THE PROTEOMIC RESPONSE OF SEA SQUIRTS (GENUS *CIONA*
CONGENERS) TO ACUTE HEAT STRESS: EVIDENCE FOR DIFFERENTIAL
THERMAL SENSITIVITIES

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ABSTRACT

The proteomic response of sea squirts (genus *Ciona* congeners) to heat stress: Evidence for differential thermal sensitivities

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The sea squirts *Ciona intestinalis* and *C. savignyi* have disparate distribution patterns, which may result from differences in their thermal tolerance limits. Because *C. intestinalis*, an almost cosmopolitan species, has a more widespread distribution, it is thought that it is better adapted to endure a wide range of temperatures. In order to compare the heat stress response between these two congeners, we studied global changes in protein expression, using a proteomics approach. To characterize the response to extreme heat stress, animals of both species were exposed to temperatures of 22°C, 25°C, and 28°C for 6 h, and then were left to recover at a control temperature (13°C) for 16 h. An additional experiment was conducted to assess the effect of mild-to-moderate heat stress including a 6 h exposure to temperatures of 18°C, 20°C, and 23°C, and a 16 h recovery at a control temperature (16°C). A quantitative analysis, using 2D gel electrophoresis and gel-image analysis, showed that in the high heat stress (HHS) experiment, 15% and 18% of the all protein spots detected demonstrated changes in expression in *C. intestinalis* and *C. savignyi*, respectively. In the low heat stress (LHS) experiment, 4% of the total number of proteins detected changed significantly in both *C. intestinalis* and *C. savignyi*. Using matrix-assisted laser desorption ionization (MALDI) tandem time-of-flight mass spectrometry, we were able to identify proteins with a 65–100% success rate, depending on species. Our results indicate that *C. intestinalis* maintains higher baseline levels of molecular chaperones and launches a quicker response to thermal stress than *C. savignyi*, suggesting it may be the more thermally tolerant of the two. In addition, actins, tubulins, and ATP-synthase F1 β -subunits were the most susceptible to proteolytic degradation, which may indicate that they have relatively higher thermal sensitivities.

Keywords: Proteomics, heat stress, *Ciona*

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I. INTRODUCTION

In 1929, Danish physiologist August Krogh gave a lecture at the 13th International Physiological Congress in Boston, Massachusetts, during which he set forth his idea that for every biological problem that scientists seek to study there exists a corresponding model organism that is perfectly suited to the question at hand. He said:

For such a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied. Many years ago when my teacher, Christian Bohr, was interested in the respiratory mechanism of the lung and devised the method of studying the exchange through each lung separately, he found that a certain kind of tortoise possessed a trachea dividing into the main bronchi high up in the neck, and we used to say as a laboratory joke that this animal had been created expressly for the purposes of respiration physiology. I have no doubt that there is quite a number of animals which are similarly "created" for special physiological purposes, but I am afraid that most of them are unknown to the men for whom they were "created," and we must apply to the zoologists to find them and lay our hands on them. (Krogh, 1929)

This concept has come to be known as "Krogh's Principle" and it has become a central concept in many fields of biology, including comparative physiology and functional genomics (Krebs, 1975).

In this study, the problem that we seek to understand is the way in which the whole organism responds to acute thermal stress, specifically at the protein level. In order

to address this broad question, two sister species of ascidian tunicate, the sea squirts *Ciona intestinalis* and *C. savignyi*, were chosen as a model study system. The evolutionary position of these two congeners places them right at the dividing line between vertebrates and invertebrates, giving their genomes the perfect “blend” of simplicity and complexity. This balance allows for a full understanding of the molecular mechanisms involved in the cellular stress response, while still resulting in conclusions that are applicable to “higher” organisms. The evolutionary proximity of the two *Ciona* congeners, in addition to differences in their distribution ranges, allow for a meaningful species comparison that highlights the molecular adaptations responsible for variation in thermal tolerance limits.

A natural extension of the Kroghian principle suggests that for every biological problem there also exists a scientific technique which is best suited to its study. In our attempt to understand the full scope of molecular changes that occur within the organism in response to thermal stress, proteomics was deemed to be the appropriate method of study. The advantage of the proteomics approach is that there are no previous assumptions made regarding the importance of a specific subset of proteins; instead

the entire complement of proteins present in the organism can be examined simultaneously, enabling the characterization of molecular mechanisms from a systems biology perspective. In other words, specific changes in the abundance of a single protein can be linked to global changes in the activity of multiple metabolic pathways. Comparing comprehensive changes across many metabolic pathways with observed changes in organismal physiology and population-level ecology allows for a complete understanding of the cellular stress response at a range of hierarchical levels, from individual molecules up to whole organism function.

In this chapter, I will discuss each of the three main components of this project: the *Ciona* study system, the problem of heat stress, and the technique of proteomics. In each of the subsequent sections, I will explore the relevant literature that has influenced current understanding in each of these areas. In addition, I will examine the relevance of the present work, from elucidating the molecular underpinnings of the chordate stress response to the implications of global climate change on marine invertebrates.

The Genus *Ciona*: *C. intestinalis* and *C. savignyi*

The genus *Ciona* is comprised of several species of ascidian tunicate, which are commonly referred to as sea squirts (Millar, 1953; Brusca and Brusca, 2003; Berná et al., 2009). Sea squirts are sessile and solitary marine invertebrate filter-feeders that occur both on natural substrates, such as rocks and eelgrass beds, and artificial substances, like dock pilings and ship hulls (Millar, 1971). Two species within the genus, *Ciona intestinalis* (Linnaeus, 1767) and *Ciona savignyi* (Herdman, 1882), are model organisms of particular interest to developmental biologists and embryologists (Nydam and Harrison, 2010). Their key evolutionary position as urochordates, in addition to their sequenced and annotated genomes, make these organisms ideal study systems for clarifying chordate phylogeny, as well as the mechanisms behind vertebrate development and evolution (Sato et al., 2003; Nydam and Harrison, 2007; Berná et al., 2009).

In addition, the *Ciona* congeners *C. intestinalis* and *C. savignyi* are classified as highly invasive species that are able to colonize a wide-variety of habitats (Carver et al., 2003; Lambert and Lambert, 2003; Carver et al., 2006; Blum et al., 2007). They have been known to outcompete

native marine invertebrates, which results in a decrease in the abundance and richness of native species (McDonald, 2004; Blum et al., 2007). This is especially true in aquaculture communities, particularly those involved in shellfish production, where *Ciona* have been shown to negatively impact the growth and abundance of many crop species (Carver et al., 2003; Carver et al., 2006; Ramsay et al., 2009). For this reason, ecological studies of the *Ciona* congeners are of particular interest for developing management strategies for aquaculture industries (Carver et al., 2003; Carver et al., 2006; Dijkstra and Harris, 2007).

In this section, we will start by discussing some of the basic ecology associated with sea squirts, mainly focusing on their reproductive strategy. Next, we will transition to a discussion of their evolutionary position and the important role that *Ciona* genomes play in studying vertebrate evolution. Lastly, we will end with an examination of population dynamics and discuss the conclusions that have been made concerning the characterization of phylogenetic relationships within the genus *Ciona*.

Ciona Ecology

Although there are some key differences between *Ciona intestinalis* and *C. savignyi*, these two closely related ascidian congeners share many aspects of their natural history and ecology. *Ciona* are hermaphroditic animals, meaning that they possess both male and female reproductive parts (Millar, 1953). Despite producing both male and female gametes, *Ciona* rely on cross-fertilization with other individuals to reproduce because they exhibit a high degree of self-sterility (Millar, 1953; Byrd and Lambert, 2005; Jiang and Smith, 2005). In addition, hybridization between *C. intestinalis* and *C. savignyi* has never been observed in nature, despite the fact that hybrids formed in the lab are able to develop to the tadpole stage (Byrd and Lambert, 2000; Berná et al., 2009).

Like other solitary ascidians, *Ciona* are broadcast spawners that eject vast quantities of sperm and eggs into the water column (Millar, 1971; Byrd and Lambert, 2000). *Ciona* are oviparous, meaning that once fertilization occurs, subsequent development of larvae takes place outside of the body (Millar, 1953; Millar, 1971). In addition to free swimming larvae, individuals also exude mucus strings consisting of fertilized eggs that attach to

nearby substrata and develop into adults (Petersen and Svane, 1995). This localized form of reproduction can have a great affect on the dispersal capabilities and subsequent distribution of *Ciona* populations (Svane and Havenhand, 1993; Petersen and Svane, 1995). In fact, researchers studying a population of *C. intestinalis* in a Danish fjord found that despite the abundance of pelagic larvae produced, few were found in the water column, resulting in highly localized dispersal and a nearly closed population structure (Svane and Havenhand, 1993; Petersen and Svane, 1995).

In order to maximize the chance of successful fertilization, *Ciona* populations exhibit synchronous spawning cued by specific environmental conditions (Millar, 1953; Millar, 1971). Light seems to be the main environmental cue that influences the time of spawning, and it has been used to induce spawning in the laboratory (Millar, 1971; Svane and Havenhand, 1993). A study by Lambert and Brant (1967) indentified a molecular mechanism for light as a trigger for spawning in *C. intestinalis*. They showed that the relative effectiveness of difference wavelengths in inducing spawning matches up almost exactly with the absorption spectrum of the chromophore cytochrome *c* (Lambert and Brant, 1967). This indicates that cytochrome

c, or some other closely related hemoprotein, may act as a receptor molecule that sends the signal to trigger spawning (Lambert and Brant, 1967).

In the field, *Ciona* are more abundant in shaded locations, suggesting that they prefer to live out of the direct sunlight (Svane and Havenhand, 1993). *Ciona intestinalis* all seem to spawn at a specific time of day, approximately one to one and a half hours before sunrise, presumably because the light conditions at this time of day allow larvae to hatch and find shaded areas to settle at midday (Millar, 1971; Svane and Havenhand, 1993).

Once the eggs are fertilized, *Ciona* embryos develop into larvae that attach themselves to a solid substratum and metamorphose into their sessile adult form (Chiba et al., 2004). During metamorphosis, the larval tail is reabsorbed, and the entire body plan is reoriented as the notochord becomes the adult ganglion and other adult tissues differentiate (Brusca and Brusca, 2003; Chiba et al., 2004). The embryonic period lasts approximately 24 hours, depending on water temperature (Svane and Havenhand, 1993). Adults are found at a variety of tidal depths, most commonly ranging from the tidal zone down to about 500 meters (Millar, 1953; Hewitt et al., 2002; Millar, 1971). Their preferred habitat varies widely from natural

substrates such as rocks, algal holdfasts, shells, and eelgrass beds, to artificial structures such as dock pilings, buoys, ship hulls, and even aquaculture equipment (Millar, 1953; Millar, 1971; Hewitt et al., 2002).

Normally, the generation time for *Ciona* is about 2-3 months; however, growth and development are also affected by temperature, and the time it takes to reach maturity has been shown to fluctuate across populations (Dybern, 1965; Millar, 1971; Chiba et al., 2004). As shown in Table 1, the number of generations produced per year varies across geographically separated populations and is influenced by water temperature (Millar, 1971). Generally, the growth rate, and therefore the number of generations produced per year, increases with increasing water temperature (Millar, 1971).

Table 1. Summary of the relationship between water temperature, depth and the number of generations per year for *C. intestinalis* across various populations (Adapted from Dybern, 1965).

Region	Depth	Temperature	No. of generations per year
Sub-Arctic.....	0–500 m	<6°C	<1
West coast of Norway.....	Ca. 100 m	6–9°C	<1
West coast of Norway in poller...	0–5 m	1–20°C	2
West coast of Sweden	15–30 m	5–15°C	1
West coast of Sweden	0–10 m	1–18°C	2
South coast of England..... (Plymouth)	Surface water	7–16°C	2(–3)
French Mediterranean coast.....	Surface water	12–26°C	At least 3
Gulf of Naples	Surface water	13–27°C	At least 3
Sub-tropical and tropical	Surface water	20–32°C	3–4 or more

The two *Ciona* congeners differ in their distinct distribution ranges. *Ciona intestinalis* is a cosmopolitan species with a nearly worldwide coastal distribution (Fig. 1a; Therriault and Heborg, 2008; Berná et al., 2009). *Ciona savignyi*, on the other hand, is a Pacific species geographically restricted to Japan and the western coast of North America (Fig. 1b; Lambert, 2003; Therriault and Herborg, 2008; Berná et al., 2009).

Figure 1. Global distribution maps of (A) *Ciona intestinalis*, and (B) *Ciona savignyi*. Grey shaded regions represent native or cryptogenic (origin unknown) ranges, while black shading represents invaded areas (Adapted from Therriault and Herborg, 2008, with *C. savignyi* information added from Lambert, 2003).

The historic distribution of *C. intestinalis* was restricted to natural populations found in Northern European waters; however, current populations are found worldwide, with the largest populations in the Mediterranean as well as the eastern and western coasts of North America (Hewitt et al., 2002). Additional populations are distributed across the western and northern seaboard of Europe, the western and eastern coasts of South America,

at Cape Verde, and off the Australian and Japanese coasts (Figure a; Millar, 1971).

Ciona savignyi are thought to be native to Japan, with the first recorded occurrence on the Pacific coast of North America in 1913 from a collection made by Ritter in Loring, Alaska (Nishikawa, 1991; Lambert, 2003). In 1937, more *C. savignyi* specimens were collected from British Columbia, and stored in the American Museum of Natural History (Lambert, 2003). These two Pacific coast specimens were at first falsely identified as *C. intestinalis* (Linnaeus, 1767), but were reexamined in 1985, when their true identity was revealed (Lambert, 2003).

Researchers believe that *C. intestinalis* have achieved their worldwide distribution, and their classification as an invasive species, due to their ability to be transported by ships via ballast waters or attached to ship hulls (Ritter and Forsyth, 1917; Svane and Havenhand, 1993; Hewitt et al., 2002; McDonald, 2004; Blum et al., 2007). However, this theory does not explain the restricted distribution of *C. savignyi*, as both species are equally able to colonize artificial substrata, and both are able to invade new habitats; instead, it suggests that differences in niche specifications may play a role in their differing

distributions (Lambert and Lambert, 2003; Zvyagintsev et al., 2007).

Recently genetic studies have suggested that distinct populations of *C. intestinalis* may be different sub-species, or perhaps even distinct species altogether (Suzuki et al., 2005; Caputi et al., 2007; Berná et al., 2009; Nydam and Harrison, 2010; Zhan et al., 2010). These findings call into question the previously established population genetic structure of *C. intestinalis*, as well as the genealogical relationships within the genus *Ciona*.

Evolutionary Importance

The phylum Chordata consists of three separate sub-phyla: Cephalochordata, Urochordata, and Vertebrata (Millar, 1971; Swalla et al., 2000; Satoh et al., 2003). Ascidian tunicates, including sea squirts within the genus *Ciona*, belong to the sub-phylum Urochordata (Millar, 1971; Corbo et al., 2001). This key phylogenetic position makes species within *Ciona* model organisms for the study of vertebrate evolution because they possess all of the basic features of vertebrate morphology and development, while retaining the genetic simplicity of an invertebrate (Corbo et al., 2001).

The first tunicates appeared in the fossil record approximately 550 million years ago (MYA), around the time of the Cambrian explosion (Dehal et al., 2002). Both ascidians and vertebrates are deuterostomes, meaning that their embryonic blastopore develops into the anus rather than the mouth, as is the case in protostomes (Dehal et al., 2002; Canestro et al., 2003). Since urochordates and vertebrates split approximately 486 MYA, vertebrates have undergone two genome-wide duplication events, which have greatly increased their genome size and led to the complexity of modern vertebrates (Moody et al., 1999; Dehal et al., 2002; Dehal and Boore, 2005; Suzuki et al., 2005; Putnam et al., 2008).

The first of these two whole genome duplications is thought to have occurred before agnaths (jawless vertebrates including hagfish and lampreys) branched from the remaining vertebrates, and the second is thought to have taken place before the cartilaginous fish-bony vertebrate split (Dehal and Boore, 2005; Putnam et al., 2008; Berná et al., 2009). This 2R hypothesis, for “two rounds” of duplication, posits that the genomic redundancy that results from a duplication event allows for some genes to develop new functions, called neofunctionalization, while other genes become specialized due to the

partitioning of old functions across several genes, termed subfunctionalization (Dehal and Boore, 2005). It is thought that neo- and subfunctionalization have allowed for the development of the complex vertebrate genome (Dehal and Boore, 2005).

Due to a lack of duplication events, basal chordates, like *C. intestinalis* and *C. savignyi*, have significantly smaller genomes containing only the basic complement of genes necessary for the development of a common chordate ancestor (Moody et al., 1999; Dehal et al., 2002). Thus, *Ciona* are model organisms for the study of vertebrate development and evolution.

Population Genetics

In 2002, Paramvir Dehal and his team were able to draft the complete genome sequence of *C. intestinalis* using sperm from a single individual and a whole-genome shotgun approach. In shotgun sequencing, overlaps between multiple copies of sequenced DNA fragments are identified and then pieced together to decipher the full DNA sequence (Dehal et al., 2002). Dehal and his team found that the *C. intestinalis* genome contains approximately 16,000 protein coding genes, which is comparable to the number found in invertebrates and is far fewer than the number found in

vertebrates (Dehal et al., 2002; Kim et al., 2007). They were also able to estimate that the total *C. intestinalis* genome consists of 160 million base pairs, which makes it the smallest of any accessible chordate genome and about 20 times smaller than the human genome (Dehal et al., 2002).

Initial attempts to sequence the *C. savignyi* genome were published in 2005 by Jade Vinson and colleagues (Vinson et al., 2005). The genome assembly for *C. savignyi* was especially challenging due to the high degree of polymorphism present in the *C. savignyi* genome (Vinson et al., 2005; Small et al., 2007). A highly polymorphic genome complicates whole-genome shotgun sequencing because divergent haplotypes are sequenced together, making it difficult to differentiate between alleles at the same locus and paralogs at different loci (Vinson et al., 2005; Small et al., 2007). The resultant non-redundant reference sequence was found to contain 174 million base pairs, with each locus represented once (Small et al., 2007).

Both *C. intestinalis* and *C. savignyi* have been found to have highly polymorphic genomes, meaning that large genetic differences exist between members of the same species (Dehal et al., 2002). In fact, the *C. savignyi* genome has a single-nucleotide polymorphism (SNP) ratio of 4.5%, which is considered to be the highest in the animal

kingdom (Vinson et al., 2005; Zhan et al., 2010). The SNP ratio of *C. intestinalis* is 1.2%, which is still large considering it is 15-fold higher than the SNP ratio in humans (Dehal et al., 2002). This high degree of polymorphism within the *Ciona* genome has traditionally been explained by a high mutation rate and the large effective population size attributed to their broadcast spawning (Kimura, 1983; Dehal et al., 2002; Hedgecock et al., 2004; Zhan et al., 2010).

However, as mentioned previously, although *Ciona* have pelagic larvae, they are unable to disperse far from their adult population (Svane and Havenhand, 1993; Petersen and Svane, 1995). This has resulted in limited natural dispersal ability and has led to nearly closed populations that have little gene flow between them (Petersen and Svane, 1995; Hewitt et al., 2002). *Ciona intestinalis* populations have historically been genetically separated, and it has been suggested that any gene flow that occurs between current populations is due to the advent of anthropogenic dispersal mechanisms (Millar, 1971; Caputi et al., 2007). Thus, the high degree of polymorphisms present in the *Ciona* genomes are most likely due solely to high mutation rate (Berná et al., 2009).

Recently, several studies have demonstrated that the species *Ciona intestinalis* is actually comprised of several morphologically identical but genetically distinct cryptic species (Suzuki et al., 2005; Caputi et al., 2007, Ianelli et al., 2007; Nydam and Harrison, 2007, 2010; Zhan et al., 2010). At least three distinct cryptic species have been identified including a Northeast Pacific/Mediterranean type (spA), a Northwest Atlantic type (spB), and a third type (spC) that is restricted to a specific location off the coast of France in the Mediterranean Sea (Fig. 2; Caputi et al., 2007, Ianelli et al., 2007; Nydam and Harrison, 2007, 2010; Zhan et al., 2010). Because there is little information available in the literature concerning *C. intestinalis* spC, the remainder of this discussion will focus on *C. intestinalis* spA and spB.

Figure 2. Distribution of cryptic species *Ciona intestinalis* sp.A (pink), sp.B (pale blue), and sp.C (red dot). White shaded circles represent populations that have yet to be classified (modified from Ianelli et al., 2007).

To determine whether different populations of *Ciona intestinalis* constitute genetically distinct species, researchers compared randomly selected shotgun sequences between British and Japanese populations and contrasted them using the BLAST (Basic Local Alignment Search Tool) algorithm (Suzuki et al., 2005). This allowed them to calculate the sequence homology between *C. intestinalis* spA and spB (Suzuki et al., 2005). They found that these two cryptic species show 13% sequence divergence, slightly less

than the 15% sequence divergence present between *Ciona intestinalis* and *C. savignyi* (Suzuki et al., 2005). These numbers are especially striking when compared to the amount of sequence divergence between humans and chimpanzees, which is estimated to be 1.24%, $\pm 0.07\%$ (Chen and Li, 2001). Since 2005, additional studies have confirmed the classification of at least two genetically distinct types of *C. intestinalis* based on significant differences between microsatellite markers and mitochondrial DNA sequences (Caputi et al., 2007; Ianelli et al., 2007).

Researchers have also been able to reconstruct a phylogeny detailing the relationships within the *Ciona* genus by comparing the amino acid sequences from 13 protein coding genes (Fig. 3; Suzuki et al., 2005). Their phylogenetic analysis confirms the existence of two distinct species within *C. intestinalis* that show little relationship to the sister species *C. savignyi* (Suzuki et al., 2005).

Figure 3. Phylogenetic relationships within the genus *Ciona*. Local bootstrap confidence values are shown on the branches and horizontal branch lengths are proportional to the estimated number of amino acid substitutions per site. Sequence divergence estimates are shown in red (modified from Suzuki et al., 2005).

Using the calculated mutation rate for the fruit fly, *D. melanogaster*, researchers were also able to calculate the divergence time, τ , between *C. intestinalis* spA and spB (Suzuki et al., 2005). They found that approximately 28–37 million years have passed since the two genetically distinct populations diverged from one another (Suzuki et al., 2005). However, because they did not directly calculate the mutation rate for *C. intestinalis*, their estimated divergence time may be inaccurate if the molecular clock of *C. intestinalis* runs significantly faster or slower than that of *D. melanogaster*. Also, it is hard to confirm their estimated divergence time because there are very few examples of tunicates in the fossil record (Suzuki et al., 2005).

Recently, efforts have been made to characterize the rate of molecular evolution within the genus *Ciona* in order to clarify phylogenetic relationships within the genus and provide a more accurate estimate of the divergence time between *C. intestinalis* and *C. savignyi* (Berná et al., 2009). Previous estimates proposed that the *Ciona* congeners, *C. intestinalis* sp. A and *C. savignyi*, diverged from one another between 200–280 million years ago (MYA), based on insect and vertebrate molecular clocks, respectively (Small et al., 2007; Berná et al., 2009). However, by comparing average amino acid distances and divergence times for many vertebrate species pairs, Berná and colleagues were able to demonstrate that the *Ciona* genomes evolve faster than that of other vertebrates, 50% faster on average, with few exceptions (Berná et al., 2009). Also, previous estimates were made prior to when the new phylogeny of chordate organisms was established, which indicates that urochordates, not cephalochordates, are the closest relatives of modern vertebrates (Delsuc et al., 2006; Putnam et al., 2008; Berná et al., 2009).

Taking all of this information into account, researchers were able to calibrate the molecular clock of ascidian evolution, and re-estimate the divergence time between the *Ciona* congeners (Berná et al., 2009). They

determined that *C. intestinalis* and *C. savignyi* diverged from one another approximately 184 (± 15) MYA (Berná et al., 2009). This means that the *Ciona* congeners diverged from one another around the same time that humans diverged from platypuses, 162–191 MYA (Berná et al., 2009). The fact that these two sister species, with nearly indistinguishable morphologies and almost identical life histories, diverged from one another nearly 200 MYA and possess such extensive genetic differences is truly astonishing. It shows how limited we are in our conventional knowledge of evolutionary processes, and makes the *Ciona* congeners an ideal study system for clarifying the mechanism and progression of chordate evolution.

In conclusion, ascidian sea squirts belonging to the genus *Ciona* are sessile and solitary marine invertebrates that can thrive on both natural substrates and artificial substances, like ship hulls (Millar, 1953; Millar, 1971; Byrd and Lambert, 2000; Berná et al., 2009). Their opportunistic nature has allowed them to take advantage of anthropogenic means of dispersal, which has contributed to their classification as invasive species (Carver et al., 2003; McDonald, 2004; Carver et al., 2006; Blum et al., 2007). *Ciona* are classified as urochordates, and as such they are a part of the subphylum within the phylum Chordata that is

most closely related to the vertebrate subphylum (Corbo et al., 2001; Satoh et al., 2003). The easy accessibility of their sequenced genomes, as well as their genomic and anatomic simplicity allow for comparisons that highlight the differences between invertebrates and early vertebrates (Corbo et al., 2001). Overall, *Ciona* is an important genus that provides a multitude of scientific research opportunities ranging from the ability to learn more about how introduced species impact native communities, to understanding more about the evolutionary relationships between vertebrates and invertebrates.

Heat Stress

Heat stress influences organisms on several levels, from their molecular physiology all the way up to their behavioral ecology (Pörtner et al., 2006; Pörtner, 2010). In order to understand the full impact of heat stress, we will start by discussing how environmental temperature affects physiological functions and why organisms have had to find a way to cope with thermal stress. Next, we will explore the history of the heat shock response and the discovery of heat shock proteins. Then, we will move beyond heat shock proteins and discuss the full cellular stress response, characterized by a “minimal stress proteome”, which includes proteins conserved across all three domains that are responsible for responding to thermal stress (Kültz, 2005). Lastly, we will conclude by relating the heat shock response to organismal ecology and discussing the relationship between a species’ thermal tolerance window and its biogeographical distribution (Somero, 2002; Tomanek, 2008; Pörtner, 2010; Tomanek, 2010).

Environmental temperature affects cellular function in two fundamental ways. First, temperature is directly related to the rate of physiological reactions, such that an increase in temperature corresponds to an increase in

the reaction rate (Somero, 1969; Gillooly et al., 2001). This effect is quantified by a temperature coefficient, known as the Q_{10} value, which expresses the temperature dependence of a physiological reaction in relation to temperature changes of 10 degrees Celsius (Somero, 1978; Gillooly et al., 2001). In other words, if the rate of a reaction doubles when the temperature is increased 10°C, it is said to have a Q_{10} of 2 (Somero, 1978; Hochachka and Somero, 2002).

Second, elevated temperatures affect the stability and integrity of macromolecular structures, including membrane lipids and proteins (Somero, 1969; Hochachka and Somero, 2002). Environmental temperature fluctuations affect the permeability and viscosity of membrane lipids, with membranes becoming more fluid at higher temperatures (Singer and Nicolson, 1972; Spector and Yorek, 1985). Temperature also affects protein structure at all levels except primary, as the peptide bonds that connect amino acids remain stable even at biologically extreme temperatures (Somero, 1978). Secondary, tertiary, and quaternary protein structure are all temperature-sensitive because they are maintained by weak bonds, such as van der Waals forces and hydrophobic interactions (Somero, 1978). These bonds are destabilized at temperature extremes,

causing proteins to unfold or denature, thus impairing their function (Somero, 1978; Baldwin, 1986).

When facing long-term exposure to elevated temperatures, cells are able to utilize various homeostatic mechanisms in order to overcome these heat-induced changes in physiological reaction rates, membrane fluidity, and protein structure. To maintain constant metabolic reaction rates over a wide range of environmental temperatures, cells are able to adjust enzyme properties in order to achieve “metabolic temperature compensation” (Somero, 1969; Hochachka and Somero, 2002). This can be accomplished by altering the enzyme-substrate (E-S) affinity, measured as the inverse of the Michaelis-Menten constant (K_m), which at the low substrate concentrations found in the cell is the key determinant of the catalytic reaction rate (Alberty, 1952; Somero 1969; Somero, 1978). Therefore, E-S affinity is inversely related to changing environmental temperature, which effectively, though not completely, compensates for thermal variations and allows for the maintenance of nearly constant metabolic reaction rates (Somero, 1969; Somero, 1978; Hochachka and Somero, 2002).

To combat the effect of fluctuating temperature on the stability of membrane lipids, cells can alter the composition of fatty acids within the membrane in order to

maintain constant fluidity and permeability (Singer and Nicolson, 1972; Spector and Yorek, 1985). When environmental temperature is increased, cells increase the proportion of saturated fatty acids, stabilizing the bonds between fatty acid tails, and keeping the membrane from becoming too fluid (Singer and Nicolson, 1972; Spector and Yorek, 1985). Maintaining the integrity of biological membranes is important in order to ensure effective transport and constant membrane permeability (Singer and Nicolson, 1972; Sinensky, 1974).

Once environmental temperatures are elevated to the point that macromolecular damage begins to occur, the cell launches a targeted response referred to as the heat shock response (for review see Lindquist, 1986; Lindquist and Craig, 1988; Feder and Hofmann, 1999). This phenomenon was first discovered nearly half a century ago in studies focusing on the fruit fly *Drosophila melanogaster* (Ritossa, 1962; Storti et al., 1980). In 1962, Ritossa found that chromosomes in the salivary glands of larval *D. melanogaster* underwent transcriptional changes when the larvae were kept at elevated temperatures. Over time it was shown that at increased temperatures, a small set of proteins were up-regulated in *Drosophila* cells, while nearly all other protein synthesis was put on hold (Storti

et al., 1980). These proteins came to be known as heat shock proteins (HSPs), and as their name implies, they can be induced in response to thermal stress (Storti et al., 1980; DiDomenico et al., 1982, Lindquist, 1986; Feder and Hofmann, 1999).

After many years of study, several well established conclusions have been made about the structure and function of heat shock proteins. First, it is apparent that HSP genes are highly conserved evolutionarily, and that heat shock proteins are present in nearly every species that has been examined thus far (Lindquist, 1986; Feder and Hofmann, 1999; Tomanek, 2011). These HSP genes have been subdivided into several families based upon sequence homology and differences in molecular weight, function, and cellular localization (Feder and Hofmann, 1999).

Second, although functions differ slightly between families, all HSPs are able to function as molecular chaperones to stabilize and repair misfolded proteins (Mohsenzadeh et al., 1994; Gottesman et al., 1997; Feder and Hofmann, 1999). Properly folded proteins sequester hydrophobic amino acid residues in their interior region, effectively protecting them from the aqueous environment of the cytosol (Gottesman et al., 1997; Feder and Hofmann, 1999; Hochachka and Somero, 2002). This structural

configuration is integral to maintaining proper protein function (Hochachka and Somero, 2002). When proteins are denatured, their hydrophobic interiors are exposed, increasing the likelihood of hydrophobic interactions between proteins, and leading to aggregations of misfolded, non-functional proteins (Gottesman et al., 1997; Feder and Hofmann, 1999; Hochachka and Somero, 2002). Molecular chaperones function by binding exposed hydrophobic amino acid groups, preventing inappropriate hydrophobic interactions, and facilitating the re-folding of damaged proteins (Feder and Hofmann, 1999; Hochachka and Somero, 2002; Tomanek, 2008). Because heat stress often results in denatured proteins, the ability of HSPs to act as chaperones makes them key players in the heat stress response, and the main indicators of stress-induced protein damage (Lindquist, 1986, Samali and Orrenius, 1998; Feder and Hofmann, 1999; Tomanek, 2008).

Heat shock proteins have also been shown to function in conjunction with ATP-dependent proteases and ubiquitin-mediated protein degradation pathways in order to degrade proteins that cannot be repaired (Parsell and Lindquist, 1993; Hayes and Dice, 1996; Fisher et al., 1997; Gottesman et al., 1997). In addition, HSPs have been implicated in the prevention of stress-induced apoptosis, or programmed

cell death, both by dampening upstream signaling events and inhibiting downstream effects, namely the caspase proteolytic cascade (Mosser et al., 1997; Samali and Orrenius, 1998). This last function may help to explain the phenomenon of thermotolerance, also known as cross-tolerance, in which cells pre-conditioned with a mild heat shock are able to withstand a level of stress that would otherwise be lethal (Moseley, 1997; Samali and Orrenius, 1998; Kültz, 2005).

The third conclusion that can be drawn from past studies is that despite their name, not all HSPs are induced by stress; some are constitutively expressed to ensure normal folding of newly synthesized proteins (Hayes and Dice, 1996; Samali and Orrenius, 1998; Feder and Hofmann, 1999). Constitutively expressed HSPs have also been found to play a role in protein transport, as the enzyme responsible for removing clathrin from coated vesicles, clathrin-uncoating ATPase, is a member of the HSP70 gene family (Lindquist, 1986). In addition, increased levels of constitutive HSPs have been associated with increased protection against not only thermal stress, but also oxidative stress in mouse, hamster, and monkey cells (Lindquist, 1986; Lindquist and Craig, 1988; Mehlen et al., 1995). In fact, past research has shown that extreme

temperature is not the only stress that causes elevated expression of HSPs. Heat shock proteins have also been implicated in the cellular response to a wide-variety of stresses including exposure to extreme ion concentrations, low oxygen environments (hypoxia), heavy metals, and other toxic substances, just to name a few (Feder and Hofmann, 1999; Kültz, 2005).

As our knowledge of heat shock proteins has increased and our experimental tools have become more sophisticated, it is no longer sufficient to show that an as yet unexamined species expresses HSPs in response to a particular stress. Instead, the focus has shifted to understanding all of the molecular mechanisms behind the entire stress response, of which HSPs are only a part (Feder and Hofmann, 1999; Kültz, 2005; Tomanek, 2011; Tomanek and Zuzow, 2010). Heat shock proteins are now referred to as “stress proteins” and the term “cellular stress response” (CSR) has been coined to describe the cell’s overall reaction to the macromolecular damage inflicted by various environmental stressors (Kültz, 2005; Wang et al., 2009). This definition highlights the fact that the CSR is not stressor-specific, but instead it describes the way a cell copes with the end result of stress: macromolecular damage, including the

deformation and functional impairment of DNA, proteins, and membrane lipids (Kültz, 2005; Tomanek and Zuzow, 2010).

In order to study the cellular stress response as a whole, researchers have gone from assaying the effect of stress on specific genes or proteins, to utilizing “-omics” techniques, such as transcriptomics, metabolomics, and proteomics, to study global expression changes in response to stress (Gracey and Cossins, 2003; Feder and Walser, 2005; Mitchell-Olds et al., 2008). The advantage of “-omics” techniques is that there are no previous assumptions made regarding the importance of a subset of genes or proteins; instead the entire complement of mRNA transcripts or proteins of an organism can be simultaneously monitored for their response to stress (Feder and Walser, 2005; Tomanek, 2011). Many studies have used microarrays to profile the transcriptomic response of various organisms to environmental stress (for review see Gracey and Cossins, 2003); however, the focus of research into the stress response is shifting to proteins and the characterization of the proteome (Feder and Walser, 2005). Proteins are the functional unit of the cell and, unlike mRNA transcripts, protein expression changes can be directly linked to changes in the molecular phenotype that occur in response

to environmental stimuli (Hochachka and Somero, 2002; Feder and Walser, 2005).

Proteomic studies have generated large data sets that have helped to elucidate the role that many proteins play in the CSR. Results from these studies have shown that a subset of proteins are remarkably well conserved across many taxa, and these are often referred to as the “minimal stress proteome” (Kültz, 2005; Petrak, 2008; Wang et al., 2009; Tomanek, 2011). These stress-induced proteins have a wide-variety of functions that suggest the involvement of many different cellular pathways in the CSR (Kültz, 2005; Wang et al., 2009; Tomanek, 2011).

One component of this response includes proteins responsible for repairing macromolecular damage, such as molecular chaperones that assist in protein folding and proteins involved in DNA damage sensing and repair (Kültz, 2005; Petrak et al., 2008; Wang et al., 2009; Tomanek, 2011). Another element of the minimal stress response are proteins involved in protein synthesis and degradation, including both ATP-dependent degradation mechanisms and ATP-independent proteases, indicating that protein turnover pathways are affected by stress (Mohsenzadeh et al., 1994; Kültz, 2005; Tomanek and Zuzow, 2010; Tomanek, 2011). Cellular energetics also play a key role in the CSR,

including proteins involved in carbohydrate and fatty acid metabolism and ATP synthesis (Kültz et al., 2005; Wang et al., 2009; Tomanek and Zuzow, 2010; Tomanek 2011). Stress also impacts cellular energetics by altering the ratio of NAD^+ to NADH , also known as the cellular redox state (Krebs and Veech, 1969). Both NAD^+ and NADH are important oxidizing and reducing agents, respectively, which must be maintained in a delicate balance to ensure continued cellular respiration and ATP production (Krebs and Veech, 1969).

Antioxidant proteins that are responsible for protecting the cell from damage caused by harmful reactive oxygen species (ROS) are also represented. Reactive oxygen species, such as superoxide anions ($\cdot\text{O}_2^-$), hydrogen peroxides (H_2O_2), and hydroxyl radicals ($\cdot\text{OH}$), form spontaneously as a by-product of the electron transport chain (ETC) and can cause serious damage to DNA and proteins (Dean et al., 1997; Chandel and Schumacker, 2000; Kültz, 2005). When a cell is undergoing nearly any type of stress, there is an increased demand for ATP, leading to an increase in electron flux through the ETC (Brand and Murphy, 1987). The increased electron flux causes higher than normal levels of ROS to accumulate, which results in increased damage to macromolecules (Rabilloud et al., 2002; Kültz, 2005; Tomanek and Zuzow, 2010).

Cytoskeletal elements are especially sensitive to damage by ROS, and there is evidence to suggest that cytoskeletal proteins may provide a buffer against the excessive oxidative damage caused by ROS (Huot et al., 1997; Dalle-Donne et al., 2001; Tomanek and Zuzow, 2010; Tomanek, 2011). Following exposure to stress, small heat shock proteins (sHSPs) are phosphorylated in order to help stabilize the actin cytoskeleton; therefore, cytoskeletal reorganization, as well as increased synthesis of sHSPs are hallmarks of the cellular stress response (Dalle-Donne et al., 2001; Rabilloud et al., 2002; Kültz, 2005; Wang et al., 2009; Tomanek and Zuzow, 2010; Tomanek, 2011). While stress induces increased synthesis of some proteins, cell cycle proteins and proteins involved in cellular growth are inhibited, most likely in order to balance the increased energy costs of repairing stress-induced molecular damage (Kültz, 2005; Wang et al., 2009; Tomanek, 2011). Lastly, if the cell is unable to cope with prolonged or extreme environmental stress, proteins are triggered that begin the cascade of events leading to apoptosis, or programmed cell death (Samali and Orrenius, 1998; Kültz, 2005; Wang et al., 2009; Tomanek, 2011).

The large number of cellular pathways involved in responding to stress gives an indication as to the inherent

complexity that characterizes the cellular stress response. Overall, the main function of the CSR is to preserve the normal function of many biological processes. This is accomplished by diverting cellular energy to producing proteins that repair and recycle macromolecules damaged by stress, and increasing energy production to meet these demands, while maintaining a balanced redox state. These major physiological functions are only a part of the overall CSR, and while the full picture is starting to emerge, the details are still not fully understood.

Thus far, we have discussed the effect of environmental stress at both the molecular and cellular levels. In order to fully understand the impact of thermal tolerance limitations on biogeographical distributions and community-level ecology, we must now discuss the physiological mechanisms that are affected by stress at the organismal level of organization (Pörtner, 2001; Pörtner, 2002). Individuals function best within a certain range of temperatures that enable successful growth and reproduction, allowing for the maintenance of population-level community structure within a natural environment (Pörtner, 2001; Pörtner, 2002; Pörtner and Knust, 2007). Outside of this thermal window, organisms begin to experience a decline in physiological function that is

closely linked to the rise in oxygen demand that occurs at extreme temperatures (Pörtner, 2001; Pörtner, 2002; Pörtner and Knust, 2007).

As mentioned earlier, as environmental temperature increases, electron flux through the electron transport chain (ETC) also increases (Brand and Murphy, 1987; Chandel and Schumacker, 2000). Because oxygen is the final electron acceptor of the ETC, the increased electron flux correlates with an increased need for oxygen within the cell (Brand and Murphy, 1987; Pörtner, 2001; Pörtner, 2002; Pörtner and Knust, 2007). At an organismal level, this increased oxygen requirement can be quantified by the concentration of oxygen present in the arterial blood supply, PO_2 (Pörtner, 2001; Pörtner, 2002). To exacerbate the problem, as temperature increases, oxygen solubility decreases, making it even more difficult to deliver sufficient oxygen to tissues (Pörtner and Knust, 2007).

As environmental temperatures rise, organisms compensate for the increased oxygen demand by increasing their heart and ventilation rates (Pörtner, 2001; Braby and Somero, 2006). Once these physiological processes reach their maximum, if temperature continues to rise, PO_2 begins to fall, creating an increasingly hypoxic environment inside the body (Pörtner, 2001; Pörtner, 2002). At a

cellular level, this creates a situation in which the cell experiences both thermal and oxidative stress simultaneously, which provides one explanation for the role of antioxidant proteins in the minimal stress response (Davidson et al., 1996; Tomanek, 2011).

The temperature at which PO_2 , and aerobic performance, starts to decline is referred to as the pejus temperature (T_p)– pejus means ‘getting worse’ in Latin (Pörtner, 2001; Pörtner, 2002; Pörtner, 2010). At thermal extremes, oxygen limitation is observed long before full functional failure, therefore decreased aerobic capacity plays an important role in setting a species’ thermal tolerance window (Pörtner, 2001; Pörtner, 2002; Pörtner and Knust, 2007; Pörtner, 2010). At temperature extremes outside of the T_p , PO_2 and physiological function continue to decline, and eventually the organism reaches its critical temperature (T_c), which indicates that the mode of mitochondrial metabolism has switched from aerobic to anaerobic (Pörtner, 2001; Pörtner, 2002; Pörtner and Knust, 2007; Pörtner, 2010). Past the T_c , an organism encounters its denaturation temperature, T_d , which is defined as the temperature at which molecular damage begins to occur (Pörtner, 2001; Pörtner, 2002; Pörtner and Knust, 2007; Pörtner, 2010). It is when an organism reaches its T_d that the cellular stress

response is launched (Pörtner, 2001; Pörtner, 2002). Pejus, critical, and denaturation temperatures characterize a species-specific thermal tolerance window, and constitute a direct measurement of a species' thermal sensitivity (Pörtner, 2001). Thus, variations in thermal tolerance windows between species can give an important indication of their expected biogeographical distributions (Pörtner, 2001; Perry et al., 2005; Pörtner and Knust, 2007; Tomanek, 2010)

One way to study thermal tolerance differences between species at an organismal level is to look at two closely related species with different distributions and characterize their thermal stress responses. In one study, proteomics was used to analyze the effect of acute thermal stress on the blue mussel species pair *Mytilus galloprovincialis* and *M. trossulus* (Tomanek and Zuzow, 2010). Several studies measuring heart rate, ubiquitin-conjugate formation, and protein thermal stabilities all suggest that *M. galloprovincialis* is a more warm-adapted species than *M. trossulus* (Braby and Somero, 2006; Tomanek and Zuzow, 2010). By comparing the stress proteomes of these two congeners, the researchers were able to pinpoint specific cellular mechanisms that differed between the two species and may provide a molecular explanation for their

observed differences in thermal tolerance (Tomanek and Zuzow, 2010).

Using a systems-level approach, Tomanek and Zuzow were able to link molecular mechanisms with observed differences in biogeographical range (Tomanek, 2008; Tomanek and Zuzow, 2010). With recent increases in temperature due to global climate change already impacting the distribution of many species, more studies like this need to be done to relate changes observed at the organismal level with cellular and molecular mechanisms (IPCC, 2007; Tomanek, 2008; Tomanek, 2010). In order to predict how species will respond to further environmental change, we must integrate our knowledge of the molecular, cellular, organismal, and community-level components of thermal tolerance, in order to create a comprehensive understanding of the impact of stress at all hierarchical levels.

Proteomics

When studying cellular stress physiology, the first question that must be answered is: what is the best molecular indicator of cellular stress? A good indicator must meet three basic criteria: sensitivity to even minute changes in the environment, fast response time, and prolonged duration within the cell (Feder and Walser, 2005). Some studies have taken a genomics approach and used mRNA transcripts as markers of cellular stress (Gracey and Cossins, 2003). This approach has drawbacks however, because mRNA is short-lived within the cell, and many mRNA signals are not transcribed into functional proteins (Abbott, 1999; Feder and Walser, 2005). In fact, the correlation between mRNA and protein levels is generally lower than 0.5 due to post-transcriptional modifications and differences in the rates of degradation (Abbott, 1999). Proteins, on the other hand, are the functional unit of the cell, and as such, they meet all three criteria for a good indicator (Feder and Walser, 2005). Proteins, or their post-translational modifications, are sensitive to even small changes in the environment, and their relative abundances change in a dynamic fashion that allows them to respond quickly to a wide variety of environmental

perturbations (Hochachka and Somero, 2002). In addition, the half-life of most stress-induced proteins is in the range of days to several weeks, making their signal detectable for a relatively long time (Tomanek and Somero, 2000). For these reasons, proteins are ideal indicators of stress and therefore are the focus of many studies of the cellular stress response (Feder and Hofmann, 1999; Tomanek, 2002; Kültz, 2005; Tomanek and Zuzow, 2010).

Proteomics is the term used to describe a wide range of techniques that all seek to quantify the full complement of proteins within the cell, or the proteome, either in response to changing environmental conditions, or to observe changes over time (Tyers and Mann, 2003; Berth et al., 2007; Nesatyy and Suter, 2007). Proteomics is a systems biology approach that employs a holistic perspective to analyze complex interactions in biological systems (Bantscheff et al., 2007). It is discovery-based, not hypothesis-driven, meaning that the end result of a proteomic study is a suite of testable hypotheses concerning the molecular workings of observed phenomena (Tomanek and Zuzow, 2010). Proteomics can be used to compare closely related organisms that have been adapted to different environmental conditions or exposed to different types of environmental stress (Tomanek and Zuzow, 2010).

Analyzing differences between the proteomes of congeneric species allows for the characterization of the physiological basis of environmental responses and adaptations from a global perspective (Tomanek and Somero, 2000; Tomanek, 2002).

Any proteomics technique can be broken down into three basic steps: protein separation, quantification, and identification. The classical approach to protein separation is two-dimensional gel electrophoresis (2-D GE), which has been used since its introduction in the mid-1970's (MacGillivray and Rickwood, 1974; Rabilloud, 2002). Generally, this technique separates proteins in two dimensions, by pH and molecular mass, utilizing SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Other separation techniques have been developed over the years, including semi-gel-free systems and shotgun gel-free liquid chromatography methods (Berth et al., 2007). These systems can be used in order to circumvent some of the intrinsic limitations of 2-D GE, including the underrepresentation of low abundance and hydrophobic membrane proteins, and the difficulty in resolving proteins in the extreme acidic and basic ranges (Rabilloud, 2002). In addition, complex biological samples contain more proteins than can be visualized on a single gel, thus

characterization of an entire proteome using 2-D GE is still not feasible (Stochaj et al., 2003). Therefore, utilizing gel-based and gel-free separation techniques in conjunction with one another can be beneficial, mainly by increasing the percentage of the entire proteome that can be visualized and characterized (Berth et al., 2007). Despite the inherent limitations, two-dimensional gel electrophoresis still allows for simultaneous quantification of up to 90% of the proteome, and is the most widely used technique for protein separation; therefore, it will be the focus of this discussion (Berth et al., 2007).

Protein Separation

The protocol associated with protein separation via 2-D GE can be divided into 4 main steps: (1) sample preparation, (2) isoelectric focusing, or 1st dimension, (3) SDS-PAGE, or 2nd dimension, and (4) staining. Many different types of samples can be analyzed using 2-D GE, from isolated organelle fractions to whole organism tissue samples (Görg et al., 2000). No matter which type of sample is used, proteins within the sample must first be fully solubilized, disaggregated, denatured, and reduced to be effectively separated (Stochaj et al., 2003).

To accomplish this, samples are homogenized in a specially formulated lysis buffer containing a neutral chaotrope, such as urea or thiourea, a zwitterionic detergent, such as CHAPS, a reducing agent, such as dithiothreitol (DTT), and carrier ampholytes that maintain an optimum pH for maximum protein solubility and minimal protein aggregation (Stochaj et al., 2003; Posch et al., 2006). The reagents present in the homogenization buffer ensure that cell membranes are quickly and thoroughly lysed, so that there is little to no protease activity. Some formulations even include protease inhibitors designed to prevent specific proteolytic interactions; however protease inhibitors are expensive and if chaotrope concentrations are high enough, they are not always required (Stochaj et al., 2003; Posch et al., 2006). Once samples are homogenized, it is sometimes necessary, especially when working with marine samples, to purify the proteins to remove salts or other interfering substances, such as nucleic acids, lipids, and phenolic compounds (Stochaj et al., 2003; Posch et al., 2006). This is accomplished by precipitating the proteins using trichloroacetic acid (TCA) and acetone, washing them with acetone to remove all traces of TCA, and then re-

solubilizing them using a salt-free rehydration buffer (Stochaj et al., 2003; Posch et al., 2006).

The last phase of sample preparation includes running an assay to determine the exact protein concentration in each sample to ensure that the amount of protein loaded on to each gel is standardized across all samples. The two most common methods of spectrophotometric protein quantitation are characterized by either Coomassie dye binding or protein-catalyzed reduction of cupric (Cu^{2+}) ions to cuprous (Cu^+) ions

(Bradford, 1976; Walker, 1996). Both of these methods are unsuitable for proteomic studies,

however, due to their incompatibility with

detergents (CHAPS and SDS) and reducing agents (DTT) present in samples prepared for 2-D GE (Krieg et al., 2005). For this reason, new methods have been developed that precipitate proteins while excluding interfering substances, which allows for quantitation based on the specific binding of copper ions to proteins (2D Quant Kit, GE Healthcare).

Figure 4. Relationship between pH and isoelectric focusing point (pI).

Once preparation is complete, proteins within the sample are separated along a pH gradient using isoelectric focusing (IEF). Proteins are amphoteric molecules, meaning that they possess either a positive, negative, or neutral net charge depending on the pH of their surroundings (Stochaj et al., 2003). The pH at which a protein has a neutral net charge is referred to as its isoelectric focusing point, or pI (Righetti, 1983). At pH values above the pI, the protein possesses a net negative charge, while at pH values below the pI, the protein has a net positive charge (Fig. 4; Stochaj et al., 2003).

In IEF, proteins are absorbed into an immobilized pH gradient (IPG) strip which has a continuous pH gradient along its length ranging from acidic to basic. This pH gradient is established by acrylamido buffers that are bound to the polyacrylamide gel within the strip to ensure that the pH gradient is immobilized and does not shift during IEF (Stochaj et al., 2003). When a charge is passed through the IPG strip, proteins situated at a pH above their pI, will be drawn towards the positive anode (Righetti, 1983; Stochaj et al., 2003). As it moves along the pH gradient, the protein becomes less and less negatively charged, and therefore is less and less attracted to the anode, until it finally comes to rest at

its isoelectric focusing point (Righetti, 1983; Stochaj et al., 2003). The reverse is true for proteins located at pH values below their pI. Over time, all of the proteins within the sample migrate to their pI, resulting in protein separation along a pH gradient (Righetti, 1983; Stochaj et al., 2003).

The pH gradient of IPG strips can be varied in order to visualize proteins with a wide range of different isoelectric focusing points (Görg et al., 2000). IPG strips with a pH gradient ranging from 4-7 are the most commonly used because the majority of proteins within the cell have isoelectric focusing points within this range (Righetti, 1983). To capture a wider range of proteins, some use IPG strips with a pH range of 3-10; however, this can sometimes result in decreased separation of proteins in the middle pH range. Strips with a very narrow pH range, sometimes spanning only 1.5 pH units, are also available for researchers interested in a specific subset of proteins (Görg et al., 2000). In addition, the length of the IPG strip can be modified to enhance protein separation, and lengths range from 7-cm up to 26-cm, with the most commonly used strips being 11-cm long (Stochaj et al., 2003).

Before being separated by molecular mass, proteins that have undergone isoelectric focusing must be

equilibrated to prepare them for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). During the equilibration process, samples are exposed to the detergent sodium dodecyl sulfate, SDS, which is an anionic surfactant that denatures proteins by binding to their peptide backbone (Stochaj et al., 2003). When SDS is bound, it imparts a negative charge that overwhelms the proteins' native charge, resulting in all proteins within the sample acquiring a net negative charge (Stochaj et al., 2003). Samples are also treated with a reducing agent, DTT, which cleaves disulfide bonds and completely denatures the protein (Stochaj et al., 2003). Lastly, samples are washed with iodoacetamide, a reagent that binds to cysteine residues and any remaining DTT to prevent disulfide bonds from reforming (Görg et al., 2000; Stochaj et al., 2003).

Once proteins are negatively charged and fully equilibrated, they are ready to be separated by SDS-PAGE. IPG strips are placed on top of polyacrylamide slab gels, which consist of cross-linked acrylamide and bis-acrylamide monomers that form a net-like matrix (Görg et al., 2000; Stochaj et al., 2003). During electrophoresis, proteins migrate vertically down the polyacrylamide gel, whose matrix structure acts as a sieve. As the negatively charged proteins are pulled towards the positive anode, larger

proteins move more slowly through the gel matrix, while smaller proteins are able to pass through more easily. Thus, the relative migration distance is inversely related to protein size, with low molecular mass proteins migrating farther than proteins with high molecular masses (Stochaj et al., 2003; Drabik et al., 2008).

Like IPG strips, polyacrylamide slab gels come in a variety of shapes and sizes. Pore size within the polyacrylamide gel can be modified by altering the percentage of acrylamide used, and gradient gels, with varying pore size throughout the gel, can be used to further increase protein separation (Stochaj et al., 2003; Drabik et al., 2008). Standard gel sizes range from 16 X 16-cm to 20 X 20-cm, with a thickness of 1-1.5mm (Stochaj et al., 2003). These small-format gels allow for rapid separation and are compatible with 11-, 13-, and 17-cm IPG strips. For maximal resolution, reproducibility, and protein load capacity, some researchers use large-format gels that are 26 X 20-cm and are compatible with 18-, 24-, or even 26-cm IPG strips (Görg et al., 2000; Stochaj et al., 2003).

After SDS-PAGE is complete, proteins within the gel are separated according to their pI and molecular mass; however, the resulting gels must be stained in order to

visualize and analyze the samples. Many different staining methods exist, and the specific characteristics of each stain, as well as the desired identification method, must be considered when deciding which stain is most suitable. Colloidal Coomassie Blue is a commonly used stain that is relatively inexpensive and easy to use (Stochaj et al., 2003; Berth et al., 2007). Another benefit is that Coomassie is compatible with mass spectrometry, as it does not interfere with peptide ionization (Stochaj et al., 2003; Berth et al., 2007). The main drawback to Coomassie is that it has low sensitivity, with a detection range limited to 8-50 ng of protein per spot (Stochaj et al., 2003; Berth et al., 2007). This means that low abundance proteins are poorly visualized, and therefore Coomassie may not be suitable for samples with small protein loads (Stochaj et al., 2003; Berth et al., 2007).

The two other staining methods are fluorescent dyes and silver staining, which both have higher sensitivities than Coomassie Blue. Fluorescent dyes have a detection range of 2-4 ng of protein, while silver staining enables detection down to 0.1 ng of protein per spot (Görg et al., 2000). While silver staining is appealing for its incredibly high sensitivity, it has several disadvantages including a complex and labor-intensive application

protocol, poor reproducibility, and susceptibility to interference (Görg et al., 2000). Some silver staining methods are also incompatible with mass spectrometry; however, protocols exist that include modifications which allow for subsequent mass spectrometry (Yan et al., 2000). Fluorescent dyes, like SYPRO ruby, are almost as sensitive as silver staining, fairly easy to use and reproducibility is not a problem. The only down-side is that fluorescent dyes are quite expensive and also require a fluorescent scanner for gel imaging; therefore the associated cost may be prohibitive (Stochaj et al., 2003). Once stained, gel images are scanned into the computer for subsequent analysis.

Protein Quantification

At this point, protein separation is complete. The next step is protein quantification, which utilizes specific computer software programs to determine the relative abundance of each protein

Figure 5. Gel image warping and fusion (modified from Berth et al., 2007).

spot across all of the treatment conditions. Many commercial software products exist for 2D gel image analysis, including PDQuest (Bio-Rad), Melanie (Genebio), SameSpot (Nonlinear Dynamics), and Delta2D (DECODON), just to name a few (Berth et al., 2007). Despite the brand of software used, quantification of proteins follows the same basic procedure. First, slight variations in the spot positions that occur across gels must be eliminated (Berth et al., 2007). This is done by a process called “warping” in which gel images are overlaid and corresponding spots in each gel are matched using vector lines to connect them. The images are then transformed, or warped, according to the vectors to produce an exact overlay (Fig. 5; Berth et al., 2007). Once spot positions on all gels are matched through warping, the computer software compiles all the matched spots into a fusion image that is representative of every gel in the experiment (Berth et al., 2007). The next step is to define the boundaries of each individual spot in a process called “spot detection”, which uses Gaussian models of spot intensity to automatically determine spot boundaries (Berth et al., 2007). Automatically detected spots are then edited by the user to ensure all boundaries represent true spots.

Once spots are defined, they can be quantified based on the pixel-density, which gives a relative estimate of protein expression (Berth et al., 2007). Spot quantities must be normalized to mitigate differences between gel images caused by small variations in protein loading, dye/staining efficiency, and inconsistencies in image scanning (Berth et al., 2007). Normalization is done by converting the raw spot volumes into relative spot volumes based on the ratio of pixels represented within a single spot, compared to the total number of pixels in the gel. In this way, spot volume is represented as a percentage of the whole, thereby eliminating the effect of small gel-to-gel differences (Berth et al., 2007).

When normalized spot volumes are compared across treatments, protein expression profiles can be generated for each spot. Expression profiles showcase the change in protein abundance that occurs for each spot across all treatments. Protein synthesis, degradation, or post-translational modifications can all result in the changes in protein abundance detected. Therefore, expression profiles allow for the identification of proteins that are involved in the cellular response to the treatment being applied (Berth et al., 2007). As in other areas of science, statistical methods are essential, in this case allowing

for the separation of real or significant changes in protein expression from fluctuations due to random chance.

The specific statistical methods required to analyze changes in protein expression vary depending on the experimental design and the number of treatment groups. Simple experiments with only one treatment and a control should use a t-test, while more complex experiments with multiple treatment factor levels should utilize a one- or two-way ANOVA. Despite the relative robustness of t-tests and ANOVAs to non-normal data distributions, the fact that spot volume variances are unequal, in addition to their inherently non-normal distribution, necessitate the use of permutations (Ludbrook and Dudley, 1998). Therefore, utilizing permutations of the data to construct a normal distribution is preferred over the traditional t- or F-statistic in proteomic and other biomedical studies (Ludbrook and Dudley, 1998).

Because the data set is so large, with anywhere from 300 to 500 protein-spot volumes being simultaneously compared, it is also sometimes necessary to correct for false discoveries. As the number of statistical comparisons increases, so does the probability of making a type I error (falsely rejecting a null hypothesis) in at least one of the comparisons (Ludbrook, 1998). This probability is known

as the family-wise error rate (FWER). Therefore, even with a low p -value, many protein spots deemed to show significant changes in their expression across treatments will in fact be false discoveries (Ludbrook, 1998). For a full understanding of this problem, a distinction must be made between the false positive rate and the false discovery rate (FDR). The false positive rate is the percentage of truly null observations that are deemed significant, and it is represented by the p value. The false discovery rate, on the other hand, is the percentage of observations deemed significant that are in fact truly null, and it is represented by the q value. In other words:

“...a false positive rate of 5% means that on average 5% of the truly null features in the study will be called significant. A FDR of 5% means that among all features called significant, 5% of these are truly null on average.” (Storey and Tibshirani, 2003)

This slight distinction explains why even with a small p -value, or a controlled false positive rate, the false discovery rate can still be a problem (Storey and Tibshirani, 2003). To remedy this situation, some have proposed that for genomewide studies especially, it may be preferable to control the FDR instead of the false positive rate, substituting a q value for the traditional p value (Storey and Tibshirani, 2003; Pawitan et al., 2005).

Despite the ability to decrease the number of declared significant proteins that are truly unchanging, controlling the FDR can make the statistical tests so stringent that few, if any, proteins are deemed reliably significant. This is a disadvantage when attempting to understand and explain complex biological phenomena. Therefore, a balance is required. If controlling the FDR comes at the expense of acquiring meaningful results, then it cannot be used. That being said, the fact that attempting to compare protein abundance across treatments for hundreds of proteins simultaneously inevitably leads to an increased number of false discoveries must be acknowledged. This problem is something that researchers are still struggling with, and no silver-bullet solution has been found as of yet. The best we can do is take our biological results with a statistical grain of salt and attempt to explain them the best we can.

Future studies should further investigate the necessity of correcting for the FDR by analyzing proteins with known changes in concentration and determining how effective various statistical methods are at picking up on the changes in abundance without introducing false positives and false discoveries. In the present study, no correction for multiple comparisons was used; instead, the

false positive rate was controlled with a p value of 0.05, allowing for a meaningful biological interpretation of the dataset. Because this is a proteomic study, where the detected proteins number in the hundreds, forgoing a multiple comparisons correction is more acceptable than it would be for a transcriptomic study, where thousands of mRNA transcripts being analyzed simultaneously.

Once it is established which proteins show significant changes in their expression across treatments, a hierarchical clustering analysis can be done to group proteins into clusters based on similarities in their expression profiles (Zvelebil and Baum, 2008). This allows for the categorization of proteins that show similar changes in their abundance across treatment groups, which may suggest that they belong to the same cellular pathway (Zvelebil and Baum, 2008). This analysis becomes more meaningful once the protein identities have been determined.

Protein Identification

The final step of a proteomic study is to determine the identity of the significant proteins and interpret their role in the cellular response to the treatment applied. Mass spectrometry (MS) is the most widely-used

method for identifying proteins of interest (Domon and Aebersold, 2006). The term “mass spectrometry” covers a wide-range of protein identification methods, and not all of them will be discussed in depth in this review. Instead, we will focus on the technique of peptide mass fingerprinting utilizing matrix-assisted laser desorption/ionization (MALDI) tandem time of flight (TOF/TOF) mass spectrometry, which is commonly paired with the 2-D GE protein separation method (Aebersold and Mann, 2003).

Generally, protein identification using MS can be broken down into 3 stages. First, proteins are digested into peptide fragments using proteases that cut at specific sites along the peptide backbone. This results in a unique set of predictable peptide fragments, which can then be used in protein identification (Aebersold and Mann, 2003; Drabik et al., 2008). The most commonly used enzyme is trypsin, which cleaves proteins at arginine and lysine residues, and results in peptides with C-terminally protonated amino acids (Aebersold and Mann, 2003; Olsen et al., 2004).

Second, the mass and abundance of each peptide fragment within the sample is determined using MS to generate a peptide mass fingerprint (PMF). Sometimes, the

most abundant peptides in the sample are further fragmented to create peptide fragment fingerprints (PFF); this is referred to as tandem mass spectrometry (MS/MS). Lastly, bioinformatics is used to identify proteins by searching PMF data, or combined PMF/PFF data, in a genomic database and matching them with protein candidates that show the same PMF/PFF upon theoretical enzymatic digestion (Drabik et al., 2008; Tomanek, 2011). In reality, this simplified workflow varies greatly depending on many factors including the mode of protein separation, and the type of mass spectrometry and database search conducted (Aebersold and Mann, 2003; Tomanek, 2011).

Every mass spectrometer consists of three main components: the ion source, the mass analyzer, and the detector (Aebersold and Mann, 2003). Because mass spectrometric measurements are carried out in the gas-phase on ionized peptides, or analytes, an ion source is required in order to volatilize and ionize the peptides present in each protein sample (Aebersold and Mann, 2003). There are two main types of ion sources used in modern MS, the first is electrospray ionization (ESI), and the second is matrix-assisted laser/desorption ionization (MALDI). Both ESI and MALDI are so-called “soft-ionization” techniques that allow for the transfer of intact ionized peptides into a gas-

phase, while preventing excessive fragmentation (Domon and Aebersold, 2006).

ESI is most commonly used to ionize peptides present in a solution, and therefore is suited for protein preparations that have been separated using liquid chromatography (Aebersold and Mann, 2003). While ESI typically results in precursor ions with multiple charges, MALDI ionizes and sublimates analytes out of a solid, crystalline matrix, resulting in singly charged precursor ions (Suckau et al., 2003; Domon and Aebersold, 2006). ESI is also able to analyze complex samples that contain a mixture of proteins, while MALDI is more suitable for less complex samples that have been pre-separated using 2-D GE (Aebersold and Mann, 2003).

After passing through the ion source, ionized peptides encounter the mass analyzer, which is responsible for measuring the mass to charge ratio (m/z) for each peptide in the sample (Aebersold and Mann, 2003). There are four main types of mass analyzer: ion trap, time-of-flight (TOF), quadrupole, and Fourier transform ion cyclotron (FT-MS) analyzers (Aebersold and Mann, 2003). The details of each type of mass analyzer have been summarized elsewhere (see Aebersold and Mann, 2003; Suckau et al., 2003; Domon and Aebersold, 2006); however, it is worth mentioning that

ESI ion sources are usually coupled to ion trap or quadrupole mass analyzers, while MALDI is mostly used in conjunction with TOF analyzers (Aebersold and Mann, 2003). As the name implies, ion trap mass analyzers trap ions in an electric or magnetic field until they can be further fragmented, usually using collision-induced dissociation (CID) (Aebersold and Mann, 2003). Time of flight analyzers, on the other hand, measure the mass of intact peptides based on their flight times over a known distance, and are recognized for their high mass accuracy and resolution (Lopez-Otin and Overall, 2002; Aebersold and Mann, 2003).

Newer mass spectrometers contain multiple mass analyzers enabling both peptide fragmentation and accurate mass measurements (Aebersold and Mann, 2003). One example is the MALDI TOF-TOF, which utilizes a MALDI ion source and contains two TOF analyzers separated by a collision cell (Fig. 6; Suckau et al., 2003). This configuration enables accurate mass measurements for PMF, and the peptide fragmentation required for MS/MS (Aebersold and Mann, 2003).

From the mass analyzer, ionized peptides move to the detector, which records the number of peptides at each m/z value (Aebersold and Mann, 2003). Once all of this

information is combined, a peptide mass fingerprint is generated for each protein sample.

Figure 6. Schematic diagram of MALDI-TOF/TOF mass spectrometer (modified from Aebersold and Mann, 2003).

A typical PMF is presented as a spectrum showing the m/z along the x-axis, and the peptide intensity, or abundance, along the y-axis (Fig. 7a; Tomanek, 2011). Using a PMF-based database search engine, such as MASCOT, the PMF can be matched to a theoretical digest extracted from a protein database (Perkins et al., 1999; Gevaert and Vandekerckhove, 2000; Tomanek, 2011). This is especially effective if the organism being studied has a sequenced and annotated genome.

Figure 7. (A) The peptide mass fingerprint (PMF) of ATP synthase F1 – subunit was obtained with matrix-assisted laser desorption ionization (MALDI) mass spectrometry. (B, *insert*) A single peptide ($M_r = 1406.68$ Da) was selected from the PMF of (B) and further fragmented (using tandem mass spectrometry, or MS/MS) into a peptide fragment fingerprint (PFF; see text for details).

In order to confirm the match, a process called tandem mass spectrometry (MS/MS) can be used to select peptides with the highest intensity in the PMF, and further fragment them to create a peptide fragment fingerprint (PFF) (Fig. 7b, Tomanek, 2010). If the peptides within the sample all come from a single protein, as is the case in MALDI-TOF/TOF MS where proteins are most often pre-separated by 2-D GE, the PFF and PMF can be combined and a single search can be conducted that contains both MS and MS/MS data (Drabik et al., 2008; Tomanek, 2011).

Each protein matched in the database is given a score representing the probability that the given match is due to random chance (Pappin, 1993; Drabik et al., 2008). If the score is above the significance threshold, which denotes a p-value less than 0.05, and two or more peptide fragments are matched, then the hit is deemed statistically significant and the protein is considered identified (Pappin, 1993). Different search engines utilize different scoring algorithms; MASCOT employs the molecular weight search (MOWSE) algorithm, which reports scores as $-10 \cdot \log_{10}(P)$, where P represents the absolute probability that the observed match is a random event (Pappin, 1993; Perkins et al., 1999). Thus, with the MOWSE algorithm, a high score means a low probability that the match is false (Pappin, 1993; Perkins et al., 1999).

Once proteins have been identified using mass spectrometry, researchers are left with the complex result of a proteomic experiment: a list of anywhere from ten to hundreds of proteins that show significant changes in their expression in response to the applied treatment (Tomanek and Zuzow, 2010). The next, and arguably most difficult, step is to determine the physiological role each protein plays in the cell, and to uncover protein interactions that exist between significantly changing proteins. This

requires a careful review of any and all literature available pertaining to the function of each identified protein. The ultimate goal is to move from an arbitrary list of proteins to a working model of cellular pathways that are responsible for responding to the stress applied.

It is in this step that the heat map, which was generated by grouping significant proteins into clusters based on similarities in their expression profiles, becomes valuable (Zvelebil and Baum, 2008). Because they share similarities in their expression patterns, the proteins within each cluster are most often a part of the same cellular pathway or response. Thus the heat map can provide clues about which proteins may be working in concert to produce the observed response.

In order to aid this process, many companies are developing so-called “pathway analysis” software that utilize various text-mining algorithms to automatically extract facts about protein functions and interactions directly from published scientific literature (Yuryev et al., 2006). One example is MedScan, a natural language processing technology distributed by Ariadne Genomics. MedScan utilizes a full-sentence parsing system in order to find relations between proteins and other cellular molecules, which are derived from the semantic and lexical

structure of single sentences taken from literature articles (Yuryev et al., 2006). This automated extraction process enables the characterization of many protein interactions, which can then be visualized using pathway generating tools to construct interaction networks (Fig. 8; Perroud et al., 2006; Yuryev et al., 2006).

Figure 8. Example of a protein interaction network generated by the pathway analysis software Pathway Architect (Stratagene). Proteins in the network, with the exception of TNF, were found to have significant changes in their expression following proteomic profiling of clear cell renal cell carcinoma (Perroud et al., 2006).

Furthermore, proteins with significant expression changes identified in proteomic experiments can be entered into the software, which will then search the literature

for functional and relational information (Yuryev et al., 2006). Using this information, biological networks can be constructed that show the relationships between significant proteins, along with additional proteins that interact with many components of the network, even if they were not directly discovered in the proteomic experiment (Fig. 8; Perroud et al., 2006). This approach links significant proteins to the cellular pathways that they comprise, highlights protein associations both within and between pathways, and places expression changes in the context of global pathway regulation (Yuryev et al., 2006). Overall, this technology is helping researchers to interpret the vast amount of information that results from discovery-based proteomic approaches, and to transform lists of significant proteins into a functional understanding of the cellular response to stress.

II. MANUSCRIPT

Abstract

The sea squirts *Ciona intestinalis* and *C. savignyi* have disparate distribution patterns, which may result from differences in their thermal tolerance limits. Because *C. intestinalis*, an almost cosmopolitan species, has a more widespread distribution, it is thought that it is better adapted to endure a wide range of temperatures. In order to compare the heat stress response between these two congeners, we studied global changes in protein expression, using a proteomics approach. To characterize the response to extreme heat stress, animals of both species were exposed to temperatures of 22°C, 25°C, and 28°C for 6 h, and then were left to recover at a control temperature (13°C) for 16 h. An additional experiment was conducted to assess the effect of mild-to-moderate heat stress including a 6 h exposure to temperatures of 18°C, 20°C, and 23°C, and a 16 h recovery at a control temperature (16°C). A quantitative analysis, using 2D gel electrophoresis and gel-image analysis, showed that in the high heat stress (HHS) experiment, 15% and 18% of the all protein spots detected demonstrated changes in expression in *C.*

intestinalis and *C. savignyi*, respectively. In the low heat stress (LHS) experiment, 4% of the total number of proteins detected changed significantly in both *C. intestinalis* and *C. savignyi*. Using matrix-assisted laser desorption ionization (MALDI) tandem time-of-flight mass spectrometry, we were able to identify proteins with a 65–100% success rate, depending on species. Our results indicate that *C. intestinalis* maintains higher baseline levels of molecular chaperones and launches a quicker response to thermal stress than *C. savignyi*, suggesting it may be the more thermally tolerant of the two. In addition, actins, tubulins, and ATP-synthase F1 β -subunits were the most susceptible to proteolytic degradation, which may indicate that they have relatively higher thermal sensitivities.

Keywords: Proteomics, heat stress, *Ciona*

Introduction

Thermal stress influences organisms on several levels, from their molecular physiology all the way up to their behavioral ecology. The effect of temperature on the rate of physiological reactions and the stability of macromolecular structures plays a role in limiting the geographic distribution ranges of organisms (Hochachka and Somero, 2002; Tomanek, 2008; Tomanek, 2010; Pörtner, 2010). Recent increases in global temperature averages and extremes due to climate change, along with the dramatic increases forecasted by climate models, have highlighted the importance of studying the thermal stress response in order to predict the effects of increased thermal stress on species distribution and, ultimately, survival (IPCC, 2007).

Many studies have been done to try to elucidate the cellular mechanisms responsible for responding to thermal stress, and parts of the heat shock response (HSR), such as the role of molecular chaperones in protecting proteins from denaturation, repairing misfolded proteins, and facilitating the degradation of irreversibly damaged proteins, are very well characterized (for review see Feder and Hofmann, 1999; Hochachka and Somero, 2002; Tomanek,

2008). In order to gain a more comprehensive understanding of the molecular response to thermal stress, several transcriptomic studies have utilized a global approach to simultaneously characterize changes in the expression of thousands of mRNA transcripts, corresponding to thousands of genes, in response to environmental stress (Gracey and Cossins, 2003; Gracey et al., 2008; Stillman and Tagmount, 2009; Lockwood et al., 2010). This systems biology perspective has allowed for the discovery of many physiological processes that serve as novel indicators for thermal stress (Gracey et al., 2008; Stillman and Tagmount, 2009; Lockwood et al., 2010).

Recent progress in the field of proteomics, another systems biology approach that allows for the simultaneous characterization of the abundance of hundreds of proteins, has made it possible to identify the majority of proteins that show significant expression changes in response to a variety of environmental stressors (for review see Tomanek, 2011). These changes in protein expression can be the result of protein synthesis, degradation, or post translational modifications, and the term expression is used broadly to refer to all three events. By quantifying the dynamic changes in protein abundance and modifications in response to shifting environmental conditions,

proteomics allows for a more direct characterization of the molecular phenotype than is possible with transcriptomics (Feder and Walser, 2005; Tomanek and Zuzow, 2010). In addition to a small number of proteomic studies that examine cellular responses to thermal stress, to our knowledge, only one other study utilizes proteomics to compare the thermal stress responses of two closely related species with varying distribution ranges, the blue mussel species pair *Mytilus galloprovincialis* and *M. trossulus*, (Tomanek and Zuzow, 2010).

In this study, we chose to examine the closely related sea squirt congeners, *Ciona intestinalis* and *Ciona savignyi*. Ascidian tunicates belonging to the genus *Ciona* are sessile and solitary marine invertebrates that reproduce by broadcast spawning and can thrive on both natural substrates, such as eelgrass beds, and artificial substances, like ship hulls (Millar, 1953; Millar, 1971; Byrd and Lambert, 2000; Berná et al., 2009). Their opportunistic nature has allowed them to take advantage of anthropogenic means of dispersal, mostly by hull-fouling, which has contributed to their classification as invasive species (Ritter and Forsyth, 1917; Svane and Havenhand, 1993; Hewitt et al., 2002; McDonald, 2004; Blum et al., 2007). Their fully sequenced and annotated genomes, genomic

and anatomic simplicity, and key evolutionary position make these modern urochordates an ideal study system for clarifying chordate phylogeny, as well as the mechanisms behind vertebrate development and evolution (Dehal et al., 2002; Satoh et al., 2003; Vinson et al., 2005; Nydam and Harrison, 2007; Berná et al., 2009).

Although these two sister species share many aspects of their natural history and ecology, one area where the two congeners differ is in their distinct distribution ranges. *Ciona intestinalis* is a cosmopolitan species with a nearly worldwide coastal distribution, while *C. savignyi* is a Pacific species geographically restricted to Japan and the western coast of North America, from British Columbia to the southern tip of California (Lambert, 2003; Therriault and Herborg, 2008; Berná et al., 2009). The relative phylogenetic proximity of the two *Ciona* congeners, in addition to differences in their distribution ranges, provide a species comparison that emphasizes the molecular adaptations responsible for variation in the cellular response to heat stress.

Several studies define the ecological range of thermal stress that one of the two *Ciona* species experiences; however, no direct species comparisons within the same life stage exist to date, so very little is known concerning

their relative thermal tolerances (Dybern, 1965; Marin et al., 1987; Therriault and Herborg, 2008). For adult *C. intestinalis*, temperature tolerance has been shown to range from -1°C to an upper threshold of 30°C; however, adult mortality increases at temperatures below 10°C, so 10°C to 30°C is a more realistic thermal range (Dybern, 1965; Therriault and Herborg, 2008). In addition, increased thermal stress, as indicated by decreased rate of algal filtration, has been observed at temperatures above 21°C in *C. intestinalis* (Petersen and Riisgård, 1992; Therriault and Herborg, 2008). Thus, the upper limit for long-term survival may actually be lower than 30°C.

The goal of the present study was to use the discovery approach of proteomics to identify differences in the acute thermal stress responses between the two *Ciona* congeners. Using existing estimates of ecological thermal tolerance range as a guide, two separate experiments were conducted to evaluate the proteomic responses of the *Ciona* congeners when exposed to extreme and moderate heat stress. The high heat stress (HHS) experiment included treatment temperatures ranging from 22°C to 28°C (13°C control), while exposure temperatures in the low heat stress (LHS) experiment ranged from 18°C to 23°C (16°C control). Using two-dimensional gel electrophoresis (2-D GE) and tandem

mass spectrometry (MS), we were able to identify the majority of the proteins that showed significant expression changes, either due to protein synthesis, degradation, or post-translational modifications (PTMs), in response to low and high levels of heat stress.

In comparing significantly changing proteins and baseline levels of molecular chaperones between species, we were able to pinpoint specific differences in their thermal stress responses, which suggest differing thermal tolerances between the two congeners. In addition, we found that some of the proteins identified in the heat stress experiments were degraded prior to separation by 2-D GE. Upon further investigation, we discovered that the extent of the proteolytic breakdown varied significantly across heat stress experiments and that some functional categories of proteins were more susceptible to degradation than others. These results indicate that proteolytic degradation plays a role in the response to extreme thermal stress, and provide an indirect measure of variations in protein thermal sensitivity.

Materials and Methods

Animal collection and maintenance

Ciona intestinalis (Linnaeus, 1767) and *C. savignyi* (Herdman, 1882) were collected subtidally from the Yacht Harbor in Santa Barbara, California, USA (34°24'13" N, 119°41'31" W). Species were differentiated from one another based on the color of the pigment spot present at the tip of the sperm duct, which is orange in *C. intestinalis* and white in *C. savignyi* (Hoshino and Tokioka, 1967). Animals were transported back to San Luis Obispo, CA in insulated coolers and were kept overnight at a control temperature (13°C for the high heat stress [HHS] experiment, and 16°C for the low heat stress [LHS] experiment) in recirculating seawater tanks. The different control temperatures used in each experiment reflect the average water temperatures at the collection site during the season in which each experiment was run, late summer for the LHS experiment, and early spring for the HHS experiment (Fig. 9).

Figure 9. Graph of surface water temperature in Santa Barbara, CA (34°40'83" N, 119°68'50" W). Arrows indicate the month during which each heat stress experiment took place, with blue denoting the low heat stress experiment and red for the high heat stress experiment. Dashed lines indicate control temperature used in each experiment.

Experimental design

High Heat Stress (HHS) Experiment

Ciona intestinalis and *C. savignyi* were placed in a temperature-controlled ice-chest with circulating seawater and aeration. The temperature was increased by 6°C h^{-1} from 13°C to 22°C , 25°C or 28°C ($n = 5$ for all groups, with the following exceptions: $n = 4$ for *C. savignyi* 13°C and 25°C). Sea squirts were kept at these temperatures for a 6 h incubation and subsequently brought back to 13°C for a 16 h recovery period (Fig. 10). Immediately after recovery, individuals were pooled into samples to obtain sufficient amounts of protein (2 *C. intestinalis* individuals per

sample and 3 *C. savignyi* per sample), and samples were flash-frozen in liquid nitrogen and stored at -80°C .

Figure 10. Experimental design for the low heat stress (dashed lines) and high heat stress (solid lines) experiments. Samples were heated at a steady rate of $6^{\circ}\text{C per hour}$ until they reached their target temperatures. After a 6 hour incubation at the target temperature, samples were cooled to their respective controls for a 16 hour recovery period, after which they were flash-frozen in liquid nitrogen ($n = 5$ for all groups, with the following exceptions: $n = 4$ for *C. savignyi* 13°C and 25°C).

Low Heat Stress (LHS) Experiment

The low heat stress experiment used the same basic design as the HHS experiment, but with different temperatures. The control temperature was 16°C , and the three treatment temperatures were 18°C , 20°C , and 23°C . As in the HHS experiment, the rate of temperature increase was 6°C h^{-1} , and sea squirts remained at their target temperature for a 6 h incubation, and were cooled back down

to 16°C for a 16 h recovery period (n = 5 for all groups; Fig. 10). Individuals were pooled into samples consisting of 2 individuals for *C. intestinalis* and 3 individuals for *C. savignyi*, and samples were flash-frozen in liquid nitrogen immediately following recovery and kept at -80°C.

Protease Inhibitor Experiment

In order to determine whether serine, cysteine, metallo-, and aspartic protease activity affects sample preparation, additional *C. intestinalis* and *C. savignyi* were collected from the Santa Barbara Yacht Harbor and were immediately flash-frozen in liquid nitrogen. For each species, half of the samples were processed with unaltered homogenization buffer (see below), while the other half were processed with homogenization buffer containing substances shown to inhibit aspartic, serine, cysteine, and metallo-proteases (see below; n = 5 for both species).

Dissection and homogenization

Frozen samples were thawed and the outer tunic membrane was dissected off of each individual. The contents of the stomach and intestine were removed, and the remaining tissues (all internal organs and structures) were pooled into ice-cold ground-glass homogenizers. The tissue

was lysed in a ratio of 1:2 of tissue to homogenization buffer (7 mol l⁻¹ urea, 2 mol l⁻¹ thiourea, 1% ASB-14, 40 mmol l⁻¹ Tris-base, 0.5% immobilized pH 4-7 gradient (IPG) buffer (GE Healthcare, Piscataway, NJ, USA) and 40 mmol l⁻¹ dithiothreitol).

For the protease inhibitor experiment samples, one tablet of complete Mini protease inhibitor cocktail was added for every 10 ml of homogenization buffer (Roche, Mannheim, Germany). This cocktail enabled inhibition of serine, cysteine, and metallo-proteases. Pepstatin, an inhibitor of aspartic proteases, was also added to the homogenization buffer (0.7 µg ml⁻¹) prior to tissue lysis.

The homogenate was subsequently centrifuged at room temperature for 30 min at 16,100 g and the supernatant was used for further processing. Proteins of the supernatant were precipitated by adding four volumes of ice-cold 10% trichloro-acetic acid in acetone and incubating the solution at -20°C overnight. After a 15 min centrifugation at 4°C and 18,000 g, the supernatant was discarded and the remaining pellet was washed with ice-cold acetone, and centrifuged again before being re-suspended in rehydration buffer (7 mol L⁻¹ urea, 2 mol L⁻¹ thiourea, 3.25 mmol L⁻¹ CHAPS (cholamidopropyl-dimethylammonio-propanesulfonic acid), 2% NP-40 (nonyl phenoxyethylpolyethoxyethanol-40),

0.002% bromophenol blue, 0.5% IPG buffer and 100 mmol L⁻¹ dithioerythritol) through vortexing. The protein concentration was determined with the 2D Quant kit (GE Healthcare), according to the manufacturer's instructions.

Two-dimensional gel electrophoresis

Proteins (800 µg) were loaded onto immobilized pH gradient strips (pH 4-7, 24 cm; BioRad, Hercules, CA, USA) for separation according to their isoelectric point (pI). We started the isoelectric focusing protocol with 12 h of active rehydration (50V), using an isoelectric focusing cell (BioRad). The following protocol was used for the remainder of the run (all voltage changes occurred in rapid mode): 250V for 15 min, 10000V for 3 h, 10000 V for 80000 Vhrs, and a 500V hold step. Once removed from the IEF cell, the strips were frozen and stored at -80°C.

Frozen strips were thawed and incubated in equilibration buffer (375 mmol L⁻¹ Tris-base, 6 mol L⁻¹ urea, 30% glycerol, 2% SDS and 0.0002% bromophenol blue) for 15 min, first with 65 mmol L⁻¹ dithiothreitol and then, second, with 135 mmol L⁻¹ iodoacetamide. IPG strips were placed on top of an 11.8% polyacrylamide gel with a 0.8% agarose solution containing Laemmli SDS electrophoresis (or running) buffer (25 mmol L⁻¹ Tris-base, 192 mmol L⁻¹ glycine,

0.1% SDS). For the high heat stress experiment, 24 cm IPG strips were run on large-format gels, 26 x 20 cm, at 30W for 30 minutes, and 100W for 4 hours (Ettan DALTsix; GE Healthcare). For the low heat stress experiment, large-format gels, 25.6 x 23.0 cm, were run at 50V for 30 minutes, then 200V for 5.5 hours (Protean Plus Dodeca Cell; BioRad). In both cases, recirculating water baths set to 12°C were used to keep running buffer cool. Gels were subsequently stained with colloidal Coomassie Blue (G-250) overnight and destained by washing repeatedly with Milli-Q water for 48 h. The resulting gel images were scanned with an Epson 1280 transparency scanner.

For the protease inhibitor experiment, the same overall protocol was followed, with a few minor deviations. A less concentrated protein solution (400 µg) was loaded onto smaller immobilized pH gradient strips (pH 4-7, 11 cm; BioRad, Hercules, CA, USA), and the 2nd dimension was run using small-format gels, 13.3 x 8.7 cm, that ran at 200V for 50 minutes (Criterion Dodeca Cell; BioRad). The buffers used were the same as those described for the high and low heat stress experiments.

Gel image analysis

Digitized images of two-dimensional gels were analyzed using Delta2D (version 4.0; Decodon, Greifswald, Germany) (Berth et al., 2007). We used the group warping strategy to connect gel images through match vectors. All images within a species were fused into a composite image (= proteome map), which represents average volumes for each spot (Fig. 7). Spot boundaries were detected within the proteome map and transferred back to all gel images using match vectors. After background subtraction, protein spot volumes were normalized against total spot volume of all proteins in a gel image (for detailed review of image analysis protocols, see Berth et al., 2007).

Mass spectrometry

Proteins that showed changes in expression in response to treatment were excised from gels using a tissue puncher (Beecher Instruments, Sun Prairie, WI, USA). Gel plugs were destained twice with 25 mmol L⁻¹ ammonium bicarbonate in 50% acetonitrile, dehydrated with 100% acetonitrile and digested with 11 ng µL⁻¹ trypsin (Promega, Madison, WI, USA) overnight at 37°C. Digested proteins were extracted using elution buffer (0.1% trifluoroacetic acid (TFA)/acetonitrile; 2:1) and concentrated using a SpeedVac

(Eppendorf, Hauppauge, NY, USA). The elution buffer containing the digested protein was mixed with 5 μ l of matrix solution (0.2 mg ml⁻¹ α -hydroxycyano cinnamic acid in acetonitrile) and spotted on an AnchorchipTM target plate (Bruker Daltonics Inc., Billerica, MA, USA). The spotted proteins were washed with a buffer containing 0.1% TFA in 10 mM ammonium phosphate, and recrystallized using an acetone/ethanol/0.1% TFA (6:3:1) mixture.

Peptide mass fingerprints (PMFs) were obtained on a matrix-assisted laser desorption ionization tandem time-of-flight (MALDI-TOF-TOF) mass spectrometer (Ultraflex II; Bruker Daltonics Inc., Billerica, MA, USA). We chose a minimum of eight peptides to conduct tandem mass spectrometry in order to obtain information about the b- and y-ions of the peptide sequence.

To analyze the peptide spectra we used flexAnalysis (version 3.0; Bruker Daltonics Inc.) and applied the TopHat algorithm for baseline subtraction, the Savitzky-Golay analysis for smoothing (with: 0.2 m/z; number of cycles = 1) and the SNAP algorithm to detect peaks (signal-to-noise ratio: 6 for MS and 1.5 for MS/MS). The charge state of the peptides was assumed to be +1. We used porcine trypsin for internal mass calibration.

To identify proteins we used Mascot (version 3.1; Matrix Science Inc., Boston, MA, USA) and combined PMFs and tandem mass spectra in a search against two databases. One is a genomic database for *Ciona intestinalis* that initially contained approximately 8,314,131 entries, representing 19,858 different gene sequences for *C. intestinalis* (Dehal et al., 2002). The other is a genomic database for *C. savignyi* containing 9,689,492 entries, representing 20,143 gene sequences (Vinson et al., 2005; Small et al., 2007). Methionine residues can become oxidized due to reactive oxygen species generated during the electrophoresis process, and carbamidomethylation of cysteine residues is a result of using iodoacetamide to keep disulfide bridges from reforming; therefore, oxidation of methionine and carbamidomethylation of cysteine were included as variable modifications (Sun and Anderson, 2004). Our search allowed one missed cleavage during trypsin digestion.

For tandem mass spectrometry we set the precursor-ion mass tolerance to 0.6 Da. The molecular weight search (MOWSE) score that indicated a significant hit was dependent on the database: Scores higher than 29 were significant ($p < 0.05$) in a search in both the *Ciona intestinalis* EST and *C. savignyi* EST. However, we only accepted positive identifications that included at least

two matched peptides in addition to a MOWSE score above the significance threshold (see Tables 1-6).

Statistical analysis

Normalized spot volumes were analyzed within Delta2D by using an analysis of variance (one-way ANOVA) within each species and with temperature as the main effect. For the one-way ANOVA a null distribution was generated using 1000 permutations to account for the unequal variance and non-normal distributions of the response variables and a p-value of 0.05 was used instead of using a multiple-comparison correction, which was deemed too strict given that the number of protein spots is much smaller in comparison to microarray experiments. A two-way ANOVA was not possible because a number of gel regions were difficult to match between species, and even for those proteins that overlapped between species it was unclear whether proteins were orthologous or paralogous homologs. Following the one-way ANOVA, *post-hoc* testing to compare treatments was conducted using Tukey's analysis ($p < 0.05$) in MiniTab (version 16). In our heat maps, we used hierarchical clustering to group proteins based on similarities in their expression patterns. To accomplish this, we used average

linking in the statistical tool suite within Delta2D, and used a Pearson's correlation metric.

Results

High Heat Stress (HHS) Experiment

Ciona intestinalis

A proteome map showing the average normalized pixel volume of all proteins detected in the 20 analyzed sample gel images is shown in Figure 11. After spot boundaries were evaluated within this composite map, 342 individual protein spots were detected. Using an ANOVA based on permutations, we determined that 51 protein spots (or 15% of the total) showed significant changes in their expression across temperature treatment groups ($p < 0.05$). Of the 51 significant protein spots, 36 spots (or 71%) were successfully identified by MALDI TOF/TOF mass spectrometry (labeled by number in Fig. 11 and listed in Table 2).

Hierarchical clustering of the identified, significantly changing proteins reveals four main clusters representing similar patterns of protein expression (Fig. 12). The first cluster of three proteins includes myosin light-chain, transglutaminase, and relaxin-3, a G protein-coupled receptor. All of these proteins show increased expression in the 22°C temperature group (Fig 12).

Figure 11. A composite gel image (or proteome map) depicting the 342 individual protein spots detected in the *Ciona intestinalis* high heat stress experiment. The proteome map represents the average normalized pixel volumes for each protein spot across all 20 sample gels analyzed. Numbered spots were those that showed changes in abundance in response to acute heat stress treatments (one-way ANOVA, $p < 0.05$) and were identified using tandem mass spectrometry (for identifications, see Table 2).

The second cluster contains 14 proteins, including an assortment of actins, actin-binding proteins, tubulins, calcium-binding proteins, and proteins involved in energy metabolism. This cluster is characterized by up-regulation in the 22°C and 25°C treatment groups (Fig 12). The third cluster consists mainly of actin isoforms, all of which show down-regulation in the 22°C and 25°C treatments, while the fourth cluster of six proteins, four actin isoforms, a

calcium-binding protein, and a G protein-coupled receptor, are all down-regulated in the 22°C and 28°C temperature groups (Fig 12).

Figure 12. HHS *C. intestinalis* – Hierarchical clustering of identified proteins using Pearson’s correlation in response to acute heat stress. Each square represents a single spot on a single gel, with each row representing a single spot across all of the gels in the experiment, and each column representing all of the spots on a single gel. Blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature treatments are labeled along the upper horizontal axis, while the right vertical axis represents the standardized expression patterns of identified proteins. The clustering shown along the left vertical axis shows four major groups of proteins with similar expression patterns.

Table 2. Protein identifications (using MS/MS) of spots changing with temperature treatment in *C. intestinalis* high heat stress experiment.

Spot ID	Protein ID	Predicted MW (kDa)	Predicted pI	Estimated MW (kDa)	Estimated pI	GenBank ID	Mascot Score	Peptide Matches	Sequence Coverage	Functional Category
1	Calregulin	48.45	4.25	49.00	4.30	GI:198432093	116	6	11%	molecular chaperone
15	ATP Synthase F1 β -subunit	55.86	5.26	31.00	6.25	GI:198421396	276	8	20%	energy metabolism
32	ATP Synthase F1 β -subunit	55.86	5.26	25.00	4.97	GI:198421396	214	6	15%	energy metabolism
28	Cytochrome C Oxidase Subunit	16.41	5.38	15.00	4.61	GI:198427831	92	5	40%	energy metabolism
26	Calcium Vector Protein Target	25.40	4.78	26.00	4.56	GI:198422600	324	7	40%	calcium-binding
31	Calcium Vector Protein Target	25.40	4.78	28.00	4.81	GI:198422600	38	2	14%	calcium-binding
33	Transglutaminase	87.14	5.76	22.00	5.17	GI:74095919	179	7	10%	extracellular matrix
4	Collagen (Type 28)	91.37	4.93	78.00	4.77	GI:198424351	121	4	11%	extracellular matrix
5	Collagen (Type 28)	91.37	4.93	79.00	4.81	GI:198424351	178	4	8%	extracellular matrix
35	α -Tubulin	50.06	4.94	22.00	5.50	GI:198422770	297	4	10%	cytoskeletal
36	α -Tubulin	50.06	4.94	21.00	5.76	GI:198422770	89	2	5%	cytoskeletal
19	β -Tubulin	49.96	4.73	35.00	5.16	GI:198424255	285	7	10%	cytoskeletal
22	β -Tubulin	49.96	4.73	34.00	4.93	GI:198424255	397	8	23%	cytoskeletal
27	Myosin LC-1 (skeletal muscle)	16.87	4.43	21.00	4.38	GI:198424414	256	3	22%	cytoskeletal
29	Cofilin 2	18.53	5.26	21.00	4.83	GI:198423127	230	4	27%	cytoskeletal
25	Alpha-actinin	102.43	5.08	36.00	4.66	GI:198426726	42	3	44%	cytoskeletal
11	Actin	41.70	5.29	42.00	5.25	GI:86610891	207	5	12%	cytoskeletal
12	Actin	41.70	5.29	43.00	5.25	GI:86610891	67	5	12%	cytoskeletal
21	Actin	41.70	5.29	33.00	5.01	GI:86610891	99	6	20%	cytoskeletal
9	Muscle actin	43.86	5.37	43.00	5.19	GI:198433784	41	4	11%	cytoskeletal
16	Muscle actin	43.86	5.37	32.00	5.66	GI:198433784	161	5	17%	cytoskeletal
17	Muscle actin	43.86	5.37	28.00	5.61	GI:198433784	160	5	15%	cytoskeletal
18	Muscle actin	43.86	5.37	31.00	5.38	GI:198433784	140	5	15%	cytoskeletal
30	Muscle actin	43.86	5.37	24.00	4.81	GI:198433784	47	3	8%	cytoskeletal
34	Muscle actin	43.86	5.37	19.00	5.24	GI:198433784	92	3	12%	cytoskeletal
7	Muscle actin (Isoform 1)	42.16	5.18	46.00	5.16	GI:198433891	138	5	14%	cytoskeletal
10	Muscle actin (Isoform 1)	42.16	5.18	44.00	5.18	GI:198433891	108	6	17%	cytoskeletal
13	Muscle actin (Isoform 1)	42.16	5.18	46.00	5.25	GI:198433891	178	5	20%	cytoskeletal
14	Muscle actin (Isoform 1)	42.16	5.18	43.00	5.90	GI:198433891	225	7	22%	cytoskeletal
20	Muscle actin (Isoform 1)	42.16	5.18	35.00	5.12	GI:198433891	329	6	20%	cytoskeletal
23	Muscle actin (Isoform 1)	42.16	5.18	34.00	4.83	GI:198433891	231	6	20%	cytoskeletal
24	Muscle actin (Isoform 1)	42.16	5.18	37.00	4.79	GI:198433891	126	5	15%	cytoskeletal
2	Relaxin-3 GPCR	51.74	8.83	58.00	4.58	GI:198436421	200	4	1%	unknown
3	Relaxin-3 GPCR	51.74	8.83	53.00	4.61	GI:198436421	115	3	1%	unknown
6	Relaxin-3 GPCR	51.74	8.83	46.00	4.93	GI:198436421	165	4	1%	unknown

Ciona savignyi

Figure 13 shows a proteome map displaying the average normalized pixel volumes for all proteins detected in the 18 analyzed sample gel images. A total of 403 individual protein spots were detected once spot boundaries were defined. Using an ANOVA based on permutations, 74 protein spots (or 18%) showed significant changes in their abundance in response to temperature stress ($p < 0.05$). Of the 74 significantly changing spots, we were able to positively identify 68 proteins (or 92%) using MALDI TOF/TOF mass spectrometry (labeled by number in Fig. 13, and listed in Table 3).

Using a hierarchical clustering analysis, the identified significant proteins were grouped into four main clusters based on similarities in their expression patterns (Fig. 14). The first cluster is comprised of six proteins, all actin isoforms, which are characterized by down-regulation in the 22°C, 25°C, and 28°C temperature groups, compared to the 13°C control group. Cluster two contains four proteins, muscle actin and three tubulins, which are up-regulated in the 22°C group, and the third cluster consists of 15 proteins that are down-regulated in the 28°C group (Fig. 14). This third cluster contains fibrillin-2 and a mix of actin and tubulin isoforms.

Figure 13. A composite gel image (or proteome map) depicting the 403 individual protein spots detected in the *Ciona savignyi* high heat stress experiment. The proteome map represents the average normalized pixel volumes for each protein spot across all 18 sample gels analyzed. Numbered spots were those that showed changes in abundance in response to acute heat stress treatments (one-way ANOVA, $p < 0.05$) and were identified using tandem mass spectrometry (for identifications, see Table 3).

Lastly, the fourth cluster, which contains 43 proteins, makes up the majority of the significantly changing proteins, and is characterized by up-regulation in the 28°C treatment group. This last cluster contains a wide-variety of proteins including heat shock proteins and other molecular chaperones, energy metabolism proteins, components of the extracellular matrix, calcium-binding proteins, and multiple isoforms of all three major

cytoskeletal elements: actins, tubulins, and intermediate filaments. Interestingly, the expression pattern seen in this cluster of up-regulation at the highest temperature treatment (28°C) is completely absent in the *C.*

intestinalis high heat stress results. Also, about 74% of the proteins identified in this cluster, 32 out of 43, have an apparent molecular mass in the gel that is lower than is predicted from the *C. savignyi* genomic database (for details, see below).

Figure 14. HHS *C. savignyi* - Hierarchical clustering of identified proteins using Pearson's correlation in response to acute heat stress. Blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature treatments are labeled along the upper horizontal axis, while the right vertical axis represents the standardized expression patterns of identified proteins. The clustering shown along the left vertical axis shows four major groups of proteins with similar expression patterns.

Table 3. Protein identifications (using MS/MS) of spots changing with temperature treatment in *C. savignyi* high heat stress experiment.

Spot ID	Protein ID	Predicted MW (kDa)	Predicted pI	Estimated MW (kDa)	Estimated pI	GenBank ID	Mascot Score	Peptide Matches	Sequence Coverage	Functional Category
3	Calregulin	48.45	4.25	57.00	4.31	GI:198432093	207	5	12%	molecular chaperone
4	Calregulin	48.45	4.25	64.00	4.37	GI:198432093	231	7	17%	molecular chaperone
30	Hsp70	71.82	5.34	26.00	6.11	GI:198420813	306	7	13%	molecular chaperone
41	Hsp70	71.82	5.34	36.00	4.68	GI:198420813	45	2	5%	molecular chaperone
43	Hsp70	75.79	5.64	34.00	4.63	GI:198435320	223	3	7%	molecular chaperone
44	Hsp70	75.79	5.64	34.00	4.68	GI:198435320	203	3	7%	molecular chaperone
40	Hsp90	69.00	4.84	34.00	4.80	GI:198427715	277	5	8%	molecular chaperone
42	Hsp90	83.21	4.60	35.00	4.64	GI:198414289	68	2	2%	molecular chaperone
29	ATP Synthase F1 β -subunit	55.86	5.26	32.00	6.37	GI:198421396	267	7	18%	energy metabolism
52	Mitochondrial MDH	35.96	6.45	24.00	5.12	GI:198434598	107	3	11%	energy metabolism
28	GADPH	35.60	6.91	36.00	6.24	GI:198433006	189	5	19%	energy metabolism
37	Calcium Vector Protein Target	25.40	4.78	32.00	4.97	GI:198422600	282	3	24%	calcium-binding
38	Calcium Vector Protein Target	25.40	4.78	31.00	4.89	GI:198422600	212	3	24%	calcium-binding
5	Ca-binding EGF-like Domain	48.58	4.63	61.00	5.01	GI:198420709	249	6	18%	calcium-binding / extracellular matrix
45	Fibrillin-2	305.02	4.42	27.00	4.54	GI:198431487	139	5	3%	calcium-binding / extracellular matrix
24	Fibronectin	107.59	5.71	40.00	5.79	GI:198424073	129	4	32%	calcium-binding / extracellular matrix
27	Fibronectin	107.59	5.71	36.00	5.82	GI:198424073	49	2	15%	calcium-binding / extracellular matrix
1	Collagen (Type 28)	91.37	4.93	94.00	5.01	GI:198424351	100	1	4%	extracellular matrix
2	Collagen (Type 28)	91.37	4.93	93.00	5.13	GI:198424351	95	1	4%	extracellular matrix
6	Intermediate Filament	58.00	5.05	51.00	4.76	GI:74096043	284	5	12%	cytoskeletal
7	Intermediate Filament	58.00	5.05	52.00	4.79	GI:74096043	367	7	18%	cytoskeletal
8	Intermediate Filament	58.00	5.05	53.00	4.83	GI:74096043	372	7	18%	cytoskeletal
26	α -tubulin	50.06	4.94	37.00	5.87	GI:198422770	73	6	17%	cytoskeletal
53	α -tubulin	50.06	4.94	22.00	5.34	GI:198422770	151	4	9%	cytoskeletal
54	α -tubulin	50.06	4.94	20.00	5.51	GI:198422770	208	3	7%	cytoskeletal
57	α -tubulin	50.06	4.94	14.00	5.68	GI:198422770	125	3	10%	cytoskeletal
58	α -tubulin	50.06	4.94	14.00	5.44	GI:198422770	77	2	5%	cytoskeletal
23	β -tubulin	49.96	4.73	43.00	6.05	GI:198424255	247	6	10%	cytoskeletal
25	β -tubulin	49.96	4.73	40.00	5.81	GI:198424255	298	9	23%	cytoskeletal
33	β -tubulin	49.96	4.73	38.00	5.33	GI:198424255	94	7	13%	cytoskeletal
34	β -tubulin	49.96	4.73	37.00	5.19	GI:198424255	243	8	17%	cytoskeletal
36	β -tubulin	49.96	4.73	37.00	5.07	GI:198424255	221	8	13%	cytoskeletal
39	β -tubulin	49.96	4.73	35.00	4.84	GI:198424255	56	3	7%	cytoskeletal
61	β -tubulin	49.96	4.73	11.00	4.85	GI:198424255	180	4	11%	cytoskeletal
68	β -tubulin	49.96	4.73	18.00	4.30	GI:198424255	39	3	4%	cytoskeletal
64	Actin	41.70	5.29	15.00	4.82	GI:86610891	276	5	17%	cytoskeletal
59	Actin	41.70	5.29	14.00	4.97	GI:86610891	161	4	13%	cytoskeletal
63	Actin	41.70	5.29	14.00	4.85	GI:86610891	272	4	13%	cytoskeletal
12	Cytoplasmic actin	41.70	5.29	46.00	5.08	GI:3036959	438	6	23%	cytoskeletal
14	Cytoplasmic actin	41.70	5.29	47.00	5.18	GI:3036959	312	5	23%	cytoskeletal
21	Cytoplasmic actin	41.70	5.29	50.00	5.30	GI:3036959	213	6	20%	cytoskeletal
22	Cytoplasmic actin	41.70	5.29	52.00	5.58	GI:3036959	274	5	20%	cytoskeletal
31	Cytoplasmic actin	41.70	5.29	32.00	5.41	GI:3036959	191	4	13%	cytoskeletal
35	Cytoplasmic actin	41.70	5.29	35.00	5.02	GI:3036959	292	6	20%	cytoskeletal
46	Cytoplasmic actin	41.70	5.29	28.00	4.85	GI:3036959	85	3	13%	cytoskeletal
56	Cytoplasmic actin	41.70	5.29	9.00	6.34	GI:3036959	76	4	17%	cytoskeletal
60	Cytoplasmic actin	41.70	5.29	11.00	4.94	GI:3036959	58	2	9%	cytoskeletal
62	Cytoplasmic actin	41.70	5.29	11.00	4.73	GI:3036959	155	3	9%	cytoskeletal
48	Muscle actin	43.86	5.37	26.00	4.81	GI:198433784	86	3	9%	cytoskeletal
9	Muscle actin (Isoform 1)	42.16	5.18	53.00	4.88	GI:198433891	245	6	20%	cytoskeletal
10	Muscle actin (Isoform 1)	42.16	5.18	54.00	4.95	GI:198433891	190	6	20%	cytoskeletal
11	Muscle actin (Isoform 1)	42.16	5.18	46.00	4.83	GI:198433891	271	7	25%	cytoskeletal
13	Muscle actin (Isoform 1)	42.16	5.18	47.00	5.14	GI:198433891	418	6	23%	cytoskeletal
15	Muscle actin (Isoform 1)	42.16	5.18	49.00	5.17	GI:198433891	216	6	23%	cytoskeletal
16	Muscle actin (Isoform 1)	42.16	5.18	49.00	5.17	GI:198433891	277	6	23%	cytoskeletal
17	Muscle actin (Isoform 1)	42.16	5.18	51.00	5.17	GI:198433891	281	6	23%	cytoskeletal
18	Muscle actin (Isoform 1)	42.16	5.18	50.00	5.25	GI:198433891	261	6	23%	cytoskeletal
19	Muscle actin (Isoform 1)	42.16	5.18	48.00	5.25	GI:198433891	137	6	19%	cytoskeletal
20	Muscle actin (Isoform 1)	42.16	5.18	49.00	5.24	GI:198433891	261	6	23%	cytoskeletal
32	Muscle actin (Isoform 1)	42.16	5.18	28.00	4.84	GI:198433891	290	6	23%	cytoskeletal
47	Muscle actin (Isoform 1)	42.16	5.18	26.00	4.81	GI:198433891	343	5	17%	cytoskeletal
49	Muscle actin (Isoform 1)	42.16	5.18	24.00	4.79	GI:198433891	209	4	17%	cytoskeletal
50	Muscle actin (Isoform 1)	42.16	5.18	25.00	4.80	GI:198433891	212	5	17%	cytoskeletal
51	Muscle actin (Isoform 1)	42.16	5.18	26.00	4.92	GI:198433891	112	3	11%	cytoskeletal
55	Muscle actin (Isoform 1)	42.16	5.18	22.00	5.88	GI:198433891	170	3	10%	cytoskeletal
65	Muscle actin (Isoform 1)	42.16	5.18	21.00	5.00	GI:198433891	194	6	17%	cytoskeletal
66	Muscle actin (Isoform 1)	43.86	5.37	11.00	4.32	GI:198433784	91	3	9%	cytoskeletal
67	Muscle actin (Isoform 1)	43.86	5.37	10.00	4.28	GI:198433784	54	2	9%	cytoskeletal

Low Heat Stress (LHS) Experiment

Since the temperatures used in the high heat stress experiment represent the extreme end of the stress spectrum, in order to assess the effect of mild-to-moderate stress on the *Ciona* congeners we decided to run another experiment using lower treatment temperatures. The two experiments were run at different times of the year, which is reflected in the distinct control temperatures used for each: 13°C, a typical water temperature for early spring in Santa Barbara, CA, was used for the HHS experiment, while 16°C, a common late summer water temperature, was used for the LHS experiment (Fig. 9). Because of the seasonal difference, and the resulting difference in control temperatures, caution must be used when drawing direct comparisons between the two experiments, even though the same methods were used throughout.

Ciona intestinalis

The proteome map showing the average normalized pixel volume of all 485 proteins detected in the 20 analyzed sample gel images is shown in Figure 15. Of the 485 total spots detected, 20 proteins (or 4%) were found to show significant expression changes across temperature treatments after running an ANOVA based on permutations

($p < 0.05$). Thirteen of the 20 significant proteins (or 65%) were positively identified through MALDI TOF/TOF mass spectrometry (labeled by number in Fig. 15, and listed in Table 4).

Figure 15. A composite gel image (or proteome map) depicting the 485 individual protein spots detected in the *Ciona intestinalis* low heat stress experiment. The proteome map represents the average normalized pixel volumes for each protein spot across all 20 sample gels analyzed. Numbered spots were those that showed changes in abundance in response to acute heat stress treatments (one-way ANOVA, $p < 0.05$) and were identified using tandem mass spectrometry (for identifications, see Table 4).

As shown in Figure 16, the hierarchical clustering analysis illustrates three main clusters of proteins with similar expression patterns. The first cluster consists of

three proteins, collagen, myosin light-chain, and a muscle actin isoform, all of which are up-regulated in the 20°C temperature group. The second cluster highlights six proteins that are all down-regulated in the 18 and 20°C treatments; these include ependymin, a proteasome subunit, and four β -tubulin isoforms. The third cluster is made up of four proteins, three actin isoforms and cAMP-dependent proteins kinase II- α , which are all up-regulated in the 18°C treatment. In the low heat stress experiment, *C. intestinalis* does not contain any clusters that are characterized by up-regulation at the highest treatment temperature, 23°C.

Figure 16. LHS *C. intestinalis* - Hierarchical clustering of identified proteins using Pearson's correlation in response to acute heat stress. Blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature treatments are labeled along the upper horizontal axis, while the right vertical axis represents the standardized expression patterns of identified proteins. The clustering shown along the left vertical axis shows three major groups of proteins with similar expression patterns.

Table 4. Protein identifications (using MS/MS) of spots changing with temperature treatment in *C. intestinalis* low heat stress experiment.

Spot ID	Protein ID	Predicted MW (kDa)	Predicted pI	Estimated MW (kDa)	Estimated pI	GenBank ID	Mascot Score	Peptide Matches	Sequence Coverage	Functional Category
2	cAMP-Dependent Protein Kinase II- α	44.24	4.55	59.00	4.55	GI:198414287	266	4	20%	cell signaling
13	Proteasome Subunit Y	23.16	4.83	23.00	5.67	GI:198417706	134	6	28%	protein degradation
1	Collagen (Type 28)	91.37	4.93	94.00	5.05	GI:198424351	115	4	4%	extracellular matrix
5	β -tubulin	49.96	4.73	36.00	5.11	GI:198424255	347	7	23%	cytoskeletal
9	β -tubulin	49.96	4.73	29.00	4.76	GI:198424255	136	4	9%	cytoskeletal
10	β -tubulin	49.96	4.73	27.00	4.67	GI:198424255	175	4	19%	cytoskeletal
11	β -tubulin	49.96	4.73	21.00	4.70	GI:198424255	65	3	4%	cytoskeletal
12	Myosin Light Chain 1	16.87	4.43	17.00	4.39	GI:198424414	288	5	36%	cytoskeletal
4	Actin	41.70	5.29	47.00	5.21	GI:86610891	402	6	23%	cytoskeletal
6	Actin	41.70	5.29	36.00	5.00	GI:198433891	201	5	17%	cytoskeletal
3	Muscle Actin (Isoform 1)	42.16	5.18	54.00	5.75	GI:198433891	496	6	23%	cytoskeletal
7	Muscle Actin (Isoform 1)	42.16	5.18	36.00	4.90	GI:198433891	276	6	20%	cytoskeletal
8	Ependymin-related Protein-1	23.48	6.30	36.00	4.61	GI:198433424	62	4	16%	unknown

Ciona savignyi

Figure 17 depicts a proteome map displaying the normalized average pixel volume of all 398 individual protein spots detected across all of the 20 analyzed sample gels. A permutation-based ANOVA showed that 17 proteins (or 4%) changed significantly in abundance in response to temperature stress ($p < 0.05$). In this case, we were able to identify all 17 of the significantly changing proteins using MALDI TOF/TOF mass spectrometry (labeled by number in Fig. 17 and listed in Table 5). In both experiments, we had a higher success rate of protein identification in *C. savignyi* than in *C. intestinalis*, which makes sense considering the genomic database for *C. savignyi* is a more complete representation of the whole genome (20,143 sequences are available in the *C. savignyi* database, as opposed to 19,858 sequences in *C. intestinalis*).

Figure 17. A composite gel image (or proteome map) depicting the 398 individual protein spots detected in the *Ciona savignyi* low heat stress experiment. The proteome map represents the average normalized pixel volumes for each protein spot across all 20 sample gels analyzed. Numbered spots were those that showed changes in abundance in response to acute heat stress treatments (one-way ANOVA, $p < 0.05$) and were identified using tandem mass spectrometry (for identifications, see Table 5).

In the hierarchical clustering analysis shown in Figure 18, the expression patterns of the 17 significant proteins cluster into three main groups. The first cluster is characterized by up-regulation in the 23°C temperature treatment, and includes two heat shock proteins, Hsp70 and Hsp90, the relaxin-3 G protein-coupled receptor, an intermediate filament, ATP-synthase α -subunit, and myc-

induced nuclear antigen, also known as MINA-53 (Tsuneoka et al., 2002). As in the high heat stress experiment, this pattern of increased protein expression in the highest temperature group is only observed in *C. savignyi*.

Figure 18. LHS *C. savignyi* - Hierarchical clustering of identified proteins using Pearson's correlation in response to acute heat stress. Blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature treatments are labeled along the upper horizontal axis, while the right vertical axis represents the standardized expression patterns of identified proteins. The clustering shown along the left vertical axis shows three major groups of proteins with similar expression patterns (see text for details).

The second cluster is comprised of three proteins, two fibrinogen C isoforms and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which all show increased expression in the 20°C treatment group. The third cluster consists of eight proteins, all isoforms of actin, which show decreased expression in the 18°C, 20°C, and 23°C temperature groups, compared to the 16°C control group (Fig. 18).

Table 5. Protein identifications (using MS/MS) of spots changing with temperature treatment in *C. savignyi* low heat stress experiment.

Spot ID	Protein ID	Predicted MW (kDa)	Predicted pI	Estimated MW (kDa)	Estimated pI	GenBank ID	Mascot Score	Peptide Matches	Sequence Coverage	Functional Category
9	Hsp70	71.85	5.06	35.00	4.71	GI:224073965	104	3	7%	molecular chaperone
10	Hsp90	69.01	4.84	19.00	4.41	GI:198427715	114	3	7%	molecular chaperone
11	ATP Synthase α -subunit	59.92	8.96	7.00	4.88	GI:74096071	220	4	9%	energy metabolism
13	GADPH	35.60	6.91	36.00	6.45	GI:198433006	89	2	8%	energy metabolism
14	Fibrinogen C	30.79	7.03	41.00	6.50	GI:198418636	130	4	16%	extracellular matrix
15	Fibrinogen C	30.79	7.03	43.00	6.51	GI:198418636	127	4	16%	extracellular matrix
2	Myc-Induced Nuclear Antigen	50.84	4.66	78.00	4.87	GI:74096461	33	2	6%	cell proliferation
1	Intermediate Filament IF-F	47.11	5.10	66.00	4.68	GI:12329935	52	2	7%	cytoskeletal
4	Cytoplasmic Actin	41.70	5.29	49.00	5.30	GI:3036959	266	6	20%	cytoskeletal
8	Cytoplasmic Actin	41.70	5.29	39.00	4.96	GI:3036959	293	6	20%	cytoskeletal
6	Muscle Actin (Isoform 1)	42.16	5.18	50.00	5.15	GI:198433891	400	5	25%	cytoskeletal
3	Muscle Actin (Isoform 1)	43.86	5.37	52.00	5.29	GI:198433784	380	6	23%	cytoskeletal
17	Muscle Actin (Isoform 1)	43.86	5.37	47.00	6.14	GI:198433784	184	7	25%	cytoskeletal
16	Muscle Actin (Isoform 1)	42.16	5.18	45.00	6.13	GI:198433891	215	6	23%	cytoskeletal
5	Muscle Actin (Isoform 1)	42.16	5.18	50.00	5.23	GI:198433891	276	7	24%	cytoskeletal
7	Muscle Actin (Isoform 1)	42.16	5.18	44.00	4.95	GI:198433891	357	7	25%	cytoskeletal
12	Relaxin Peptide Receptor-3	51.74	8.83	29.00	6.01	GI:198436421	34	1	0%	unknown

Proteins by Functional Category

Molecular Chaperones

Another way to visualize our results is to group proteins into categories based on their function, and to compare heat maps organized by functional category across species and experiments. In Figure 19, a heat map is presented that depicts the expression patterns of significantly changing molecular chaperones, including the heat shock proteins hsp70 and hsp90, as well as calregulin, an endoplasmic reticulum-resident, calcium-binding chaperone (Nash et al., 1994). Molecular chaperones that were identified but did not show significant changes in their expression across treatments are shown for comparison.

Figure 19. Heat maps of molecular chaperones identified across both species and experiments. Proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature 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 molecular chaperones. Proteins followed by "n.s." (not significant) do not show significant changes in their expression in response to temperature treatment and are shown for comparison.

In both the high and low heat stress experiments, the heat shock proteins hsp70 and hsp90 only show significant changes in abundance in *C. savignyi*; their expression does not change significantly in *C. intestinalis*. In the high heat stress experiment, *C. savignyi* up-regulates hsp expression in the 28°C treatment, while in the low heat

stress experiment, hsp70 is up-regulated at 18°C and hsp90 at 20°C.

In addition, we identified one of the hsp70 isoforms in both species, so we were able to compare heat shock protein levels between species and experiments. As shown in Figure 20, this particular isoform of hsp70 only shows significant changes in expression in *C. savignyi* in the high heat stress experiment, where it is up-regulated in the 28°C treatment. Thus, baseline levels of this hsp70 isoform were lowest in HHS *C. savignyi*, and after up-regulation in the 28°C treatment, the amount of protein is comparable to the baseline levels found in *C. intestinalis* in both heat stress experiments, as well as the baseline level for *C. savignyi* in the low heat stress experiment.

Calregulin does not show significant changes in expression in the low heat stress experiment; however, in the high heat stress experiment, it is apparent that *C. intestinalis* up-regulates calregulin at 22°C, while *C. savignyi* does not show up-regulation of calregulin until the 28°C treatment group. This trend of *C. intestinalis* increasing protein abundance at a lower temperature than *C. savignyi* is seen for several other proteins throughout both experiments (see below). We were also able to identify one of the calregulin isoforms in both species, which allowed

us to compare calregulin expression across both species and experiments. This isoform of calregulin showed significant expression changes in both species in the HHS experiment, but was not significant in either species in the LHS experiment (Fig. 21). In the HHS experiment, baseline levels of calregulin are higher in *C. intestinalis* than they are in *C. savignyi*, and even after up-regulation (which occurs at 22°C in *C. intestinalis*, and 28°C in *C. savignyi*), the protein abundance is still higher in *C. intestinalis* (Fig. 21).

Figure 20. Expression profile graphs showing the relative expression of one hsp70 isoform (spot number 41 in HHS *C. savignyi*) across both species and experiments. Spot numbers are not shown for proteins that do not show significant changes in expression. Spot volumes were obtained by normalizing against the volume of all proteins, and means $\pm$ 1 s.e.m. are shown (N=5 for all groups, except 13°C and 25°C in HHS *C. savignyi* where N=4). Treatments with significant differences in expression level are marked with different letters.

Figure 21. Expression profile graphs showing the relative expression of one calregulin isoform (spot number 01 in HHS *C. intestinalis*, and spot number 03 in HHS *C. savignyi*) across both species and experiments. Spot numbers are not shown for proteins that do not show significant changes in expression. Spot volumes were obtained by normalizing against the volume of all proteins, and means $\pm$ 1 s.e.m. are shown (N=5 for all groups, except 13°C and 25°C in HHS *C. savignyi* where N=4). Treatments with significant differences in expression level are marked with different letters.

Extracellular Matrix and Calcium-binding Proteins

Several components of the extracellular matrix (ECM) showed significant changes in abundance across both species in both experiments. As shown by the heat maps in Figure 22, two ECM proteins, collagen and transglutaminase, are up-regulated in the 22°C and 25°C treatment groups in HHS *C. intestinalis*. In HHS *C. savignyi*, collagen, fibronectin, and calcium-binding EGF domain, are not up-regulated until 28°C. With collagen expression, we once again see a trend of increased abundance at a lower temperature in *C. intestinalis* when compared to *C. savignyi*.

Figure 22. Heat maps of extracellular matrix proteins identified across both species and experiments (HHS = high heat stress, LHS = low heat stress). Once again, proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature 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 extracellular matrix proteins.

Unlike the other significant ECM proteins in HHS *C. savignyi*, fibrillin-2, a glycoprotein made up of multiple calcium-binding EGF domains and responsible for protecting against extracellular proteolysis, shows down-regulation from the control group. In the LHS experiment, *C. intestinalis* shows significant up-regulation of collagen in the 20°C treatment, while *C. savignyi* doesn't show significant changes in the expression of collagen. Instead, fibrinogen C, a protein involved in forming fibrin clots and restructuring the ECM in response to tissue damage

(Alberts et al., 2008), is up-regulated in the 20°C treatment in LHS *C. savignyi*.

Another subset of proteins were classified based on their ability to bind to calcium. Despite their various functions (see discussion), these proteins were grouped together based on their shared trait of being regulated by calcium (Fig. 23). Interestingly, calcium-binding proteins only show significant expression changes in the HHS experiment, none were found to change in the LHS experiment.

Figure 23. Heat maps of calcium-binding proteins identified across both species in the high heat stress experiment (HHS). Proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature 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 calcium-binding proteins. No calcium-binding proteins were identified in the low heat stress experiment.

In HHS *C. intestinalis*, calregulin is up-regulated in the 22°C and 25°C treatments, while in HHS *C. savignyi*, calregulin is not up-regulated until the 28°C treatment. Calcium-vector protein target, a protein with an unknown

function, also shows this trend as one isoform is up-regulated in the 22°C and 25°C treatments in HHS *C. intestinalis*, but not until the 28°C treatment in *C. savignyi*. An additional isoform of Ca²⁺ vector protein target shows up in HHS *C. intestinalis*, and it is not up-regulated until the 28°C treatment. The position of these two isoforms in the HHS *C. intestinalis* gels show that they have similar molecular masses, but different isoelectric focusing points, which may indicate a post-translational protein modification (Fig. 11). Lastly, some of the significantly changing ECM proteins in HHS *C. intestinalis* also bind calcium, including: fibrillin-2, fibronectin, and calcium-binding EGF domain.

Energy Metabolism Proteins

As shown in Figure 24, a few proteins involved in energy metabolism show significant changes in their expression across treatments for both species in the HHS experiment, and for *C. savignyi* in the LHS experiment. In the HHS experiment, both *C. intestinalis* and *C. savignyi* differentially express ATP synthase F1 β -subunit, a subunit of the protein embedded in the inner mitochondrial matrix. In *C. intestinalis*, ATP synthase F1 β -subunit is characterized by up-regulation in the 22°C and 25°C

treatments, while in *C. savignyi* it is significantly up-regulated in the 28°C treatment. This constitutes another example of a protein that is up-regulated at a lower temperature in *C. intestinalis* than in *C. savignyi*. ATP synthase F1 β -subunit also shows up in LHS *C. savignyi*, where it is up-regulated in the 23°C temperature treatment.

Figure 24. Heat maps of energy metabolism proteins identified across both species and experiments (HHS = high heat stress, LHS = low heat stress). Proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature 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 energy metabolism proteins. No energy metabolism proteins were identified in the *C. intestinalis* low heat stress experiment.

A subunit of cytochrome C oxidase, complex IV of the electron transport chain, was found to be significantly up-regulated in the 22°C treatment in HHS *C. intestinalis*. Another mitochondrial protein, malate dehydrogenase (MDH), the enzyme responsible for converting malate into oxaloacetate in the Krebs cycle and also associated with

the malate-aspartate shuttle (Minárik et al., 2002), showed differential expression in HHS *C. savignyi* where it is characterized by significant up-regulation at 28°C.

Lastly, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was differentially expressed in *C. savignyi* in both the high and low heat stress experiments. In the HHS experiment, GAPDH was up-regulated in the 28°C treatment group, while in the LHS experiment it was down-regulated at 23°C. Overall, in the high heat stress experiment, differentially expressed energy metabolism proteins are up-regulated at 22°C and 25°C in *C. intestinalis*, while they are not up-regulated until 28°C in *C. savignyi*.

Cytoskeletal Elements

All three major components of the cytoskeleton, intermediate filaments, tubulin monomers, and actin, were found to be differentially expressed in at least one of the heat stress experiments. Intermediate filaments only showed significant changes in abundance in *C. savignyi*, in both the high and low heat stress experiments. In the HHS experiment, 3 isoforms of intermediate filament A (IF-A) are up-regulated in the 25°C and 28°C temperature

treatments, while in the LHS experiment, one IF-A isoform is up-regulated at 23°C (Fig. 25).

Figure 25. Heat maps of intermediate filaments identified across both experiments (HHS = high heat stress, LHS = low heat stress). Proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature 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 intermediate filament isoforms. No intermediate filaments were identified in *C. intestinalis* in either experiment.

Both α - and β -tubulin monomers showed significant changes in their expression in the HHS experiment in both *C. intestinalis* and *C. savignyi*, while only β -tubulin monomers were differentially expressed in the LHS experiment, and only in *C. intestinalis* (Fig. 26). In the HHS experiment, *C. intestinalis* has the same pattern of down-regulation in the 28°C temperature treatment for both α - and β -tubulin isoforms. In HHS *C. savignyi*, two main expression patterns are apparent for the tubulins: one group of β -tubulin isoforms are characterized by up-regulation in the 28°C treatment group, while another group containing five α -tubulin and two β -tubulin isoforms are down-regulated in the 25°C and 28°C treatment groups. These

two distinct expression patterns suggest different roles for the α - and β -tubulin monomers in the *C. savignyi* cellular response to high heat stress. In LHS *C. intestinalis*, four β -tubulin isoforms show significant down-regulation in the 18°C and 20°C treatments. No α -tubulin monomers were identified as being differentially expressed in LHS *C. intestinalis*, and no tubulin monomers at all were found to have significant expression changes in the *C. savignyi* LHS experiment.

Figure 26. Heat maps of tubulin isoforms identified across both species and experiments (HHS = high heat stress, LHS = low heat stress). Proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature 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 tubulin isoforms. No tubulin isoforms were identified in the *C. savignyi* low heat stress experiment.

The last of the three cytoskeletal elements, actin, is differentially expressed for both species in both experiments, and the expression patterns are more diverse than the previously discussed functional categories. Part of the diversity stems from the fact that actin is the most abundant protein in the cell, and the number of actin monomers found to be differentially expressed far exceeds any of the other identified proteins.

In HHS *C. intestinalis*, the expression patterns of actin isoforms and actin-binding proteins can be broken down into two main clusters: the first contains 11 actin isoforms and is characterized by down-regulation in the 22°C and 25°C temperature treatments (Fig. 27). The second cluster contains six actin isoforms, in addition to myosin light chain and two actin-binding proteins, cofilin-2 and α -actinin. This cluster shows significant up-regulation in the 22°C and 25°C treatment groups (Fig. 27).

In HHS *C. savignyi*, the multitude of actin isoforms can once again be broken into two clusters (Fig. 27). The first cluster shows up-regulation in the 28°C treatment, and it contains 17 actin isoforms. The second cluster consists of 16 actin isoforms, which are characterized by down-regulation in the 28°C treatment; the exact opposite

pattern of the first cluster. No actin-binding proteins showed significant expression changes in HHS *C. savignyi*.

In the LHS experiment, both species only had a handful of actin isoforms that showed differential expression (Fig. 28). For *C. intestinalis*, myosin light chain and one muscle actin isoform were up-regulated in the 20°C treatment, while the three remaining actin isoforms were up-regulated in the 18°C temperature group. For *C. savignyi*, all of the differentially expressed actin isoforms, eight in all, had the same expression pattern of down-regulation from the 16°C control.

Figure 27. Heat maps of actins and actin-binding proteins identified across both species in the high heat stress experiment (HHS). Proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature 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 actins and actin-binding proteins.

In summary, in both the high and low heat stress experiments, *C. intestinalis* shows either up- or down-regulation of actin isoforms at the intermediate temperature treatments, 22°C and 25°C in the HHS experiment, and 18°C and 20°C in the LHS experiment. *Ciona savignyi*, on the other hand, is characterized by up- or down-regulation actin monomers at the most extreme temperatures, 13°C and 28°C in the HHS experiment, and 16°C and 23°C in the LHS experiment.

Figure 28. Heat maps of actins and actin-binding proteins identified across both species in the low heat stress experiment (LHS). Proteins were clustered using Pearson's correlation, and blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature 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 actins and actin-binding proteins.

Estimated vs. Predicted Molecular Masses

After identifying the significantly changing proteins for each species in both experiments, a discrepancy was noted between the estimated molecular mass of the identified protein, which is approximated based on the relative position of the protein spot in the gel, and the predicted molecular mass, which is calculated based on the amino acid sequence of the positively matched protein. For several of the proteins identified, especially in the high heat stress experiment, the estimated molecular mass is considerably smaller than the molecular mass predicted by the matched amino acid sequence, indicating proteolytic degradation of proteins, possibly due to an artifact of sample preparation (Fig. 29).

In order to determine whether species and level of heat stress affect the differences between estimated and predicted molecular mass, a two-factor ANOVA was conducted, revealing a significant effect of heat stress on the difference between estimated and predicted molecular mass ($p=0.024$). By looking at the raw differences, we are able to assign directionality to the observed differences in molecular mass, and a negative average indicates that the estimated molecular mass values are mostly smaller than the predicted molecular masses. The boxplot shown in Figure 30

illustrates that, on average, the difference between the estimated and predicted molecular mass is more negative in the HHS experiment than it is in the LHS experiment, indicating that more proteins are degraded in the HHS experiment.

Figure 29. Scatterplots showing the estimated and predicted molecular mass (MM) of significantly changing proteins identified in both species and experiments. Protein identity is indicated by the shape and color of the data points, as shown in the legends. Dotted lines represent the line $y=x$, where the data points should fall if estimated MM is equal to predicted MM. (Note: fibrillin-2 was removed as an outlier from the HHS *C. savignyi* graph).

Figure 30. Boxplot showing the averages of the differences in estimated and predicted molecular mass (MM) across both species and experiments (two-factor ANOVA). There was a significant effect of heat stress on the average of the differences between estimated and predicted MM ($p < 0.05$).

However, some proteins representing distinct functional categories were not equally affected by this breakdown. Actins, tubulins, and ATP synthase F1 β -subunit seem to be the most affected across both experiments and species, while the calcium-binding proteins, including calcium-vector protein target, calregulin, and calcium-binding EGF domain, are largely unaffected (Fig. 29). In addition, other proteins including GAPDH, collagen, myosin light chain, and intermediate filaments, also seem to be unaffected.

In order to determine whether the observed differential protein breakdown was an effect of thermal stress, a two-factor ANOVA was conducted comparing the estimated and predicted molecular masses of actin monomers, many of which were identified in all four projects, between species and experiments. This test revealed a significant effect of heat stress level on the amount of breakdown in the actin monomers, with no significant effect of species or interaction between species and heat stress level ($p=0.018$). This indicates that actin monomers undergo more proteolytic degradation, on average, in the high heat stress experiment. It is difficult to determine whether the same is true for tubulin monomers and ATP synthase F1 β -subunits because these proteins were not identified in all four projects and relatively few monomers were identified overall.

Protease Inhibitor Experiment

To evaluate the role of protease activity occurring during sample preparation, a third experiment was conducted. Non-heat stressed samples from each species were split into two groups, and were processed with and without compounds known to inhibit the activity of four of the main

classes of proteases: cysteine, serine, aspartate, and metalloproteases (Chou and Shen, 2008).

Figure 31 shows a proteome map containing the average normalized pixel volume of all 345 detected individual protein spots across the 12 analyzed *C. intestinalis* sample gels. Of the 345 total protein spots, 26 (or 8%) were found to have significant changes in their expression after running a t-test based on permutations ($p < 0.05$). Seventeen of the 26 significant spots (65%) were identified using mass spectrometry (labeled by number in Fig. 31 and listed in Table 6). As shown in the hierarchical clustering analysis (Fig. 32), some, but not all, of the significantly changing proteins in this experiment overlap with proteins from the heat stress experiments, including ATP synthase F1 β -subunit, calcium vector protein target, α - and β -tubulins, along with a few actin isoforms.

Figure 31. A composite gel image (or proteome map) depicting the 345 individual protein spots detected in the *Ciona intestinalis* protease inhibitor experiment. The proteome map represents the average normalized pixel volumes for each protein spot across all 12 sample gels analyzed. Numbered spots were those that showed changes in abundance in response to treatment with protease inhibitors (t-test, $p < 0.05$) and were identified using tandem mass spectrometry (for identifications, see Table 6).

Figure 32. Heat map showing hierarchical clustering (Pearson's correlation) of proteins identified in the *C. intestinalis* protease inhibitor experiment. Blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature treatments are labeled along the upper horizontal axis, while the right vertical axis represents the standardized expression patterns of identified proteins. The clustering shown along the left vertical axis shows two major groups of proteins with similar expression patterns.

Table 6. Protein identifications (using MS/MS) of spots changing with protease inhibitor treatment in *C. intestinalis*.

Spot ID	Protein ID	Predicted MW (kDa)	Predicted pI	Estimated MW (kDa)	Estimated pI	MOWSE Score	Peptide Matches	Sequence Coverage	Functional Category
6	ATP Synthase F1 β -subunit	55.86	5.26	24.00	6.43	292	5	10%	energy metabolism
7	ATP Synthase F1 β -subunit	55.86	5.26	24.00	6.48	65	10	23%	energy metabolism
8	Calcium Vector Protein Target	25.40	4.78	30.00	4.41	58	7	40%	calcium-binding
10	HMG Transcription Factor SoxC	58.61	8.59	37.00	4.71	46	6	21%	transcription factor
9	Dolichyl-diphosphooligosaccharide glycosyltransferase	48.85	5.90	29.00	4.89	88	9	67%	protein modification
14	Peptidylglycine α -hydroxylating monooxygenase	44.74	8.84	34.00	6.73	66	11	28%	protein modification
15	Villin 1	66.46	6.23	42.00	5.94	58	5	40%	cytoskeletal
16	α -tubulin	50.06	4.94	43.00	5.78	85	8	28%	cytoskeletal
17	β -tubulin	49.96	4.73	40.00	6.08	116	2	4%	cytoskeletal
1	Actin	41.70	5.29	50.00	5.48	67	5	12%	cytoskeletal
2	Actin	41.70	5.29	38.00	5.03	102	2	7%	cytoskeletal
3	Actin	41.70	5.29	38.00	5.10	104	2	7%	cytoskeletal
4	Actin	41.70	5.29	35.00	5.27	187	3	9%	cytoskeletal
5	Muscle Actin	43.86	5.37	55.00	5.51	281	5	16%	cytoskeletal
11	Muscle Actin Isoform 1	42.16	5.18	60.00	5.54	141	4	12%	cytoskeletal
12	Muscle Actin Isoform 1	42.16	5.18	46.00	5.18	336	5	15%	cytoskeletal
13	Muscle Actin Isoform 1	42.16	5.18	39.00	5.07	130	4	12%	cytoskeletal

For *C. savignyi*, a total of 315 individual protein spots were detected across the 10 analyzed sample gels and their average normalized pixel volume is displayed in the proteome map in Figure 33. As in *C. intestinalis*, 8% of the total (24 spots) showed significant changes in abundance according to a permutation-based t-test ($p < 0.05$). We were able to identify 16 of the 24 significant spots (or 67%, labeled by number in Fig. 33 and listed in Table 7), and the hierarchical clustering analysis is shown in Figure 34. Once again, some, but not all, of the proteins that change in abundance in response to the use of protease inhibitors are the same as those that change in response to heat stress, namely mitochondrial malate dehydrogenase, calcium vector protein target, intermediate filament, β -tubulin, and a few actin isoforms (Fig. 34). It is interesting to

note that although calcium vector protein target is up-regulated in the protease inhibitor treatment, there was no observed difference between its estimated and predicted molecular masses in any of the experiments.

Figure 33. A composite gel image (or proteome map) depicting the 315 individual protein spots detected in the *Ciona savignyi* protease inhibitor experiment. The proteome map represents the average normalized pixel volumes for each protein spot across all 10 sample gels analyzed. Numbered spots were those that showed changes in abundance in response to treatment with protease inhibitors (t-test, $p < 0.05$) and were identified using tandem mass spectrometry (for identifications, see Table 7).

Figure 34. Heat map showing hierarchical clustering (Pearson's correlation) of proteins identified in the *C. savignyi* protease inhibitor experiment. Blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature treatments are labeled along the upper horizontal axis, while the right vertical axis represents the standardized expression patterns of identified proteins. The clustering shown along the left vertical axis shows two major groups of proteins with similar expression patterns: either up- or down-regulation.

Table 7. Protein identifications (using MS/MS) of spots changing with protease inhibitor treatment in *C. savignyi*.

Spot ID	Protein ID	Predicted MW (kDa)	Predicted pI	Estimated MW (kDa)	Estimated pI	MOWSE Score	Peptide Matches	Sequence Coverage	Functional Category
8	Mitochondrial MDH	35.96	6.45	27.00	5.50	172	4	11%	energy metabolism
16	Dihydrolipoamide dehydrogenase	53.51	6.65	63.00	6.48	115	2	4%	energy metabolism
14	Cold-inducible RNA-binding Protein	16.49	5.25	19.00	5.99	474	6	61%	cold stress
15	Superoxide-dismutase	15.70	5.93	18.00	6.12	271	2	19%	oxidative stress
7	Calcium Vector Protein Target	25.40	4.78	34.00	5.26	425	4	30%	calcium-binding
9	Calcium Vector Protein Target	25.40	4.78	28.00	5.08	334	3	22%	calcium-binding
2	Intermediate Filament Fb	84.12	5.36	61.00	5.02	125	27	37%	cytoskeletal
10	β-tubulin	49.96	4.73	23.00	5.26	454	5	15%	cytoskeletal
4	β-tubulin	49.96	4.73	38.00	5.31	345	7	13%	cytoskeletal
11	Actin	41.70	5.29	19.00	5.25	185	3	8%	cytoskeletal
13	Actin	41.70	5.29	15.00	5.51	108	2	5%	cytoskeletal
12	Muscle Actin	43.86	5.37	14.00	5.03	137	1	4%	cytoskeletal
1	Muscle Actin	43.86	5.37	72.00	5.33	365	5	16%	cytoskeletal
5	Muscle Actin	43.86	5.37	36.00	5.12	191	4	12%	cytoskeletal
3	Muscle Actin Isoform 1	42.16	5.18	47.00	5.23	374	5	15%	cytoskeletal
6	Muscle Actin Isoform 1	42.16	5.18	34.00	5.19	316	4	17%	cytoskeletal

Discussion

Introduction

Few species comparisons of proteomic responses to environmental stress exist to date, especially for the marine environment (for review see Tomanek, 2011; Tomanek and Zuzow, 2010). The present study provides a comparison of two sea squirt species that are well-defined in terms of their genome sequences due to the multitude of scientific research opportunities they provide, ranging from the ability to learn more about how introduced species impact native communities, to understanding more about the evolutionary relationships between vertebrates and invertebrates (Corbo et al., 2001; Satoh et al., 2003; McDonald, 2004; Blum et al., 2007). However, very little is known about their tolerances to environmental stresses such as temperature and salinity (Dybern, 1965; Marin et al., 1987; Therriault and Herborg, 2008). Although several studies define the range of thermal stress that one of the two species experiences, no direct comparisons of the same life stages exists to date. Here we employed a discovery approach, using proteomics to compare the response of *Ciona intestinalis* and *C. savignyi* to thermal stress.

In a previous species comparison of the proteomic stress response in the blue mussel congeners, *Mytilus galloprovincialis* and *M. trossulus*, we set out to describe the criteria for comparing proteomes (Tomanek and Zuzow, 2010). Briefly, observed changes in protein spot abundance can be due to protein synthesis, degradation, or post-translational modifications, and the term expression is used broadly to describe changes in protein abundance caused by any of these events. Spots that overlap in their position within a 2D gel and were also identified as the same protein can be directly compared. Also, conclusions based on several protein identifications are considered more robust than those based on only a single identification.

The results of this study show several distinct expression patterns between the two species. One possible limitation to our interpretation of these results is that because we used the whole organism, we cannot distinguish protein expression differences between tissues. The whole organism was used to overcome the difficulty of separating out a single tissue from *Ciona* with enough protein for 2DGE. Despite the lack of tissue-specificity, we are still able to compare the distinct species responses to acute thermal stress.

Another difficulty results from the fact that the two heat stress experiments were conducted during two different seasons, early spring for the high heat stress (HHS) experiment and late summer for the low heat stress (LHS) experiment, and thus cannot be directly compared. The seasonal acclimation differences, which are reflected in the two different control temperatures used, may contribute to expression differences at similar temperatures between the two experiments, for example differences between expression at 22°C in the HHS experiment and expression at 23°C in the LHS experiment. However, by looking at expression patterns within each experiment, and comparing general trends, not specific temperatures, across the two experiments, we are able to indirectly compare the acute responses to extreme and moderate heat stress.

Functional expression differences between species and experiments

Our results indicate that the two species differ in terms of the temperature at which they increase the abundance of molecular chaperones, extracellular matrix proteins, calcium-binding proteins, cytoskeletal elements and proteins involved in energy metabolism. This is especially true in the high heat stress experiment (HHS)

where *C. intestinalis* up-regulates these proteins in the 22°C and 25°C treatments, while *C. savignyi* only shows increased abundance in the 28°C treatment group. For cytoskeletal elements that show changes in expression in both species, tubulins and actin isoforms, *C. intestinalis* tends to up- or down-regulate these proteins at intermediate temperatures, 22°C /25°C in HHS and 18°C/20°C in the low heat stress experiment (LHS), while *C. savignyi* shows changes in abundance at the extreme temperatures, 13°C/28°C in HHS and 16°C/23°C in LHS. Intermediate filaments only showed changes in abundance in *C. savignyi*, although they follow the pattern of up-regulation at the highest treatment temperatures, 28°C in HHS and 23°C in LHS. These expression differences between the two species span a wide-range of functional categories and suggest that *C. intestinalis* is able to respond to acute heat shock more quickly, and at a lower temperature than *C. savignyi*.

Another interesting difference in protein expression was observed between the high and low heat stress experiments. In both species, calcium-binding proteins only show significant changes in abundance under high heat stress conditions. This may indicate that intense thermal stress, above 23°C, triggers calcium-signaling cascades. Calcium signaling is an important mechanism for regulating

several molecular pathways and cellular processes, including cell proliferation, excitability, exocytosis, and transcription (Berridge et al., 2003; Clapham, 2007). Calcium has also been shown to play an important role in mitochondrial dysfunction, where a calcium overload can increase the number of reactive oxygen species generated during cellular respiration, and trigger cytochrome *c*-mediated apoptosis (Brookes et al., 2004). Even though we cannot pinpoint the specific role of calcium in the acute heat stress response of the *Ciona* congeners, the appearance of several calcium-binding proteins in the high heat stress experiment only indicates that calcium-signaling is one mechanism that is sensitive to thermal fluctuations and may be involved in dealing with extreme thermal stress.

Species-specific differences in thermal sensitivities

When comparing the number of significantly changing protein isoforms between the high and low heat stress experiments, which differ in both temperature range and control temperature, it is obvious that for both species, a greater number of proteins change in the HHS experiment. For *C. intestinalis*, 51 protein spots changed significantly in HHS versus 20 under LHS conditions, and for *C. savignyi*, 74 spots changed in HHS versus only 17 spots in the LHS

experiment. Many of these additional protein isoforms were found to have a lower molecular mass than was predicted based on their matched amino acid sequence, suggesting some degree of heat-induced proteolytic degradation. This indicates that the extreme treatment temperatures used in the high heat stress experiment may be approaching lethal thermal tolerance limits, where proteins become unstable and are more susceptible to degradation.

Importantly, the comparison of two different species at two different thermal ranges shows statistical evidence for a tiered response of protein degradation to heat stress (Fig. 30). The fact that the direction and magnitude of the differences between estimated and predicted molecular mass vary across species and experiments indicates that the observed proteolytic breakdown is a part of a biological response to thermal stress. If the degradation was due solely to an artifact of sample preparation, the average differences between the estimated and predicted molecular mass should be no different across species or experiments (Fig. 30).

Additionally, certain classes of proteins, namely actins, tubulins, and ATP synthase F1 β -subunit isoforms, were more affected by proteolytic degradation across both species and experiments (Fig. 29). Actin monomers in

particular showed significantly more proteolytic breakdown under high heat stress conditions when compared to the amount of breakdown observed in the low heat stress experiment. This indicates that these particular proteins are more sensitive to breakdown in response to thermal stress.

Further proof that the observed proteolytic degradation is not solely an artifact of sample preparation comes from the experiment we conducted to compare protein expression differences in response to protease inhibitors. Although some of the significantly changing proteins identified in this experiment overlapped with proteins found in the heat stress experiments, far fewer proteins were detected overall, including limited numbers of actin and tubulin isoforms, and only one or two proteins involved in energy metabolism and calcium-binding. It is worth noting that the identified calcium-binding proteins, calcium vector protein target isoforms, showed no differences between their estimated and predicted molecular masses, suggesting that they were not affected by the proteolytic activity that affected other functional categories. This indicates that the intact calcium-binding proteins identified in the protease inhibitor experiment are not showing significant changes in the expression

because of proteolytic breakdown that occurred during sample preparation, but are instead playing an active role in the cellular response to the stress posed by proteases.

Species-specific strategies to respond to environmental stress

In order to fully understand the responses of organisms to thermal stress and the resulting ecological consequences, we must integrate the study of molecular and cellular physiology with the study of whole-organism and population-level responses (Pörtner et al., 2006). Therefore, it is important to attempt to link the observed molecular mechanisms driving the cellular stress response with their larger ecological implications. Overall, the fact that *Ciona intestinalis* is able to respond more quickly to heat stress, as seen in both the high baseline levels of hsp70 and lower temperatures at which several proteins are up-regulated, in addition to the greater effect of degradation on significantly changing proteins in *C. savignyi*, suggests that *C. intestinalis* is more thermally tolerant than *C. savignyi*.

This thermal tolerance stems from the fact that *C. intestinalis* seems to invest more in maintaining protective mechanisms on a daily basis, and can launch a quick thermal

response at the first sign of stress. *Ciona savignyi*, on the other hand, waits longer to recruit a molecular response, which may result in more protein damage overall. These different strategies in dealing with thermal stress may also help to explain the wider distribution range observed in *C. intestinalis*.

Studies of congeneric blue mussel species (genus *Mytilus*) have also illustrated the importance of timing when comparing the heat shock responses of two closely related species (Tomanek and Somero, 2000). The present study only examined the heat shock response at a single time point, 16 hours after exposure to thermal stress, and therefore may be missing key pieces of the puzzle. Future studies would benefit by following the time course of the *Ciona* stress response over an extended period, and evaluating the changing response to thermal stress over time.

Future Directions

The present study may in fact raise more questions than it definitively answers, and therefore it provides a framework that will serve as the foundation for many future experiments. The questions posed concerning the methodology of 2-dimensional gel electrophoresis and the possibility of

proteolytic degradation as an artifact of sample preparation are an interesting outcome of this study. Additional studies should be conducted to conclusively determine whether or not protein samples are susceptible to degradation during the separation process. These studies would confirm a key assumption of proteomics: that significant changes in protein abundance or spot placement are in fact the result of a cellular response to treatment, and not solely based on differential protein breakdown occurring during sample preparation.

In order to further elucidate the ecological differences in thermal tolerance between the *Ciona* congeners, future studies should focus on exploring the organism-level effects of heat stress and comparing them between species. Once more is known about how increased temperature affects whole organism function, we can begin to fully understand the underlying molecular mechanisms of the cellular stress response in the context of species-level differences in thermal tolerance. Future studies should also explore the effect of seasonal acclimation on the thermal stress response of *Ciona* congeners. These studies would provide key information about how the molecular response to heat stress changes over time and across seasons.

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