	T-complex 1	Molecular chaperone	✓						
37	Small HSP 24.1	Molecular chaperone						✓	
46	Proteasome, type-β	Protein degradation				✓		✓	
50	Proteasome, type-β	Protein degradation							
67	78 kDa glucose-regulated protein	Molecular chaperone							
70	78 kDa glucose-regulated protein	Molecular chaperone							
76	Calreticulin	Molecular chaperone						✓	
Discriminant Analysis				✓	✓	✓		✓	

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
65	Major vault protein	Nuclear transport							
66	Major vault protein	Nuclear transport					✓		

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
25	Cystathionine γ-lyase	Oxidative stress	✓					✓	✓
55	Peroxisome oxidin-5 (mitochondrial)	Oxidative stress	✓					✓	✓
75	Thioredoxin	Oxidative stress							
Discriminant Analysis			✓					✓	✓

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
26	Mitogen-activated protein kinase	Signal transduction				✓			
33	Guanine nucleotide-binding protein, type-β	Signal transduction						✓	
34	Tripartite motif containing protein 2	Signal transduction			✓				
35	N(G,N(G)-dimethylarginine dimethylaminohydrolase 1	Signal transduction	✓						
47	Rho GDP-dissociation inhibitor 1	Signal transduction						✓	
78	Cdc42	Signal transduction	✓					✓	✓
Discriminant Analysis			✓					✓	✓

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
14	Rab GDP dissociation inhibitor 2	Vesicular transport	✓	✓			✓	✓	✓
51	Ras-related protein Rab-2	Vesicular transport				✓		✓	

Table S4. Overview of Tukey pair-wise comparisons in *M. trossulus*.

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
	SIRT3	Sirtuin					✓	✓	
	SIRT5	Sirtuin			✓				

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
21	α -aminoadipic semialdehyde dehydrogenase	Amino acid metabolism	✓					✓	✓
32	D-octopine dehydrogenase	Amino acid metabolism					✓		✓

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
16	Tubulin α -3 chain	Cytoskeleton							✓
18	β -tubulin	Cytoskeleton	✓				✓		
19	β -tubulin	Cytoskeleton					✓		
28	Actin	Cytoskeleton	✓					✓	
29	Radial spoke head 1	Cytoskeleton			✓				
43	Radial spoke head 9	Cytoskeleton				✓		✓	
45	F-actin-capping protein, type- α	Cytoskeleton				✓		✓	
54	Short-chain collagen C4	Cytoskeleton						✓	
70	Dynein heavy chain 10 (axonemal)	Cytoskeleton				✓		✓	
72	Coactosin	Cytoskeleton			✓				
73	Profilin	Cytoskeleton	✓			✓			
85	Tubulin α -1C chain	Cytoskeleton	✓						
195	Actin	Cytoskeleton		✓		✓			
200	Actin	Cytoskeleton							
209	β -tubulin	Cytoskeleton				✓	✓	✓	✓
223	Tropomyosin	Cytoskeleton							
	Discriminant Analysis	Cytoskeleton	✓	✓	✓	✓	✓	✓	✓

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
10	Phosphoenolpyruvate carboxykinase (cytoplasmic)	Energy metabolism					✓		
17	Dihydropyridyl dehydrogenase (mitochondrial)	Energy metabolism	✓					✓	✓
22	4-hydroxybutyrate CoA-transferase	Energy metabolism							✓
26	Ornithine aminotransferase	Energy metabolism							
35	UDP-glucose 4-epimerase	Energy metabolism				✓			
48	3-hydroxyisobutyrate dehydrogenase (mitochondrial)	Energy metabolism							✓
63	Cytochrome c reductase	Energy metabolism							
166	NADP-dependent isocitrate dehydrogenase	Energy metabolism					✓		
226	Malate dehydrogenase (cytoplasmic)	Energy metabolism							
	Discriminant Analysis	Energy metabolism	✓	✓			✓	✓	✓

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
42	Heavy metal binding protein	Heavy metal binding				✓			
46	Heavy metal binding protein	Heavy metal binding						✓	
49	Heavy metal binding protein	Heavy metal binding	✓						✓
50	Heavy metal binding protein	Heavy metal binding				✓		✓	
	Discriminant Analysis	Heavy metal binding				✓		✓	✓

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
41	C1q domain containing protein (MgC1q6)	Immune defense				✓		✓	

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
52	Carbonic anhydrase	Inorganic ion transport		✓				✓	

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
5	Cystatin B	Molecular chaperone			✓		✓		
7	Endoplasmic	Molecular chaperone					✓		
9	78 kDa glucose-regulated protein	Molecular chaperone							
27	Calreticulin	Molecular chaperone							✓
47	Proteasome, type- α	Protein degradation		✓		✓			
58	Proteasome, type- β	Protein degradation	✓				✓		
66	Translationally controlled tumor protein	Molecular chaperone		✓		✓			
68	Translationally controlled tumor protein	Molecular chaperone				✓		✓	
114	78 kDa glucose-regulated protein	Molecular chaperone							
	Discriminant Analysis	Protein homeostasis	✓	✓		✓	✓	✓	✓

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
1	Major vault protein	Nuclear transport					✓		

No.	Name	Functional Category	Significant differences						
			CTL vs. MND	CTL vs. NAM	CTL vs. SUR	NAM vs. NAM/MND	SUR vs. SUR/MND	NAM/MND vs. MND	SUR/MND vs. MND
11	Tripartite motif-containing protein 2	Signal transduction			✓		✓		
30	Mitogen-activated protein kinase 1	Signal transduction							✓
39	Ser/thr-protein phosphatase 1, type- β	Signal transduction			✓				
59	Rho GDP-dissociation inhibitor 1	Signal transduction					✓		✓
78	Calcyphosin	Signal transduction							
260	14-3-3 protein γ	Signal transduction		✓					
	Discriminant Analysis	Signal transduction		✓	✓		✓		✓

Figure S1: A General Linear Model was run for all bands to determine differences in average SIRT3 and SIRT5 calibrated volumes between (a) species: *M. galloprovincialis* and *M. trossulus* and (b) treatment groups. Treatment groups are abbreviated as follows: Control (CTL), Nicotinamide (NAM), Nicotinamide + Menadione (NAM/MND), Suramin (SUR), Suramin + Menadione (SUR/MND), Menadione (MND).

Figure S2: Western blot analysis of SIRT3 calibrated volumes between treatment groups of (a) *M. galloprovincialis* and (b) *M. trossulus*. Treatment groups are abbreviated as follows: Control (CTRL), Nicotinamide (NAM), Nicotinamide + Menadione (NAM/MND), Suramin (SUR), Suramin + Menadione (SUR/MND), Menadione (MND).

(b)

Figure S3: Western blot analysis of SIRT5 calibrated volumes between treatment groups of (a) *M. galloprovincialis* and (b) *M. trossulus*. Treatment groups are abbreviated as follows: Control (CTL), Nicotinamide (NAM), Nicotinamide + Menadione (NAM/MND), Suramin (SUR), Suramin + Menadione (SUR/MND), Menadione (MND).

(a)

(b)

Figure S4: Heat maps of proteins involved in protein homeostasis identified across both species: (a) *M. galloprovincialis* and (b) *M. trossulus*. Proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume. The treatments are labeled along the upper horizontal axis of each heat map, while the

right vertical axis represents the standardized expression patterns of the identified proteins.

Figure S5: Heat maps of proteins involved in ROS scavenging and redox regulation (oxidative stress) identified across *M. galloprovincialis*. Proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume. The treatments are labeled along the upper horizontal axis of each heat map, while the right vertical axis represents the standardized expression patterns of the identified proteins.

Figure S6: Heat maps of proteins involved in energy metabolism identified across both species: (a) *M. galloprovincialis* (b) *M. trossulus*. Proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume. The

treatments are labeled along the upper horizontal axis of each heat map, while the right vertical axis represents the standardized expression patterns of the identified proteins.

Figure S7: Heat maps of cytoskeletal proteins identified across both species: (a) *M. galloprovincialis* (b) *M. trossulus*. Proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume. The treatments are labeled along the upper horizontal axis of each heat map, while the right vertical axis represents the standardized expression patterns of the identified proteins.

(a)

Figure S8: Heat maps of signaling proteins identified across both species: (a) *M. galloprovincialis* (b) *M. trossulus*. Proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume. The treatments are labeled along the upper horizontal axis of each heat map, while the right vertical axis represents the standardized expression patterns of the identified proteins.

Figure S9: Heat map of immune defense proteins identified in *M. galloprovincialis*. Proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume. The treatments are labeled along the upper horizontal axis of each heat map, while the right vertical axis represents the standardized expression patterns of the identified proteins.

Figure S10: Heat map of heavy metal binding proteins identified in *M. trossulus*. Proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume. The treatments are labeled along the upper horizontal axis of each heat map, while the right vertical axis represents the standardized expression patterns of the identified proteins.

(a)

(b)

Figure S11: Expression profile graphs showing the relative expression of proteins involved in cytoskeletal dynamics in (a) *M. galloprovincialis* and (b) *M. trossulus*. Spot volumes were obtained by normalizing against the volume of all proteins, and means $\pm$ 1 SEM are shown (N=6 per treatment: *M. galloprovincialis*; N=4-6 per treatment: *M. trossulus*). Treatments with significant differences in expression level are marked with different letters. Treatment groups are abbreviated as follows: Control (C), Nicotinamide (N), Nicotinamide + Menadione (N/M), Suramin (S), Suramin + Menadione (S/M), Menadione (M).