THE PROTEOMIC RESPONSE OF SEA SQUIRTS (GENUS *CIONA*
CONGENERS) TO HYPOSALINITY STRESS

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ABSTRACT

The proteomic response of sea squirts (genus *Ciona* congeners) to hyposalinity stress

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The ascidian species *Ciona savignyi* and *C. intestinalis* are invasive species but show interspecific differences in their population response to hypo-saline stress associated with heavy winter-run off events that are predicted to become more frequent due to climate change. Despite an almost world-wide distribution, *C. intestinalis* seems to be more susceptible to hypo-saline stress than the geographically more limited *C. savignyi*. Given that the genomes of both species are fully sequenced, we were able to compare their proteomic response to both acute and chronic salinity to characterize the mechanisms that are responsible for setting tolerance limits to hyposaline conditions in these two congeneric species. For the acute hypo-saline stress experiment, we exposed each species to decreasing salinities, 100%, 85% and 70% full-strength seawater, for 6 hours followed by a 4-hour recovery at 100%. In the chronic salinity stress experiment, each species was kept at 100% or 85% with individuals removed for analysis during a 16-day time course. Organisms were dissected to remove the tunic, and 2D SDS-PAGE was performed to separate proteins and characterize changes in protein abundances. In the acute experiment, we determined 5% and 19% of the proteins to be significantly changing abundance in *C. savignyi* and *C. intestinalis*, respectively, due to the treatment effect. For both species in the chronic experiment, we determined over 40% of the proteins to be significantly changing abundance given the treatment, time, or interaction effect. Analysis of these proteins with MALDI TOF-TOF mass spectrometry has identified numerous proteins implicated in cellular stress responses, including energy metabolism (glycolysis, ATP & NADH production), cytoskeletal restructuring, and protein turnover, providing insights into the intense cellular restructuring that occurs following hypo-saline exposure.

Keywords: Proteomics, hyposalinity stress, *Ciona*

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I. INTRODUCTION

Hyposaline conditions result in extensive cellular readjustments due to the osmotic influx of water into cells. This increases cellular volume, requiring the cellular scaffold to be restructured (Pedersen et al., 2001). Unfortunately, the frequency and intensity of the precipitation events that cause these fluxes in salinity are expected to increase as a function of climate change (IPCC, 2007). As mean temperature of a region increases, the frequency of heavy to extreme rain events (40–100 mm daily amount) increases even without a net increase in average precipitation (Karl & Trenberth, 2003). Freshwater input has been implicated in setting vertical zonation patterns in species distribution along gradients from rivers to estuaries to intertidal communities (Witman & Grange, 1998; Rutger & Wing, 2006) and these communities are subject to massive die-offs following intense rains (Goodbody, 1961). The impacts of intense rainfall are found most dramatically in estuarine environments, where freshwater input can arise from watersheds covering great expanses of land.

In order to ascertain the impact of these extreme precipitation events on distribution ranges on the

distribution of marine invasives, we have chosen to investigate the physiological response to hypo-saline stress of three species of the genus *Ciona*. *C. intestinalis* and *C. savignyi* are two species of ascidians, sessile hermaphroditic invertebrate chordates, commonly known as sea squirts. Both species of *Ciona* are prevalent species in estuarine environments of harbors and marinas worldwide (Lambert and Lambert, 1998) and are an emerging model system for genotoxic and proteomic studies (Sato et al., 2003). They occupy a key phylogenetic position, the urochordates, meaning that they sit at the bridge between the invertebrate and vertebrate lineages. As such, their genome is representative of a simplified vertebrate genome, lacking the gene duplications present in higher vertebrates, which can make it difficult to characterize the molecular pathways in the cell (Dehal et al., 2002).

Proteomics has emerged as a powerful tool to describe cellular responses to osmotic challenge. It has been successfully used to track fluctuations in protein abundance in osmoregulatory organs of sharks (Lee et al., 2006; Dowd et al., 2008; Dowd et al., 2010) and fish (Ky et al., 2007; Cheng et al., 2009), but few studies have

applied proteomics to tracking global protein abundance in marine invertebrates.

Krogh's Principle describes that for any given biological question, there is a study system that is "created", or ideal to answer that question (Krebs, 1975). In this light, *Ciona* serves as a Kroghian system for studying the impact of extreme rain events on coastal marine ecosystems. As mentioned previously, *Ciona* has a number of features that make it an ideal study system for utilizing proteomics to track responses to hyposaline stress including, most importantly a global distribution along with a sequenced and annotated genome.

The goal of this study is to utilize proteomics to characterize protein abundance in *Ciona* following exposure to acute and chronic hyposalinity stress. This will better characterize the effects of hyposalinity on marine organisms and their cells control and acclimate to chronic osmotic fluxes. This research will also increase our understanding of the environmental limits of these marine invertebrates to respond to the impending changes to their ecosystem due to climate change.

This introduction will focus on describing the three main components of this project: the ecology and genetic

structure of the *Ciona* study system, the challenges posed by hyposaline stress, and the technologies that make up the foundation of a proteomics approach. For each of these sections, the relative literature will be summarized with the goal of illustrating a complete picture of the questions of this study and the tools employed to unveil the answers.

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

Ciona are a genus of solitary hermaphroditic ascidian, commonly known as sea squirts. Originating in northern Europe, they have spread to a nearly worldwide coastal distribution through anthropogenic means by ship hull fouling (Lambert & Lambert, 1998). In these new environments, *Ciona* are very successful at occupying free substrate, thereby excluding native species and overall reducing species richness in these new ecosystems. As a result, there is much interest in the ecology of *Ciona* in order to discover ways to eliminate it from regions where it is invasive (Blum et al., 2007).

Much of the ecology of *C. intestinalis* and *C. savignyi* is shared, but key differences will be specified in these sections. This section will discuss how the physiology and ecology of these organisms have contributed to their worldwide dispersal. It will also focus on how population-level genetic variation is contributing to the divergence between the sister species *Ciona intestinalis* and *C. savignyi* along with the development and evolution of two new subspecies of *C. intestinalis*.

Anatomy & Physiology

All species of adult ascidians are immobile and affixed to a substrate. They filter feed and respire by funneling water into an incurrent siphon through ciliary action, followed by prompt expulsion by “squirting” water back into the environment through a separate excurrent siphon angled away from the body. Through the pharyngeal basket, a structure that functions like a gill, *Ciona* is able to filter small particulate matter extracted from water through a mucus sheet, which serves as a filter with pores between 0.5-2 μm wide (Lambert, 2005). The mucus sheet covering the branchial filter carries an electrostatic charge to attract food particles, with pores small enough to eliminate bacteria from filtrate but large enough to permit organic molecules to pass, which are then driven down into the gut to be digested.

Adult *C. intestinalis* have an average filtration rate of 50 mL/hr/g wet weight (Kustin et al., 1974). Summing this across a group, large populations of *Ciona* are capable of filtering huge volumes of water every day. Petersen and Riisgard (1992) concluded that a particular Scandinavian population of *C. intestinalis* filters a volume equivalent to that of a shallow Danish fjord daily. Numerous ascidian

species are colonial, with individuals grouping together to share a single excurrent siphon, but each with its own incurrent siphon. *C. intestinalis* and *C. savignyi* are solitary species, and while numerous individuals may group together on the same substrate, they are fully independent of one another.

Adult individuals are protected from predation and physical damage by a tough outer covering called a test, or tunic. The tunic is made of cellulose, the basic building block of fiber in plants (Smith & Dehnel, 1971). Sea squirts are the only animals that possess a cellulose synthase gene, which is likely the result of a lateral transfer from a bacterium in the early urochordate lineage (Nakashima et al., 2004). The tunic is relatively thin when the individual is relaxed and extended, restricting settlement by juveniles of other species, but becomes considerably thicker as the organism contracts toward the substrate when sensing contact with an external object. Additionally, the openings on both siphons tighten and close in response to physical contact or extensive stress lending individuals a behavioral avoidance strategy, potentially limiting exposure to hypo-saline conditions in the external environment.

Abundance & Distribution

Originating in Northern Europe (originally named by Charles Linnaeus in 1767), *C. intestinalis* now inhabits sites worldwide, including Australia, Japan, South Africa and the Pacific and Atlantic coasts of North and South America (Lambert & Lambert, 1998; Lambert & Lambert, 2003; Therriault & Herborg, 2008) [Fig. 1]. *C. savignyi* is a Pacific species found in Japan and the west coast of North America (Lambert, 2003). The ability of *Ciona* to successfully invade so many different regions, from sub-arctic seas to temperate zones, the tropics and further south, is a testament to the broad adaptability that *Ciona* exhibit in these environments. This can be attributed to many factors, including their complex life cycle, competitive behaviors, and polymorphic genetic structure.

Figure 1. Global distribution maps of of *Ciona intestinalis* (A) and *C. savignyi* (B). Black designates invaded range while gray indicates native range or cryptogenic (unknown origin) range. See Evolution & Selection for more information. (Figure adapted from Therriault & Herborg, 2008. *C. savignyi* information taken from Lambert, 2003).

The global distribution of the *Ciona* congeners has occurred via the colonization of harbors through ship hull fouling over several hundred years (Lambert, 2005). This expansion occurred despite a limited ability to disperse during the larval stage. Dispersal in *C. intestinalis* is attained by the drifting of spawned, fertilized eggs and by free-swimming larvae. Development of fertilized eggs

occurs over 24 h, at which point tadpole larvae hatch and search for suitable substrate. Generally, these larvae persist in the water column for minutes or hours (but can live in the laboratory for up to six days) (Svane & Havaenhand, 1992). This results in local dispersal, therefore colonization outside of the bounds of the original population is typically rare (Petersen & Svane, 1995) resulting in populations are typically closed with either limited or no natural immigration or emigration.

The harbor environment provides protection for these sensitive species, with man-made structures greatly increasing the amount of surface area available for *Ciona* to colonize. In nature *Ciona* can be found on a wide variety of substrata, including rock, shells, and other inorganic matter but also on eelgrass of the genus *Zostera* and some algae (Dybern 1963). However, floating marina docks provide the ideal settling space, rising and falling with the tides, and as such are not subjected to tidal exposure. As such, non-indigenous ascidians often form the dominant portion of sessile filter-feeding invertebrates on harbor and marina structures (Lambert & Lambert, 1998).

When *Ciona* colonize a new site, the point of origin can be determined through temporal data of the species

present in harbors in the region. For example, in the harbors of southern California, it was concluded that *C. intestinalis* was first introduced in San Diego Bay from Europe. This conclusion was reached based on the fact that a high abundance of *C. intestinalis* were observed in San Diego which shared morphology with European counterparts (Ritter & Forsyth, 1917). *C. savignyi* was identified in 1913 on the Pacific coast of North America at Loring, Alaska (Nishikawa, 1991) with its first identification in southern California (Long Beach) in 1985 (Hoshino & Nishikawa, 1985; Lambert & Lambert, 1998). *Ciona* have since spread from San Diego to other harbors along the California coastline (as far north as San Francisco Bay) through ship hull fouling (Lambert & Lambert, 1998). The detection of *Ciona* in harbors is generally limited to the spring and summer booms in population growth. In a large harbor during summer, *C. intestinalis* populations may number in the millions, owing to the vast surface area available for colonization due to man-made structures. Massive die-offs following hypo-saline conditions brought on during winter rains dramatically lower detection probability (unpublished observations by Tomanek & Koman). Larvae produced by individuals deep enough in the water column (approximately 2 m below the surface) recolonize harbors in the spring

(MacGinitie, 1939; Lambert & Lambert, 2003). Despite the seasonal fluctuations in population size, it is believed that *C. intestinalis* and *C. savignyi* are now permanent residents in the harbors of southern California (Lambert & Lambert, 1998).

Demography

In *Ciona*, both lifespan and frequency of reproductive cycles are dependent

on environmental temperature (Dybern, 1965). For *C. intestinalis*, average lifespan is slightly less than one year in Mediterranean waters. However, in subarctic regions with temperatures below

Figure 2. Mean abundance of *S. clava* and *C. intestinalis* by sampling year. Stages of establishment, invasiveness, and nuisance are indicated for *C. intestinalis*. (reproduced from Ramsay et al., 2007)

5 °C, individuals can obtain lifespans of several years. The average number of generations per year increase with increasing average year water temperature, with subarctic species averaging less than one mating period per year and

Mediterranean or tropical species averaging 3-4 generations per year. This frequency of spawning events is considered a key factor enabling *Ciona* to achieve great population densities shortly after introduction to a new environment (Ramsay et al., 2008).

The pace with which *Ciona* invades and eliminates direct competitors was documented in a study by Ramsay et al. (2007), where invasiveness of *C. intestinalis* was tracked in an estuary at Prince Edward Island in Canada (Fig. 2). Within two years of the arrival of *C. intestinalis*, the population overtook and outcompeted the solitary ascidian species *Styela clava*. The authors reason that the temperature regulation of the ascidian reproductive cycle to be primarily responsible for the success of *C. intestinalis* over *S. clava*. In the waters around Prince Edward Island, the reproduction cycle of *C. intestinalis* starts when the water temperature reaches 8 °C, while *S. clava* starts when water temperatures exceed 12 °C. They estimate that this 4 °C gap in temperatures allows *C. intestinalis* to begin spawning a full month before *S. clava*, giving it a significant recruitment advantage. Temperature may control the timing and frequency of reproductive events, but maximal reproductive

output is linked closely to the abundance of food production in the habitat allowing offspring to mature while food availability is the highest. For Mediterranean individuals (including those in southern California), this occurs in late summer/early autumn.

The most common competitors of *Ciona* for food in the water column are mussels and other bivalves, though *C. intestinalis* appear to have the advantage. *C. intestinalis*, which often grow on harbor structures crowded by other sessile filter feeders such as mussels, are capable of attaining lengths up to 15 cm enabling them to extend further into the water column than their non-ascidian neighbors (Lambert & Lambert, 1998). Additionally, the mucus net of *Ciona* allows it to filter smaller particles from water than mussels and other competitors, enabling *Ciona* to extract more food from environments with low concentrations of food particles (Petersen, 2007). In San Francisco Bay, Blum et al. (2007) demonstrated that on PVC panels lowered into the water column, abundance of *C. intestinalis* was negatively correlated with the abundance of other native species. Additionally, overall species richness was negatively correlated with *C. intestinalis* abundance. As seen in the

Prince Edwards Island example (Ramsay et al., 2007), this suggests that in environments where *C. intestinalis* is invasive, recruitment by *C. intestinalis* may be more successful due to timing of reproductive output, more efficient foraging (filtering), and a displacement of suitable substrate for other species. One explanation for this reduction in species richness lies in the outer tunic of *Ciona*. While native species like mussels do indeed take up free substrate, their shells serve as additional settling substrate for other species. Contrarily, the thin, flexible tunic of *Ciona* resists fouling by other species with the exception of certain algae.

Within harbors, *Ciona* are subject to the effects of pollution. Perhaps due to their incredible filtration capabilities, *Ciona* are capable of removing and sequestering heavy metals. Even as *Ciona* remains an invasive competitor to native harbor biota, they may have the positive effect of removing heavy metals and other toxic pollutants from harbors and could potentially be used as biological monitors for pollutants in harbors worldwide (Monniot et al., 1986). Other stresses to which *Ciona* are subject throughout the year are temperature and salinity stress. Low water temperature during the winter combined

with lowered salinity due to rain and snow run-off reduces abundance of *C. intestinalis* (Carver et al. 2006). It is likely that the severity of winter environmental conditions govern the predominance of summer breeding in *Ciona* populations.

Mating System

Ciona intestinalis is widely used as a model system in developmental biology, so while much is known about the mating system of this species, *C. savignyi*'s mating system is less defined. While there may be differences in the timing of mating stages between the two, the method of mating is expected to be similar.

Ciona are hermaphroditic solitary broadcast spawners, conserving energy and longevity of sperm by delaying sperm activation prior to contact with the egg (Yoshida et al. 2003). Individuals are simultaneous hermaphrodites; both sperm and eggs are produced at the same time. Individuals are self-sterile, and the vitelline coat around eggs serves as a molecular barrier to fertilization by self-sperm (Murabe & Hoshi, 2002). Hybridization between *C. intestinalis* and *C. savignyi* has not been observed in the field (Byrd & Lambert, 2000; Berna et al., 2009).

Mating typically occurs during the summer, with a late summer peak. A study by Bernt Dybern (1965) sampled the number of *C. intestinalis* larvae in the water column monthly over the course of three years. This study found that larvae were present from May through September, with a peak in abundance during May. While it is believed that the breeding season lasts through the summer, the actual peak of breeding is likely to vary from population to population.

Spawning in the field can occur at any time during the day (Svane & Havaenhand, 1992), however, in laboratory conditions spawning is controlled by light (Lambert & Brandt, 1967; Yamaguchi, 1970). Experiments by Lambert & Brandt (1967) elucidated the role of light in controlling spawning in *C. intestinalis*, showing that light induced spawning in 78.3% of the individuals tested following a dark acclimation period, with gametes being shed approximately 4 minutes following exposure to light. Additionally, the testing of different wavelengths of light to induce spawning was identical to the absorption spectrum of cytochrome c, suggesting that this may be the chromophore involved in triggering the signal to spawn.

It is suggested that this sensitivity to light allows the larvae to hatch during daylight to better locate shaded conditions. Tadpole larvae are negatively phototactic (light-avoiding) and generally choose shaded regions for settlement, minimizing exposure to solar radiation and algal overgrowth as they mature (Svane & Young, 1989). These regions are typically the vertical or lower surfaces of rocks, but in harbors can be the underside of boats, docks or sheltered harbor pilings (Millar, 1971).

Sperm is released into the water column and fertilizes eggs. Fertilized eggs are also slightly adhesive, making them capable of attaching to substratum (Svane & Havenhand, 1993). Some of the egg-containing mucus strings will be released intact, at which point, if they land on suitable substrate, the larvae will hatch and settle in relatively close contact with one another. As a result, larva hatch close to the adults and are more likely to adhere to local substrates which leads to the formation of dense lawns of solitary individuals, restricting space for competing species to settle.

The life cycle of *C. intestinalis* from embryonic development to sexually mature adult is short—approximately 1-2 months (Corbo et al., 2001). Once an egg

is fertilized, it undergoes a rapid period of embryogenesis (~18 h.) to become a free-swimming, non-feeding tadpole (Dehal et al., 2002). Tadpole larvae are relatively simple, consisting of only six basic tissues. The tadpoles must then attach to a substrate, at which point they undergo metamorphosis into the more recognizable adult form capable of feeding.

Larvae and juveniles are subject to predation from a variety of sources, including gastropods, crabs, and fish. Petersen and Svane (1995) showed that a native population of *C. intestinalis* in a large fjord in Denmark were most likely limited in dispersal outside of the original population boundaries by predation at the larval stage. They demonstrated that while the hydrodynamics of the fjord would distribute larvae and fertilized eggs to all parts of the fjord, there was no larval recruitment outside of the original population boundary, and as such, larval predation was likely the major factor restricting dispersal. This led them to reason that predation at the egg or larval stages by a species of jellyfish, *Aurelia aurita*, was the limiting factor in dispersal.

Population Level Genetic Variation

As discussed above, dispersal of *C. intestinalis* is typically limited to the bounds of the original parent population. Recruitment and settlement outside of the established population is rare. Anthropogenic means of transport have enhanced the spread of *Ciona* to harbors and estuaries around the world. In closed populations, the propensity for variation between source populations and founding populations is large.

The publication of the sequenced and annotated *C. intestinalis* genome (the seventh animal genome sequenced!) in 2002 has provided insights into population genetics for this species (Dehal et al., 2002). The sequencing revealed that the *C. intestinalis* genome is comprised of nearly 16,000 protein coding genes. Likewise, the *C. savignyi* genome is 174 Mb long with roughly the same number of protein-coding genes as seen in *C. intestinalis* (Small et al., 2007). Overall, the *C. intestinalis* genome is about 20 times smaller than the human genome.

Following the sequencing of the *C. intestinalis* genome, a comprehensive expressed sequence tag library and database was developed (Satou et al., 2005), a library of the mRNA transcripts isolated and reversed transcribed from

individuals. Such expressed sequence tag libraries are useful for determining the proteins that are expressed in an individual. One of the details that was gleaned from the genome was the incredible level of single individual polymorphism, 1.2% of nucleotides differing between alleles. This is nearly 15 times the amount of individual polymorphism found in humans. For contrast, *Ciona savignyi* has a single individual polymorphism of 4.5% (Vinson et al., 2005). The high level of polymorphism in *C. savignyi* is likely the result of a large effective population size, along with the continuous level of anthropogenic introductions of new individuals (Small et al., 2007).

Evidence suggests that the polymorphisms arise from its high recombination rate (Kano et al., 2006). It is likely that this same observation applies to populations of *C. intestinalis*, along with other marine invertebrates that display high levels of polymorphisms. Perhaps a high level of polymorphism or heterozygosity in an invasive species facilitates the exploration of new environments. A recent study by Aguirre & Marshall (2011) demonstrated that *C. intestinalis* populations with greatest sibling diversity had greater performance (measured by metamorphic success, post-metamorphic survival and metamorphic size), suggesting

that high levels of heterozygosity in a population leads to greater potential for resource partitioning and less competition between individuals.

Evolution & Selection

The sequencing of both *Ciona* genomes has opened the doors for genetic comparisons of populations around the world. *Ciona* belong to Class Ascidiacea, which is part of the urochordate, or tunicate, lineage and has recently supplanted cephalochordates as the closest living relative to vertebrates (Delsuc et al., 2006). As such, they sit at a critical position on the evolutionary tree bridging the gap between chordates and early vertebrates, and the ascidian tadpole has emerged as a model system for studies in vertebrate developmental biology (Dehal et al., 2002; Satoh et al., 2003).

A study by Silva and Smith (2008) comparing polymorphism in numerous invasive and native species in the Santa Barbara and Ventura harbors, including *C. intestinalis*, showed a negative correlation between the age of the population and its calculated level of heterozygosity over time. The authors suggest that invasive species originally have high levels of polymorphism, with alleles advantageous to the new

environment becoming stabilized over time, lowering polymorphism. Nevertheless, the high polymorphism in *C. intestinalis* likely allows the population to cope with environmental perturbations unique to the new habitat. This high polymorphism likely helps *Ciona* to counter the effects of limited dispersal and closed populations. Limited dispersal is often detrimental because it effectively reduces the amount of genetic variation in a population and would potentially lead to a decrease in overall fitness (Svane & Havenhand, 1993). The high degree of polymorphism may help counter the effects of limited dispersal by retaining a high degree of variability within a closed population lacking introductions of individuals from other populations (Svane & Havenhand, 1993).

The *Ciona* genome is roughly 5% the size of the human genome. This offers advantages in the use of *Ciona* for genetic studies, as its genome is essentially a simplified version of a vertebrate genome, possessing a similar set of genes, but lacking the numerous gene duplication events and redundancies that lead to complexity in vertebrate genomic data sets (Dehal et al., 2002).

The modern global range of *C. intestinalis* provides adequate separation of populations to promote divergence. A massive morphological and genetic study by Caputi et al. (2007) has led to the conclusion that *C. intestinalis* is exhibiting subspecies-level divergence. It is accepted that there are two subspecies, *C. intestinalis* Species A

Figure 3. Geographical distribution of *C. intestinalis* sp. A (pink) and sp. B (pale blue). Empty ovals indicate uncharacterized records of *C. intestinalis* populations (Caputi et al., 2007).

and Species B.

Sp. B is limited to the North Atlantic, while Sp. A has a wider spread, inhabiting the Mediterranean, Southern Atlantic, and Pacific regions (Fig. 3). The two subspecies have limited morphological distinctions, noted primarily in differences between patterns of pigmentation of the vas deferens; their distinction was supported mainly by genetic differences. The two subspecies appear to be sexually isolated and do not interbreed in the laboratory nor were hybrids found at the mouth of the English Channel, where the two overlap.

Studies of the sequence homology between three populations of *C. intestinalis*, one from Britain, one from California, and another from Japan, identified significant divergence between Pacific and Atlantic populations (Suzuki et al., 2005). There were minimal differences between individuals from California and Japan, but 13% divergence between the British population and the Pacific populations. This is notable as the divergence between *C. intestinalis* and its sister species *C. savignyi* is 15%. Suzuki and his team successfully produced hybrids between the Japanese and British individuals that grew to adulthood, but they did not test the reproductive viability of the hybrids. The

genetic separation of these two subspecies was confirmed by great differences in the mitochondrial genomes of the two (Ianelli et al., 2007).

A study by Nydam and Harrison (2010) genetically tested a number of loci between Sp. A and Sp. B and a third species, *Ciona roulei*. Their findings confirmed the peculiar genetic separation of Sp. A and Sp. B, despite morphological similarity. Surprisingly, they noted that Sp. B and *C. roulei* were genetically indistinguishable based on the loci tested, yet these two species are morphologically distinct. No cross-fertilization experiments have been performed to determine whether Sp. B and *C. roulei* are capable of interbreeding. They also addressed the question of polymorphism, demonstrating that Sp. B has higher heterozygosity than Sp. A. The authors believe that high levels of polymorphism in these species, especially Sp. B as the native and more restricted subspecies, cannot be explained by large effective population size alone. This idea counters what has been discussed in the above sections.

Such divergence between the two subspecies of *C. intestinalis* is notable, considering that the non-native Sp. A likely had its first founding individuals outside of

the North Atlantic roughly 1000 years ago with the rise of global shipping. The emergence of these two reproductively isolated subspecies within such a short time period begs the question: How fast does *C. intestinalis* evolve? A study by Berna, Alvarez-Valin, and D'Onofrio (2009) calculated genome-wide interspecies distances between vertebrate species representative of different classes, along with *C. intestinalis* (and its sister species, *C. savignyi*). This analysis concluded that *C. intestinalis* evolves at a higher rate than vertebrates (by Tajima's relative rate test), with ~85% of *Ciona* genes evolving at significantly faster rates than their vertebrate counterparts.

The authors cite Petersen et al. (2004) to explain the remarkable rate of evolution in ascidians, where the genome duplication phenomenon comes to the forefront. Two rounds of whole genome duplication took place between the ascidian lineage and the modern vertebrate lineage. Traditionally, it has been believed that more duplication would permit faster evolution because one gene copy would be more free to accept mutation than a gene for which only one copy is present in the genome. This study seems to go against this assumption. Petersen et al. (2004) believe that vertebrate

genomes evolve more slowly precisely because they have duplicate genes. More genes means more proteins leading to higher numbers of protein-protein interactions. This implies that a higher percentage of the amino acid sequence would be key to governing these interactions, and mutations in these sequences would be strongly selected against. *Ciona*, in theory, with their simple genome (containing minimal gene duplications), have limited protein-protein interactions and would therefore be more free to accumulate mutations in their genes, as their proteins would have fewer critical sequences dedicated to protein-protein interactions.

Conclusion

Ciona have managed to colonize harbors, marinas, and estuaries worldwide through their adaptability to tolerate a wide range of stressors, their ability to outcompete other organisms for both space and resources, and the timing of their breeding season. Despite all this, their populations are typically isolated and have very limited dispersal outside of the original population. Their worldwide distribution has been achieved through anthropogenic means, through fouling on ship hulls, another reminder of the human impact on marine environments.

There appear to be evolutionary consequences from such a worldwide distribution, with European Atlantic populations exhibiting genetic divergence from Pacific populations. The identification of *C. intestinalis* Species A and Species B has been made possible through breakthroughs in genomics, specifically the sequencing of the *C. intestinalis* genome. However, the development of these two subspecies is a consequence of the high level of polymorphism in *Ciona* populations and a potentially faster rate of mutation within individuals of a population. This divergence is an exciting prospect, akin to watching evolution happen in real-time, but more evidence is needed to determine whether these two subspecies are reproductively isolated from one another.

The simple genome (relative to vertebrates) of *Ciona* makes this genus an ideal study system. The *C. intestinalis* genome is considered to be one of the best annotated of all animal genomes (Satoh & Levine 2005). *Ciona* genomes resemble vertebrate genomes for they contain a similar functional set of genes, but they are simpler as they exhibit less redundancy, possessing less than half the number of protein-coding genes seen in vertebrates. As a result, *Ciona* at a genetic level provide a more useful

model for genomic studies than their more complex vertebrate cousins. Since *Ciona* lends itself so well to study, it will hopefully surrender the knowledge that will help limit its spread into pristine marine environments and aid in the rehabilitation of habitats where it has already successfully invaded.

Hyposalinity Stress

Ecological & Physiological Impact

In the open sea, salinity declines from 37‰ at the tropics to 33‰ at the poles, varying little. As a result, most marine organisms in the open ocean are stenohaline and do not experience salinity fluctuations. On the coast, however, salinity can vary widely in regions where freshwater and seawater mix such as fjords, estuaries, and river mouths. Rainwater run-off often yields a low-salinity layer which can both govern species distributions in coastal marine ecosystems and act as a challenge to survival among sessile marine animals.

Witman & Grange (1998) describe a rocky subtidal community in the southwestern fjords of New Zealand where heavy rains create a low-salinity layer the depth of which (2-10 meters) governs vertical zonation patterns among sessile invertebrate communities. Within the surface layer persist organisms tolerant to freshwater while species tolerant only to seawater persist deeper beneath the layer. Predator surveys demonstrated that predatory sea stars and urchins moved up with the low-salinity layer into shallows previously buffered from predation during the heavy rain season. Intertidal ecosystems that receive regular

influxes of freshwater from natural or anthropogenic sources display sharp declines in species abundance and diversity compared to neighboring areas that maintain salinities close to that of full-strength seawater (Rutger & Wing, 2006). When coastal marine communities are comprised of species intolerant of freshwater, massive die-offs of individuals occurs following freshwater influx. In the case of a harbor in Jamaica, tropical rains dramatically increased the flow of rivers into the harbor killing off most animals at the depth of 60 cm, with solitary and colonial ascidians being the most affected (Goodbody, 1961). Only the ascidian species with the toughest tunics had individuals that survived within the low-salinity layer.

When coastal waters begin to turn brackish, sessile organisms are the most affected as they cannot move to escape the decline in salinity. Stenohaline osmoconformers such as mussels and ascidians employ behavioral strategies to avoid exposing their tissues to hyposaline waters. A study by Shumway (1978) demonstrated that *C. intestinalis* reduce squirting rate as salinity gradually decreases, eventually tightly closing their siphons when salinity dropped below 60% of full strength seawater. When salinity declines abruptly, squirting activity immediately ceases

and siphons shut. Similar behavior is seen in mussels (*Mytilus edulis*) where they close their shell valves in response to declining salinity (Davenport, 1979). This symmetry of behavioral patterns to salinity fluctuation allows these animals prioritize high-energy activities (e.g. feeding and O₂ uptake) during periods of high salinity (low potential stress) and minimize energy consumed during periods of low salinity (high potential stress) (Davenport, 1985). Despite siphon closure, ascidians exposed to hyposaline media over several hours are subject to influx of water via osmosis from extracellular fluid into cells, increasing volume. A study by Sims (1984) on individuals of the solitary tunicate genus *Styela* showed significant increases in relative weight gain over the course of exposure to 50% seawater (between 18.4% and 39.0% depending on species), with further increase limited only by the inflexible tunic. The author hypothesizes that these observed differences in weight gain govern the ability for different species to colonize and survive areas with chronic low salinity levels.

Cell Volume Sensors

The phenomenon of cell volume fluctuation under hypoosmotic conditions is governed by the composition of

extra- and intracellular fluid. Outside of the cells nearly all osmolarity is due to inorganic ions while within the cells osmotic pressure is generated (<60-70%) by organic molecules too big to breach the cell membrane but small enough to create osmotic pressure (Davenport, 1985). These organic osmolytes are typically non-essential amino acids such as glycine, alanine, proline, glutamate, taurine, aspartate and glutamine (Gilles, 1978). Marine animals exposed to dilute seawater have extracellular fluid that becomes reduced in concentration followed by an inward flow of water from dilute extracellular fluid into the relatively more concentrated intracellular fluid. Since the cell membranes of animal cells are flexible, swelling occurs (Davenport, 1985).

Cell volume is returned to its normal state by a series of processes called regulatory volume decrease (RVD), which trigger the loss of osmotically active solutes. Before RVD can be instigated, the cell must be able to monitor its size and detect fluctuations in cell volume. One of the proposed method by which cells detect volume changes is by sheer mechanical force on the lipid bilayer via mechanosensitive channels, embedded within the membrane and sensitive when the membrane expands following the influx of fluid (Sucharev & Anishkin, 2004). There are

two main types, one where changes in the force generated by the bilayer open the channel and the other where the channel is tethered to an immobile extracellular matrix or intracellular cytoskeletal proteins. In the latter case, force is placed on the channel through the tether proteins. Once the channel is open, osmolytes can exit the cell into the extracellular fluid. The lipid bilayer may also contribute to volume signaling through the same tethering to cytoplasmic proteins. As the bilayer expands, force is placed on the cytoplasmic protein via the tether, changing protein conformation, potentially exposing sites for post-translational modification and/or altering enzyme activity allowing the signal to propagate.

Another sensor of cell volume may be intracellular ionic strength. As cell volume increases, the intracellular ionic strength decreases as water dilutes the cytoplasm. Studies have shown that the $I_{Cl,swell}$ channel, a channel implicated in organic osmolyte efflux, requires more or less swelling to become active depending on the intracellular ionic strength. Initial ionic strength acts as a set-point, and a subsequent reduction in ionic strength triggers its activation (Voets et al., 1999).

Another signal of changing cell volume is macromolecular crowding, initially proposed by Zimmerman

and Harrison (1987), where an increase in volume would free up space to increase the diffusion of reactants and alter the frequency of association and dissociation events between macromolecules and substrates (Choe & Strange, 2007). A study by Colclasure and Parker (1992) demonstrated that volume-regulated ion transporters in dog red blood cells were regulated by the concentration of intracellular proteins and not the actual change in volume.

Regulatory Volume Decrease

Sensing the volume change is just the beginning of the cell's recovery. These initial sensor mechanisms pave the way to a host of signaling pathways that help the cell recover and adapt to hyposaline conditions. The process by which the cell returns itself to its original volume after swelling is named regulatory volume decrease (RVD). The action of RVD is the product of the activation of numerous signaling pathway components, including kinases, eicosanoids, intracellular Ca^{2+} , and the Rho-GTP proteins associate with the cytoskeleton. All these pathway components eventually activate channels and transporters that remove excess solutes from the intracellular environment.

Predominant signal transduction proteins in RVD are the kinases. Specifically, members of the Src kinases, a class of cytoplasmic tyrosine kinases that have been implicated in regulatory volume decrease pathways. Mouse red blood cells, knockout for the Src kinases Fgr and Hck, displayed threefold greater K-Cl cotransporter activity, suggesting that Fgr and Hck inhibit a phosphatase that activates the cotransporter (De Franceschi, 1997). Additionally, another class of kinases, the extracellular-signal-regulated kinases 1 & 2 (ERK1/2) have been shown to be activated by hypotonicity (Pasantes-Morales, 2006). Many of the activities of these and other types of kinases seem to be specific to both species and cell type, and the actual mechanism(s) by which these kinases exert their effects in the cell have yet to be uncovered (Choe & Strange, 2007).

Another form of signal transduction in RVD uses eicosanoids such as arachidonic acid, generated from hydrolysis of cellular phospholipids by enzymes with phospholipase A2 (PLA₂) activity. Arachidonic acid is further metabolized by lipoxygenases into leukotrienes, which have been shown to regulate the activity of organic and inorganic ion efflux pathways (Jakab et al., 2002). Other metabolites of arachidonic acid (e.g. prostaglandins)

have been linked to regulating a number of different inorganic ion channels and transporters (Hoffman et al., 2009).

Intracellular Ca^{2+} , one of the most common second messengers, has been observed to increase during swelling, but the precise mechanisms that these changes trigger in RVD are unclear (Lang et al., 1998). These changes in intracellular Ca^{2+} are governed by transient receptor potential (TRP) channels, which draw in Ca^{2+} from the extracellular environment. Removal of extracellular Ca^{2+} or inhibition of TRP channels in human keratinocytes eliminates the RVD response in those cells (Becker et al., 2005).

Lastly, changes to the cytoskeleton are also characteristic of RVD. Rho GTP-binding proteins, small signaling proteins that are inactive when GDP-bound and active when GTP-bound, have key roles in regulating the polymerization of the actin cytoskeleton (Choe & Strange, 2007). In one case, overexpression of one type of Rho GTP-binding protein, RhoA, resulted in an increase in the rate of RVD in NIHT3T cells (Pedersen et al., 2002).

The cytoskeleton, the structure of which is governed by these Rho GTP proteins, seems to link to many of the RVD signaling pathways previously mentioned, beyond just the

aforementioned stretch-activated ion channels (Pedersen et al., 2001). For kinases, a number of protein kinase C (PKC) isoforms are translocated to and regulated by the F-actin cytoskeleton (Blobe et al., 1996) when PKC is gactivated by hypo-osmotic stress in Erlich ascyte tumor cells (Larsen et al., 1994). Phospholipase A₂ is translocated to the cytoskeleton upon its activation (Janmey, 1998) and phosphatidyl inositol 4,5 bisphosphate (PtdIns(4,5)P₂) increases during osmotic swelling in EATC, interacting with gelsolin and profilin to enhance F-actin disassembly (Hoffmann & Pedersen, 1998). This action may be key in the disassembly of the cortical actin cytoskeleton, requisite for the transport of vesicle-mediated insertion of osmolyte transport proteins into the cellular membrane (Pedersen et al., 2001; Downey et al., 1995).

Global Warming and Rain Events

As seen with regulatory volume decrease, most cells have volume sensing and signaling pathways that help the cell adapt and counter the effects of hyposaline conditions. Unfortunately, the frequency and intensity of precipitation events that cause these fluxes in salinity are expected to increase as a function of climate change

(IPCC, 2007). As mean temperature of a region increases, the frequency of heavy to extreme precipitation events (40–100 mm daily amount) increases even without a net increase in precipitation (Karl & Trenberth, 2003). The impacts of intense rainfall are found most dramatically in estuarine environments, where freshwater input can arise from watersheds covering great expanses of land. The Chesapeake Bay, which has a watershed across five states, is expected to have change of up to $\pm 7\%$ given a change in annual Susquehanna River flow of 10% (Najjar et al., 2010). Analysis of Washington State shows a projected increase in winter-flow with a decrease in summer-flow (Elsner et al., 2010). Studies on the precipitation of the California coast suggests increased precipitation in some regions (Groisman et al., 2005). Combine the impact of hyposalinity stress on coastal ecosystems with other stressors enhanced by climate change (e.g. ocean acidification and heat stress), and marine invertebrate communities will likely have to test the limits of their cellular stress responses in the coming decades.

Proteomics

The proteome encompasses the entire set of expressed proteins in an organism, and is subject to modification by the dynamic changes that occur in the internal and external environment (Lopez, 2007). Proteomics provides the techniques to directly track and identify the abundances of most proteins within an organism's proteome in response to specific conditions.

Studying the proteome of an organism offers particular advantages over merely studying the transcriptome. This is primarily because not all mRNA transcripts are translated into proteins, with mRNA to protein Pearson correlation coefficients ranging between 0.46 and 0.76 (Hack, 2004). An advantage of transcriptomics is that the technology is relatively simple and easily automated (Abbott, 1999). However, results from genomics studies are limited by the aforementioned mRNA-to-protein correlation issue and that one mRNA can give rise to multiple protein isoforms with a myriad of post-translational modifications (Abbott, 1999). As a result, transcriptomics can tell us which genes are actively transcribed but not directly determine if protein abundance changes (Feder & Walser, 2005).

Proteins are ideal candidates for measuring the impact of stress on the cell. They are sensitive to environmental perturbations in that their relative abundance within the cell can change dynamically with the severity of stress (Hochachka & Somero, 2002) and most stress-induced proteins do not degrade for days to several weeks, enhancing detectability of their signal (Tomanek & Somero, 2000). Proteins have been successfully used to describe the cellular stress response in numerous studies (Feder & Hofmann, 1999; Tomanek, 2002; Kultz, 2005; Tomanek & Zuzow, 2010; Serafini et al., 2011; Campanale et al. 2011).

Proteomics is a holistic approach that aims to provide a comprehensive quantitative understanding of all the proteins in a biological system (Bantscheff et al., 2007). Like most other systems biology approaches, it is discovery-based as opposed to hypothesis-driven, yielding a collection of testable hypotheses regarding the system under investigation (Tomanek & Zuzow, 2010). Comparative proteomics seeks to look at similarities and differences of proteomic responses to stress within closely-related organisms adapted to different environmental conditions or exposed to different environmental stressors (Tomanek & Zuzow, 2010).

While there are a number of proteomic workflows to choose from, all are comprised of three main steps: separation, quantification, and identification of proteins. The most widely-used forms of the protein separation in proteomic studies are gel-based, wherein proteins are extracted from tissues and separated by isoelectric point and molecular weight in two-dimensional sodium-dodecylsulfate-polyacrylamide gel electrophoresis (2D-SDS-PAGE), followed by the use of gel image analysis software that determines which proteins are significantly changing abundance between treatments (Berth et al., 2007). 2D-gel-based proteomics offers the advantages of being able to produce high volumes of gels in parallel fashion and a number of easy-to-use gel image analysis software programs available in the market (Berth et al., 2007). Gel-free approaches, such as high pressure liquid chromatography (HPLC), reverse phase chromatography (RPLC), ion exchange chromatography (IEC), and size exclusion chromatography (SEC) are often used to feed into or enhance gel-based approaches to make up for its inherent limitations, such as underrepresentation of hydrophobic or extremely acidic/basic proteins and low abundance proteins (Lovric, 2011). 2D-gel-based approaches are most common in bottom-

up analyses, where one is interested in looking at the total proteome, and will be the focus of this section.

Protein Separation

Three main steps encompass the separation of proteins: sample preparation, isoelectric focusing (1st Dimension), SDS-PAGE (2nd Dimension).

The goal of sample preparation is to simplify the complex cellular environment down to raw protein. A successful sample preparation technique should make the following criteria: (1) as much of the original protein as possible should be retained, (2) contaminants such as salts, lipids, and nucleic acids must be removed as far as possible, (3) post-translational modifications should be retained, and (4) resulting sample composition should be compatible with subsequent procedures in the proteomic workflow (Lovric, 2011).

During sample preparation protein must be freed from the cellular environment, solubilized and denatured. This is achieved by homogenizing tissue in a buffer containing a neutral chaotrope, in the case of our protocol, urea or thiourea (Görg et al., 2004). A high concentration of urea is sufficient to lyse cells even without mechanical force and disrupt the water shell around proteins to cause them

to denature and lose their structure. Also in this buffer is a zwitterionic detergent, like 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), which can disassemble lipid membranes and free membrane-bound proteins into the solution (Görg et al., 2000). Carrier ampholytes maintain optimal pH of the solution for maximal protein solubility while reducing agents such as dithiothreitol (DDT) or dithioerythritol (DTE) eliminate disulfide bridges to dissociate proteins from one another (Görg et al., 2000). Denatured, solubilized proteins are sensitive to the actions of proteases, so sometimes protease inhibitors can be added to the mix. Protease inhibitors are generally expensive and can lead to difficulties in accurately determining protein concentration (many inhibitors are small peptides) and are unnecessary if the chaotropes present in the buffer are high enough to denature the proteases themselves.

The resulting mixture is a complex solution of all the components of the cell: lipids, nucleic acids, salts and other osmolytes, and our proteins of interest. The protein solution can be cleaned up of these unwanted substances through precipitation with trichloroacetic acid (TCA) and acetone, followed by resolubilization in salt-free rehydration buffer (Görg et al, 2000).

Before proceeding to 2D-GE, the protein concentration of each sample must be determined in order to control the protein load used. A number of kits exist to determine protein concentration, but few are compatible with the type of solutions contained in the homogenization buffer used for sample preparation. The 2D-Quant Kit (GE Healthcare) was developed to circumvent those issues and allows for quantification through the selective binding of peptides to copper ions.

At this point a number of pre-fractionation techniques can be applied, if needed. Unlike with nucleic acids and PCR, there is no amplification step for proteins, so prefractionation techniques work to reduce the complexity of the sample and enrich certain groups of proteins from the rest (Görg et al., 2000). Some of the prefractionation techniques on the market include the Rotofor (Biorad, Hercules, CA), which utilizes carrier-ampholyte focusing to separate proteins into individual compartments and the Gradiflow (Gradipore), an electrokinetic membrane apparatus that can purify proteins by mobility, pI and size (Righetti et al., 2003). Additional column fractional techniques exist to isolate proteins by post-translational modification or to enrich low-abundance proteins within a sample.

The first dimension of 2D-GE consists of isoelectric focusing, the separation of proteins by pI along a stable pH gradient. The pI, or isoelectric point, is the pH at which the net charge (governed by the various charged amino acids that make up the protein) is zero. Because of the variation of amino acid make-up of the polypeptide chain, this intrinsic charge is unique for each protein. Isoelectric focusing is typically carried out on ready-made immobilized pH gradient (IPG) strips, which yield more consistent separation than laboratory-made IPG gels (Görg et al., 2000). IPG strips are available in multiple lengths corresponding to the size of the 2nd-Dimension SDS-PAGE gel (11cm, 18cm, 24cm) and wide (e.g. 3-12), medium (e.g. 4-7) and narrow (e.g. 4.5-5.5) pH ranges (Görg et al., 2004). The chosen pH range of a strip greatly impacts the resolution of proteins, as the narrower the pH range the fewer proteins will be visible on a gel. Wider pH ranges retain more proteins but with less resolution, while narrow pH ranges lose more protein but greatly resolve proteins with pI's within the narrow pH range. Medium range IPG 4-7 strips are the most commonly used, as most cellular proteins have pI's within this range (Righetti et al., 1983). During IEF, proteins are loaded onto an IPG strip, and a charge is run through the strip, with the cathode (+)

on the acidic end. Proteins at a region of the strip that is more acidic than their pI possess a net positive charge and migrate away from the cathode toward the basic end of the strip. As it approaches the region of the strip that matches its pI, individual positive charges are replaced by negative charges until the protein has a net charge of zero and stays in place in the strip (Alberts et al., 2007). The opposite occurs for proteins that start at region of the strip that is more basic than their pI.

Following IEF, the IPG strips must be equilibrated to ease transfer of proteins from the first dimension to the second dimension separating gel and allow the proteins to fully bind to SDS. Incubation of strips for 15 minutes in an equilibration buffer of Tris-HCl (pH 8.8) with sodium dodecylsulfate (SDS), dithiothreitol (DTT) (reducing agent), and urea and glycerol (reduce protein association with the charged groups present in the IPG strip gel) followed by a second incubation in the same buffer with DTT replaced by iodoacetamide (IAA) (acetylates reduced sulfhydryl groups generated from DTT) is sufficient to prepare proteins for the 2nd Dimension (Görg et al., 2004).

The 2nd Dimension is SDS-PAGE, the separation of proteins by their molecular weight. This is carried with the equilibrated IPG strip placed on top of a

polyacrylamide gel of similar length as the strip. A charge is established and proteins, overwhelmingly negatively charged through their binding to SDS, flow down the gel towards the positive cathode. Polyacrylamide is formed of a matrix of cross-linked acrylamide subunits, forming a net or sieve that the proteins must migrate through on their way toward the cathode (Alberts et al., Mol Bio Cell, p. 517). Large molecular weight proteins navigate the polyacrylamide matrix more slowly than smaller proteins, resulting in larger proteins near the top of the gel and smaller proteins near the bottom. Pore size of the polyacrylamide gel can be modified by controlling the percentage of acrylamide used when casting the gels.

Gel Staining

After the 2nd dimension, the gels must be stained in order to visualize the proteins within the gel. Protein stains are either calorimetric (e.g. colloidal Coomassie Brilliant Blue, silver stain) or fluorescent (e.g. SYPRO Ruby, Flamingo). Stains vary in their sensitivity and dynamic range, but all should be compatible with mass spectrometry if one wishes to identify proteins in the gel. Colloidal Coomassie Brilliant Blue (CBB) is an organic dye that complexes with arginine, lysine, tyrosine and

histidine residues (Ball & Karuso, 2007). It can detect down to ten nanograms per spot but with a wide dynamic range of about 2.5 orders of magnitude. Silver stain involves the binding of silver ions to the peptide core of the protein followed by the precipitation of metallic silver (Ball & Karuso, 2007). It is among the most sensitive stains available, but still has a low dynamic range and has limited compatibility with mass spectrometry, though newer recipes overcome the compatibility issue but with a loss of sensitivity (Yan et al., 2000). SYPRO Ruby (Invitrogen) is a fluorescent stain that, like CBB, associates with cationic residues but is more sensitive than CBB and has a greater dynamic range (Ball & Karuso, 2007). Flamingo (BioRad) is a newer fluorescent stain that is a coumarin-cyanine dye hybrid that associates with the SDS shell around proteins and has similar sensitivities to silver and SYPRO Ruby (Ball & Karuso, 2007).

One of the complaints about 2D-GE is a supposed lack of consistency between gels. This criticism is largely dependent on one's lab skills, and is somewhat alleviated by the availability of pre-cast IPG strips and polyacrylamide gels, along with pre-made buffers. A technique that has been developed to address these concerns is two-dimensional difference gel electrophoresis (2D

DIGE). Instead of running one sample per gel, protein samples from unique treatments are labeled with different fluourescent cyanine dyes and then run on the same gel (Marouga et al., 2005). The gels are visualized by fluorescence imaging to detect differences in abundance between the samples due to treatment.

Protein Quantification

While running tens to hundreds of 2D gels for a given experiment may be a daunting task, the process of analyzing and comparing gel images is often more time-consuming. Fortunately, a number of software options exist to simplify and streamline the process of gel image analysis, such as Delta2D (DECODON, Greifswald, Germany), PDQuest (BioRad, Hercules, CA) and Decyder 2D (GE Healthcare) (Berth et al., 2007). High quality digital image scans (typically .TIF format) of the gels are loaded into the software and grouped together by treatment. Image warping follows, whereby variation in positional spot location is corrected by establishing match vectors between identical spots between images (Berth et al., 2007). Inescapable variation in sample composition and gel runs lead to fluctuation in spot migration in the gel, but warping lines images up 1:1 without sacrificing spot density information. After

warping is complete, a fusion image is generated using the match vectors as the cue for combining all the separate gel images into a single picture. This fusion image is essentially an average of all the gels in the experiment, displaying all spots in the gels, even those that only show up in a few images.

At this point, spot boundaries are detected on the fusion image, and these boundaries are transferred along the match vectors back to the original gel images (Berth et al., 2007). The unit of measurement for protein abundance in a given spot is spot volume, given by the area and the pixel density of the spot (Berth et al., 2007). It is not appropriate to use the raw spot volumes for quantification, as variance in protein loading, image exposure time and staining efficiency produce variation in spot volume even within gels of the same treatment. Spot volume is normalized by taking the raw spot volumes and relating them to spot volumes on the same or other gels (Berth et al., 2007).

Once spot volumes are quantified and normalized across all gels, the change in protein abundance across treatment for each spot, the protein expression profile, can be generated. These protein expression profiles are the product of a number of factors, including protein

synthesis/degradation and post-translational modification. Statistically significant changes within a protein expression profile indicate proteins that are changing in response to a given treatment. Statistical tests are generally the standard t-test for simple experiments and one- and two-way ANOVAs for more complex experiments with multiple treatments. These tests are carried out in permutations in order to account for the lack of normality in the data but more importantly to account for the non-normal variances within each spot (Ludbrook & Dudley, 1998).

In genomics studies, especially those using microarrays, running multiple simultaneous statistical tests on thousands of genes increases the chance of false positives. The goal in analyzing -omics datasets is to identify as many significant genes as possible while incurring a low proportion of false positives (Storey & Tibshirani, 2003). This is typically done by restricting the size of the p-value, a measure of the false-positive rate (FPR), the number of truly null observations deemed significant. A measure of significance that may be more appropriate for -omics studies is the q-value, a measure of the false discovery rate (FDR). The FDR is a measure of the proportion of genes deemed significant that are truly null,

or the significant features that are actually false leads. The q-value is advantageous over the p-value as the q-value provides a direct measure over the significant features of an experiment.

While limiting the FDR by using a stringent q-value has the advantage of ensuring that genes that are labeled statistically significant are indeed so, it may be too restrictive on 2D-GE proteomics studies, where only a few hundred detected proteins are tracked versus thousands in a microarray study. Most proteomic studies control the FPR, using p-values ranging from 0.01 to 0.05. While the p-value concept is not suitable for microarray studies (Pawitan et al., 2005), the jury is not out as to whether it is ineffective for the much smaller 2D-GE proteomics analyses.

After one determines the statistically significant changing proteins in an experiment, it is necessary to elucidate patterns of abundance among these proteins. Hierarchical clustering generates heat maps that groups protein abundance profiles by similarity, forming clusters of similarly changing proteins that can be used to identify outliers or patterns of expression within an experiment (Berth et al., 2007). Principal component analysis (PCA), a method of multivariate analysis, serves to simplify the dimensionality of a large dataset and identify the primary

components, or projections, of that data that explain most of the variability within the cloud of data (Berth et al., 2007). Data points are given loading values (or coefficients, values that the standardized variables must be multiplied by in order to fit on a given component) that can characterize the proteins whose expression patterns contribute most to the separation of the data. For example, if one component may assign large loading values to proteins that all have roles in cell division or the cell cycle, that would lead the investigator to attribute the cell cycle as having a major influence on protein levels in that experiment (Berth et al., 2007).

Protein Identification

Knowledge of protein abundance changes is only of use when one knows the identity of the protein in question. In proteomics, the most efficient method of identifying proteins is mass spectrometry. A few classes of mass spectrometers exist on the market, including the one used in this study, MALDI TOF/TOF. Another major class of mass spectrometer is ESI HPLC, or electrospray ionization high-pressure liquid chromatography. In this highly-efficient methodology, peptides are identified as they exit a liquid separation or fractionation step (Lovric, 2011). This is

useful for shotgun proteomics, but is more difficult to apply with 2D-GE proteomics, where MALDI TOF/TOF mass spectrometry is the preferred method of protein identification (Geveart & Vandekerckhove, 2000).

Matrix-assisted laser desorption/ionization (MALDI) tandem time of flight (TOF/TOF) mass spectrometry is carried out in three main steps. The first step involves digesting the proteins into smaller peptide fragments utilizing a protease that cuts at specific sites along the amino acid chain. Typically, this enzyme is trypsin, which cleaves at the C-terminal side of arginine and lysine residues (Olsen et al., 2004). Trypsin breaks proteins down in unique but predictable (if the organism has a sequenced genome) fragments that are used for identification (Lovric, 2011).

The second step is the generation of a peptide mass fingerprint (PMF), using the MS. A PMF is a measure of both the mass and abundance of all the peptides in a sample mixture. The most abundant peptides can be further broken down using tandem mass spectrometry (MS/MS) to yield peptide fragment fingerprints (PFFs) that yield information about the amino acid make-up of an individual peptide (Fig. 4).

Finally, the PMF and PFFs are combined and searched against a genomic database to be matched up against the PMF/PFF data from theoretical enzymatic digestions of proteins (Lovric, 2011). Successful identification depends on a number of factors, including molecular weight of the protein, the error rate of the genomic database and initial purity of the protein sample (Suckau et al., 2003).

components that make up every mass spectrometer are the ion source, mass analyzer and detector (Suckau et al., 2003). The ion source is where detectable gas-phase ions are generated from a solid or liquid sample (Westermeyer et al., 2008). The two methodologies are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). ESI is used to ionize peptides within a solution and forms multiply charged ions (Westermeyer et al., 2008).

MALDI on the other hand, requires one to embed the peptides within a crystalline matrix that absorbs light at the wavelength of the laser, typically α -cyano-4-hydroxy cinnamic acid for a tryptic digest (Westermeyer et al., 2008). The laser causes desorption and ionization of matrix and analyte, followed by acceleration of the ionized peptides by the voltage plates toward the mass analyzer. In contrast to ESI, MALDI yields singly-charged ions, either by protonation or deprotonation.

The mass analyzer serves to measure the mass to charge ratio (m/z) of peptides as they are ionized off of the target. Four types of mass analyzers include the linear time-of-flight (TOF) analyzer, triple quadrupole, quadrupole ion trap and the Fourier transform ion cyclotron. The MALDI TOF-TOF mass spectrometer includes

two TOF analyzers separated by a collision cell (Suckou et al., 2003). In this way, the first TOF analyzer is used for precursor ion selection followed by high-energy collisions in the collision cell. The ions are then resolved by the second TOF analyzer. This allows for high sensitivity in both MS and MS/MS modes (Westermeier et al., 2008). ESI mass spectrometers take advantage of other analyzers like the quadrupole ion trap, where precise combination of constant voltage and radiofrequency voltage traps ions in a helium-filled chamber, releasing them from the chamber in order from low to high m/z .

The detector functions to detect the abundance of peptides that register at a given m/z ratio. For MALDI, where each peptide is singly charged, the m/z is a given for each hit. However, in ESI where molecules can carry multiple charges, deconvolution algorithms must be applied to determine the true mass of a given protein from multiple charge states (Westermeier et al., 2008).

This pattern of m/z ratios for individual peptides within a sample is the peptide mass fingerprint (PMF). A PMF is a graph with m/z range along the x-axis and intensity along the y-axis. Peptides of high abundance show up as tall peaks within the PMF, and vice versa with low-abundance peptides. These PMFs are submitted to MASCOT

(or Phenyx), a database that includes theoretical PMFs of *in silico* digests of proteins from sequenced organisms in order match the experimentally-obtained PMF to a protein identity (Westermeier et al., 2008).

Tandem mass spectrometry (MS/MS) is used to strengthen the identification. In this process, high-abundance peptides are individually further fragmented to yield a peptide fragment fingerprint (PFF). In the case of the MALDI TOF/TOF mass spectrometer this is achieved by diverting away all peptides but the one of interest and fragmenting that peptide alone within the collision cell (Westermeier et al., 2008; Suckau et al., 2003). The PMF combined with the PFFs in MASCOT greatly increase the confidence of identity.

The MASCOT database scores protein hits by the molecular weight search (MOWSE) algorithm. For a given organism, MASCOT denotes a threshold for significance, where scores above the threshold are deemed to have p-values less than 0.05 (Westermeier et al., 2008). A MOWSE score above threshold along with at least two matched peptide fragments (PFFs) yields a statistically significant hit and an identification for a given protein (Perkins et al., 1999; Gevaert & Vandekerckhove, 2000). A hit can be confirmed further by the use of a decoy, or false protein,

database. The false database is typically generated by reversing or shuffling the amino acids within each protein (Link & LaBaer, 2009; Elias & Gygi, 2007). The decoy and actual databases are concatenated together and the PMF/PFF data is compared against both simultaneously and the database determines the percent of identified proteins that are false hits. Failure to register in the decoy database but obtaining a statistically significant hit in the actual database further confirms protein identification.

Once a list of identified proteins is generated from the mass spectrometer, it is the burden of the researcher to filter through the scientific literature to determine the role of the proteins in question. More importantly, it is the relationships between proteins that are of most interest, and this is where the aforementioned hierarchical clusters and principal component analyses come into play. Proteins whose protein expression profiles group together in a cluster often play a part in the a broader cellular pathway. Linking protein identifications to their loading values within the components of a PCA can uncover functional relationships between proteins from a seemingly complex dataset.

Another recent tool that seeks to elucidate relationships between identified proteins is the advent of

pathway analysis software. Such software uses text-mining algorithms to peruse published scientific literature to identify functional and interaction information between proteins (Yuryev et al., 2006). In the case of MedScan (Ariadne Genomics Inc., Rockville, MD, USA), a full-sentence parsing system is used to identify protein-protein binding regions, protein modifications, promoter binding regions, and various regulatory information for sets of proteins. Researchers at Ariadne Genomics assure that MedScan uncovers 100% detection of facts repeated more than once in the body of literature searched (Yuryev et al., 2006). Results from pathway analysis software can be visualized as a web network of protein interactions. A software-generated web of protein interactions does not replace the human mind as the most important tool in proteomics studies. The literature must still be perused to verify the value in these supposed associations. As much as technology has enabled the generation of the massive datasets in systems biology, a list of proteins and their protein expression profiles is useless without taking the extra steps to make statements as to the importance of these fluctuations within the broader context of the initial experiment.

II. MANUSCRIPT

Abstract

The ascidian species *Ciona savignyi* and *C. intestinalis* are both invasive species that show interspecific differences in their population-level response to hypo-saline stress associated with heavy winter-run off events that are predicted to become more frequent due to climate change. Despite an almost world-wide distribution, *C. intestinalis* seems to be more susceptible to hypo-saline stress than the geographically more limited *C. savignyi*. Given that the genomes of both species are fully sequenced, we were able to compare their proteomic response to both acute and chronic salinity to characterize the mechanisms that are responsible for setting tolerance limits to hyposaline conditions in these two congeneric species. For the acute hypo-saline stress experiment, we exposed each species to decreasing salinities, 100%, 85% and 70% full-strength seawater, for 6 hours followed by a 4-hour recovery at 100%. In the chronic salinity stress experiment, each species was kept at 100% or 85% with individuals removed for analysis during a 16-day time course. Organisms were dissected to

remove the tunic, and 2D SDS-PAGE was performed to separate proteins and characterize changes in protein abundance. In the acute experiment, we determined 5% and 19% of the proteins to be differentially changing in *C. savignyi* and *C. intestinalis*, respectively, due to the treatment effect. For both species in the chronic experiment, we determined over 40% of the proteins to be changing abundance given the treatment, time, or interaction effect. Analysis of these proteins with MALDI TOF-TOF mass spectrometry has identified numerous proteins implicated in cellular stress responses, including energy metabolism (glycolysis, ATP & NADH production), cytoskeletal restructuring, and protein turnover, providing insights into the intense cellular restructuring that occurs following hypo-saline exposure.

Keywords: Proteomics, hyposalinity stress, *Ciona*

Introduction

Hyposaline conditions result in extensive cellular readjustments due to the osmotic influx of water into cells. This increases cellular volume, requiring the cellular scaffold to be restructured (Pedersen et al., 2001). Frequency and intensity of precipitation events that can cause extreme fluctuations in salinity are expected to increase as a function of climate change (IPCC, 2007). As the mean temperature of a region increases, the frequency of heavy to extreme (40–100 mm daily amount) events are predicted to increase even without a net increase in precipitation (Karl & Trenberth, 2003). The impacts of intense rainfall are found most dramatically in estuarine environments, where freshwater input can arise from watersheds covering great expanses of land. The effects of freshwater input have been implicated in setting vertical zonation patterns of marine invertebrates that make up fouling communities (Witman & Grange, 1998; Rutger & Wing, 2006). Furthermore, coastal communities can be subject to massive die-offs following intense rains (Goodbody, 1961).

In order to assess the impact of these extreme precipitation events on distribution ranges of marine

invasives, we have chosen to investigate the physiological response to hypo-saline stress of three species of the genus *Ciona*. *C. intestinalis* and *C. savignyi* are two species of ascidians, sessile hermaphroditic invertebrate chordates, commonly known as sea squirts.

Through centuries of ship hull fouling, both *Ciona* species are presently found at harbor pilings and marinas around the world (*C. intestinalis*) and the Pacific coast of North America and Japan (*C. savignyi*) (Lambert and Lambert, 1998). Both *C. intestinalis* and *C. savignyi* are considered invasive (and now permanent) residents along the Pacific coast of North America, where they often form the dominant portion of sessile filter-feeding invertebrates (Lambert & Lambert, 1998), capable of rapidly displacing native ascidian species (Ramsay et al., 2007). *C. intestinalis* has been so successful in establishing a nearly world-wide distribution that it has undergone subspecies divergence, with the two subspecies designated *C. intestinalis* Sp. A (representing Pacific, southern Atlantic, and Mediterranean populations) and *C. intestinalis* Sp. B (representing populations still residing in the original northern European Atlantic regions) (Suzuki et al., 2005; Caputi et al., 2007; Ianelli et al., 2007) (**Fig. 5**). This speciation

event is likely due to the confluence of the wide distribution of *C. intestinalis*, high individual polymorphism, and faster rate of evolution compared to vertebrates (Satou et al., 2005; Vinson et al., 2005; Berna et al., 2009).

Figure 5. Global distribution maps of *Ciona intestinalis* (A) and *C. savignyi* (B). Black designates invaded range while gray indicates native range or cryptogenic (unknown origin) range. See Evolution & Selection for more information. (Figure adapted from Therriault & Herborg, 2008. *C. savignyi* information taken from Lambert, 2003).

Ciona are susceptible to fluctuating salinity conditions that occur during heavy winter rain and run-

offs. Massive die-offs of *Ciona* populations occur during these episodes of prolonged hyposaline conditions during the winter, later followed by recolonization during the spring by individuals deeper in the water column (Lambert and Lambert, 1998). Studies of their embryos have suggested that the two species differ in their tolerance to hyposaline stress, which may be a factor enabling *C. savignyi* to expand its distribution range earlier than *C. intestinalis* in harbors where the two species compete (Lambert and Lambert, 2003).

As *Ciona* are sessile, they cannot employ behavioral avoidance other than closing their siphons during stressful salinity conditions (< 60% full strength seawater), and must cope with whatever changes occur in their environment (Shumway, 1978). Similar behavior is seen in mussels (*Mytilus edulis*) where they close their shell valves in response to declining salinity (Davenport, 1979). This symmetry of behavioral patterns to salinity fluctuation allows these animals to maximize feeding and O₂ uptake during periods of high salinity and minimize energy expenditure during periods of low salinity (Davenport, 1985). Despite siphon closure, ascidians exposed to hyposaline media over several hours are subject to influx

of water via osmosis from extracellular fluid into cells, increasing volume. Individuals of the solitary tunicate genus *Styela* showed significant increases in relative weight gain over the course of exposure to 50% seawater (between 18.4% and 39.0% weight gain, depending on species), with further increase limited only by the inflexible tunic (Sims, 1984).

Dilute seawater reduces the solute concentration in the extracellular fluid of marine animals leading water to flow via osmosis from the dilute extracellular fluid into the high solute environment of the intracellular fluid. Since the cell membranes of animal cells are flexible, swelling occurs (Davenport, 1985). Cell volume is returned to its normal state by a series of processes called regulatory volume decrease (RVD), which trigger active efflux of osmotically active solutes, organic osmolytes or inorganic ions by which water then follows (Okada et al. 2001).

To our knowledge there have been no studies to track the physiological effects of hyposaline stress on the cells of adult *Ciona*. To do so, we have focused on the proteomic response to acute and chronic hypo-salinity stress. Proteomics has been successfully applied to elucidate key

cellular pathways implicated in response to environmental stressors in marine invertebrates (Tomanek, 2011; Tomanek & Zuzow, 2010; Serafini et al., 2011; Campanale et al. 2011).

The proteome encompasses the entire set of expressed proteins in an organism, and is subject to modification by the dynamic changes that occur in the internal and external environment (Lopez, 2007). Proteomics provides the technique to directly track and identify the abundance of proteins within an organism's proteome that respond to specific conditions. Studying the proteome of an organism offers particular advantages over merely studying the transcriptome as not all mRNA transcripts are translated into proteins, with mRNA to protein Pearson correlation coefficients ranging between 0.46 and 0.76 (Hack, 2004; Feder & Walser, 2005).

As both species of *Ciona* can inhabit a variety of environments globally (and often co-inhabit the same harbors), they provide an opportunity to compare the evolutionary differences in their responses to the same stressor. Additionally, their proximity to vertebrates on the evolutionary tree provides insights into the evolutionary origins of the cellular stress response to salinity stress. Their relatively simple sequenced genomes

function as a vertebrate framework and provide an ideal system to study the full complement of proteins mobilized in response to salinity stress. For these reasons, *Ciona* serve as a Kroghian system for studying the salinity stress response (Krebs, 1975).

This study used two-dimensional proteomics to analyze the responses of two *C. intestinalis* subspecies (Sp. A & Sp. B) and *C. savignyi* to acute and chronic hyposaline stress in order to yield both an intra- and inter-species comparison of their responses. The acute exposure experiments were carried out in 2007 and 2009, and processed the whole organism in order to look at changes in the entire complement of proteins in all tissues.

We subsequently performed a chronic exposure designed and carried out in 2010. The chronic exposure enabled us to track changes in the proteome over time in response to the stress, yielding two experimental 'tempos', tracking protein abundances over the 24 h circadian cycle and the broader changes over several days. We eliminated gut and digestive tissues from processing in order to limit the complexity of the sample given the more complex nature of the experiment.

Comparisons of the proteomes within each species across different levels of salinity enabled the identification of proteins altered in abundance in response to hyposaline stress. These identifications provide us with insights into the nature and evolutionary origins of the vertebrate acute salinity stress response and the diversity of the stress response within the *Ciona* genus.

Materials and Methods

Animal collection and maintenance

Acute Exposure

Specimens of both *Ciona intestinalis* and *C. savignyi* were collected on March 29th, 2007 from the Santa Barbara Yacht Harbor in Santa Barbara, CA, USA (34°24'N, 119°41'W).

C. intestinalis Sp. B specimens were collected on September 14th, 2009 from Grimstad Harbor outside Bergen, Norway (60°18'N, 5°15'W). Specimens were stored in seawater from the site during transit. At the lab, *Ciona* were kept in tanks with seawater from their respective collection site (13°C, 923 mOsm, Santa Barbara / 15°C, 704 mOsm, Bergen) prior to the experiment.

Chronic Exposure

Specimens of both *Ciona intestinalis* and *C. savignyi* were collected on August 24th, 2010 from the Santa Barbara Yacht Harbor in Santa Barbara, CA, USA (34°24'N, 119°41'W). Specimens were stored in seawater from the site during transit. At the Cal Poly Pier at Avila Beach, CA, USA (35°11'N 120°43'W) *Ciona* were divided equally into two tanks, with a plastic mesh dividing each tank in half,

separating the two species spatially. Each tank contained 110 individuals of both species. Tanks were filled with 20 L filtered seawater from the pier, and the temperature of the tanks was controlled with a water bath and a coil with flow-through raw seawater from the bay. Aerators with airstones maintained aeration, and 5 mL of Phytofeast (Reed Mariculture Inc., Campbell, CA) algae mix was added to each tank to feed the animals. Filtered seawater was replaced daily and new Phytofeast was added daily as well. Tanks were covered with tarps at all times to limit exposure to UV radiation.

Experimental design

Acute Exposure

Ten organisms from each species were randomly separated into three treatment groups: a 100% control and 85 and 70% experimental. The osmolarity for the three treatments is was measured as follows: 100% (923 mOsm Santa Barbara / 704 mOsm Bergen), 85% (785 mOsm Santa Barbara / 602 mOsm Bergen) and 70% (646 mOsm Santa Barbara / 504 mOsm Bergen) (**Fig. 6**). Note that these levels are relative to the salinity at the site, with the Bergen collection site

almost 200 mOsm less than the Santa Barbara site. Beakers were filled with 1 L of water from the collection site at 100% or diluted down to 85% and 70% salinity with MilliQ water (0.2 μ m filter), aerated and temperature-controlled at 13°C. The 85% and 70% groups were transferred to the 85% beaker, and after a 30-min acclimation period, to avoid shock, the 70% group was transferred into the 70% beaker. The three treatment groups (100%, 85%, 70%) were maintained at their target salinity for 6 h. Following exposure to hyposaline conditions, the specimens were brought back to 100% salinity stepwise to recover for 4 h. Immediately following the 4 h recovery ten *Ciona* specimens per group were paired to a sample (N=5 for all groups) and frozen in liquid nitrogen and stored at -80°C. The recovery step allowed for any remaining mRNA transcripts to complete translation into protein, yielding a more comprehensive perspective of the organisms' response to hyposaline stress.

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Chronic Exposure

110 organisms each from both species were randomly separated into two treatment groups: a 100% (~1000 mOsm) control and 85% (~850 mOsm) (**Fig. 7**). Tanks were filled with 20 L of filtered seawater and animals were allowed to acclimate in the tanks for three days prior to the experiment. The experiment began at 0800 h on the third day. Twelve animals from both species from each tank were removed and frozen on aluminum foil over dry ice (t=0 h)

and subsequently kept at -80 °C. The water was replaced in both tanks, but with the 85% tank filled with filtered seawater diluted with MilliQ water to 85% of full-strength seawater (~850 mOsm). The experiment continued in a time course with each time point double the length of the time point that preceded. Animals were removed from the tanks at 6, 12, 24, 48 h. and 4, 8, and 16 d. Tank water and algae food were replaced daily, and the collected animals were screened for signs of vitality, e.g. siphon closure, squirt reflex and body contraction. With the exception of the 6 and 12 h time points, all other collection times occurred at 0800 h to account for diurnal variation.

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Tissue Sample Preparation

For the acute exposure, whole organisms were thawed and then dissected to remove the fibrous outer tunic. Tissues were homogenized using cooled ground-glass homogenizers with a 1:2 ratio of tissue to homogenization buffer [7M urea, 2M thiourea, 1% ASB-14 (amidosulfobetaine-14), 40 mM Tris-base, 0.5% immobilized pH 4-7 gradient (IPG) buffer (GE Healthcare, Piscataway, NJ, USA) and 40mM

dithiothreitol]. Homogenized tissues were held for 1 h at room temperature to allow proteins to dissolve, then immediately centrifuged for 30 min at 16,100 g. The supernatant was decanted and saved.

For the chronic exposure, the tunic was removed from the thawed organisms. In order to limit the complexity of the sample, the organisms were then dissected to remove the top half of the animals (siphons, body wall, and pharyngeal basket) away from the rest of the organs (mainly digestive tissue, e.g. stomach and intestines) for homogenization. Tissues were homogenized as described above.

Ice-cold 10% TCA/Acetone was added to the supernatants in a 1:4 ratio to precipitate the protein. Protein mixtures were stored at -20°C overnight and then centrifuged at 4°C for 15 min at 18,000 g. The supernatant was discarded, and cold 100% acetone was added to wash out any remaining contaminants from the protein pellet. Solutions were centrifuged once more for 15 minutes at 18,000 g. The resulting supernatant was discarded and the protein pellet was suspended in rehydration buffer [7M urea, 2M thiourea, 2% CHAPS (cholamidopropyl-dimethylammonio-propanesulfonic acid), 2% NP-40 (nonyl-phenosylpolyethoxylethanol-40), 0.002% Bromophenol Blue,

0.5% ampholyte and 100mM DTE (dithioerythreitol)]. We vortexed the protein mixtures and stored them at 4°C overnight followed by a centrifugation at room temperature for 10 min., 16,100 g. Supernatant was decanted and stored at -80°C.

The protein concentration of each sample was determined using the 2-D Quant Kit (GE Healthcare) according to the manufacturer's instructions.

Two-dimensional gel electrophoresis

For samples from the acute exposure, a total of 800 µg protein per lane was added to 24cm, pH 4-7 ReadyStrip IPG strips (Bio-Rad, Hercules, CA, USA). Isoelectric focusing was conducted in a Protean IEF Cell [Bio-Rad] using the following running conditions: Rapid increase to 250 V for 15 min, 10,000 V for 3 h, 10,000 V for 80,000 V/hr, then holding at 500 V for 24 h. Strips were frozen overnight at -80 °C after the run was complete.

For samples from the chronic exposure, a total of 400 µg protein per was added to 11 cm, pH 4-7 ReadyStrip IPG Strips (Bio-Rad, Hercules, CA, USA). We ran the strips in a Protean IEF Cell [Bio-Rad] using the following running

conditions: passive rehydration (5 h), active rehydration at 50 V (12 h), 500 V (1 h), 1000 V (1 h), 8000 V (2.5 h). Strips were frozen overnight at -80°C following the run.

Equilibration buffer [375 mM tris-base, 6 M urea, 30% glycerol, 2% sodium dodecylsulfate (SDS), 0.002% bromophenol blue] mixed with 10 mg/mL DTT was added to the strips and incubated for 15 min. We then incubated the strips with equilibration buffer mixed with 135 mM iodoacetamide for 15 min. Strips were then added to the top of 12% SDS-polyacrylamide gels with 0.8% agarose solution containing Laemmli SDS electrophoresis running buffer (25 mM tris-base, 192 mM glycine, 0.1% SDS).

For the acute exposure, the gels were placed in the Ettan DALT Six electrophoresis unit [GE Healthcare] with a circulating water bath at 12 °C and run at 50 V for 30 min, and 200 V for 4.5 h. Gels in the chronic exposure were placed in a Criterion Dodeca electrophoresis unit [Bio-Rad] at 200V for 55 min at 10 °C.

We stained the gels overnight in colloidal Coomassie Blue (G-250) stain and destained by washing multiple times with MilliQ water for at least 48 h. They were then digitally scanned with a transparency image scanner (Epson 1280, Long Beach, CA, USA).

Gel image analysis

Digitally scanned gel images were analyzed with Delta2D analysis software (version 3.6; Decodon, Greifswald, Germany) . Gel images were linked via match vectors by a group warping strategy which yielded a fused composite image representing mean volumes of each spot. We used the composite image, or proteome map, to detect spot boundaries which were transferred back onto the other gel images via match vectors. We subtracted the background from the image and normalized the protein spot volume against the total spot volume of all the proteins within a gel image.

Statistical Analysis

Delta 2D (version 3.6; Decodon, Greifswald, Germany) was used to analyze normalized spot volume with analysis of variance (one-way ANOVA) within each species and with salinity as the main effect. We generated a null distribution using 1000 permutations to account for unequal variance and non-normal distributions of the protein spots, using a p-value of 0.05. Gel regions were difficult to

match between species, so a two-way ANOVA or other statistical tests between the gels of different species were not possible. We used average linking with Pearson's correlation metric to create hierarchical clusters within Delta2D. Principle component analyses (PCA) of samples in the acute exposure were made within Delta2D to determine the contribution of salinity to the variation in protein abundance. Fisher's LSD post-hoc test was used to compare treatment means of protein spots significantly changing under the ANOVA.

Mass Spectrometry

Proteins that changed in abundance due to treatment were excised from gels with a tissue puncher (Beecher Instruments Inc., Sun Prairie, WI). Destaining buffer (25mM ammonium bicarbonate in 50% acetonitrile) was added twice to remove Coomassie stain from the gel plugs. We dehydrated the plugs with 100% acetonitrile and digested the proteins overnight with 11 ng μL^{-1} of trypsin (Promega, Madison, WI, USA) at 37 °C. Digested peptides were eluted from the gel plug with extraction buffer [0.1% trifluoroacetic acid (TFA)/acetonitrile; 2:1] and concentrated in a SpeedVac (Thermo Fisher Scientific,

Waltham, MA, USA). We mixed the peptide solution with 5 μ L matrix solution (0.2 mg/mL α -hydroxycyano cinnamic acid in acetonitrile) and plated it on an Anchorchip™ target plate (Bruker Daltonics Inc., Billerica, MA, USA). Plated peptides were washed with 0.1% TFA and recrystallized with the addition of an acetone/ethanol/0.1% TFA (6:3:1) solution.

Mass spectrometry was performed using a MALDI TOF-TOF mass spectrometer ['Ultraflex II', Bruker Inc.]. We obtained a peptide mass fingerprint (PMF) for each sample with tandem MS carried out on the eight most intense peaks to obtain information about the b- and y-ions of the peptide sequence.

Spectra were processed and analyzed using flexAnalysis (version 3.0; Bruker Daltonics Inc.) with the TopHat algorithm for baseline subtraction, the Savitzky-Golay analysis for smoothing (0.2 m/z ; number of cycles=1), and the SNAP algorithm for peak detection (signal-to-noise ratio: 6 for MS and 1.5 for MS/MS). We assumed the peptide charge state to be +1 and used porcine trypsin for internal mass calibration.

Proteins were identified using Mascot (version 3.1; Matrix Science Inc., Boston, MA, USA) with PMFs and tandem

mass spectra combined to search against the *C. intestinalis* EST library (representing 13,464 gene sequences) and the *C. savignyi* EST library (representing approximately 15,000 gene sequences). We included oxidation of methionine and carbamidomethylation of cysteine as the only variable modifications within the search. One missed trypsin digestion cleavage was allowed, and the tandem MS tolerance was set to 0.6 Da. A molecular weight search (MOWSE) score was deemed significant if it was higher than 27 in the *C. intestinalis* EST library or 28 in *C. savignyi* ($p < 0.05$).

Results - Acute Exposure

C. intestinalis Sp. A (Santa Barbara, CA) significant proteins

Using a proteome map generated from all gels in the experiment, a total of 347 spots were detected. Of these,

65 (19% of total) were determined to show significant changes in protein abundance across the treatment groups (ANOVA, $p < 0.05$). We were able to identify 55 (85%) of those spots (**Fig. 8; Table 1**).

Principal component analysis (PCA) was used to elucidate patterns of protein abundance changes between the three treatments for *C. intestinalis* Sp. A (**Fig. 9**). The first principal component (PC1) explained 37.22% of the variation between the samples and separates the 85% salinity group from the 100 and 70% groups. The PCA assigns each protein a loading value for each component, a coefficient typically ranging from -2 to +2 that can be used as a measure for each protein's contribution to the distribution of samples along the component. Typically, proteins with positive loadings significantly increase abundance in one or more treatments and vice versa for proteins with negative loadings. Proteins with the highest positive loadings (greater than 1.00) are as follows: fourteen actin isoforms, three α -tubulin isoforms, five β -tubulin isoforms, and two calcium vector protein target protein isoforms (CaVPTs). All of these proteins were shown to be up-regulated in the 85% treatment compared to 100 and 70%. Proteins with negative loadings in PC1 (lower than -

1.00) include: one actin, one α -tubulin, one collagen, FK506 binding protein and a proteasome α -6 subunit. All of these proteins were shown to be down-regulated in the 85% treatment compared to 100 and 70% in *C. intestinalis* Sp. A. The second principal component (PC2) explained 12.59% of the variation between the samples and separates samples within the 85% treatment. Proteins with the highest positive loadings (greater than 1.00) include: nine actin isoforms, one α - and one β -tubulin, one CaVPT protein, and peroxiredoxin 6. With the exception of peroxiredoxin 6, all these proteins are cytoskeletal in function, with all except for a few actins increasing in the 85% treatment. Proteins with negative loadings in PC2 (lower than -1.00)

include: three actins, one α - and one β -tubulin, cofilin 2, one CaVPT protein, peptidase M1, and a proteasome α -5 subunit. All proteins except the proteasome α -5 subunit were up-regulated in the 85% treatment relative to the others.

Hierarchical clustering sorted the identified proteins into two clusters containing proteins that display similar patterns of protein abundance changes across treatments (**Fig. 10**). The first cluster contains 35 proteins including numerous actins and tubulin isoforms, CaVPT, cofilin 2, peroxiredoxin 6, peptidase M1 and 20S proteasome α -5 subunit. These proteins display increased abundance in the 85% treatment. The second cluster contains 20 proteins, including several actin and tubulin isoforms, collagens, HSP70, ATP-synthase β subunit, FK506 binding protein 9, capping, 20S proteasome α -5 subunit, and proteasome α -6 protein isoform 2. These proteins display decreased abundance in the 85% treatment.

Ciona intestinalis Sp. A

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Table 1. Protein identifications (using MS/MS) of spots changing with salinity treatment in *C. intestinalis* Sp. A acute hyposalinity stress experiment.

Spot ID	Protein ID	MM Estimated (kDa)	pI Estimated	MM Predicted (kDa)	pI Predicted	GenBank ID	Mascot Score	Peptide Matches	Sequence Coverage (%)	Functional Category
1	Collagen, type XXVIII	98	4.6	91.4	4.93	GI: 198424351	31	6	18	Cytoskeletal
2	Collagen, type XXVIII	80	4.4	91.4	4.93	GI: 198424351	87	6	8	Cytoskeletal
3	Collagen, type XXVIII	75	4.4	91.4	4.93	GI: 198424351	47	3	3	Cytoskeletal
4	FK506 binding protein 9	66	4.8	65.1	4.94	GI: 198425823	31	3	12	molecular chaperones
8	Tubulin α -1 chain	52	5.2	50.1	4.94	GI: 198422770	73	3	8	Cytoskeletal
9	Actin	49	5	41.7	5.29	GI: 86610891	31	1	2	Cytoskeletal
10	Tubulin α -1 chain	45	6.3	50.1	4.94	GI: 198422770	81	6	20	Cytoskeletal
11	Muscle Actin Isoform 1	45	5.1	42.2	5.18	GI: 198433891	117	8	26	Cytoskeletal
12	β -tubulin	43	6.3	50	4.73	GI: 198424255	41	5	13	Cytoskeletal
13	Actin	39	4.7	41.7	5.29	GI: 86610891	157	3	10	Cytoskeletal
14	Actin	39	4.9	41.7	5.29	GI: 86610891	112	4	14	Cytoskeletal
15	Actin	39	5	41.7	5.29	GI: 86610891	139	3	11	Cytoskeletal
16	Tubulin α -1 chain	41	5.4	50.1	4.94	GI: 198422770	144	3	8	Cytoskeletal
17	β -tubulin	41	6	50	4.73	GI: 198424255	258	7	17	Cytoskeletal
18	Muscle Actin Isoform 1	39	5.2	42.2	5.18	GI: 198433891	149	4	14	Cytoskeletal
19	β -tubulin	39	5.5	50	4.73	GI: 198424255	32	3	8	Cytoskeletal
20	Actin	37	5.6	41.7	5.29	GI: 86610891	39	1	7	Cytoskeletal
21	Cappin, muscle Z-line, α 2	39	6.5	32.3	5.95	GI: 198422219	80	4	21	Cytoskeletal
22	CsMA-1 isoform 1	38	4.6	43.9	5.37	GI: 198433784	49	3	9	Cytoskeletal
23	β -tubulin	33	5	50	4.73	GI: 198424255	73	1	2	Cytoskeletal
24	CsMA-1 isoform 1	32	5.7	43.9	5.37	GI: 198433784	92	3	10	Cytoskeletal
25	ATP synthase β subunit	32	6.4	55.9	5.26	GI: 198421396	215	8	20	energy metabolism
27	HSP70 cognate	32	6.8	71.9	5.34	GI: 198420813	38	4	9	molecular chaperones
29	ATP synthase β subunit	31	6.6	55.9	5.26	GI: 198421396	225	8	16	energy metabolism
31	PsmA6-prov protein isoform 2	30	6.6	27.9	6.35	GI: 198422472	59	3	20	protein degradation
32	Peroxisredoxin 6	30	6.4	24.7	6.22	GI: 198428584	36	1	4	oxidative stress
33	CsMA-1 isoform 1	30	5.9	43.9	5.37	GI: 198433784	69	2	6	Cytoskeletal
34	CsMA-1 isoform 1	29	5.9	43.9	5.37	GI: 198433784	83	2	6	Cytoskeletal
35	Actin, cytoplasmic	29	6	41.7	6.42	GI: 198424621	91	3	8	Cytoskeletal
36	Actin	31	5.4	41.7	5.29	GI: 86610891	84	5	15	Cytoskeletal
37	Muscle Actin Isoform 1	31	5	42.2	5.18	GI: 198433891	40	2	7	Cytoskeletal
38	β -tubulin	31	4.6	50	4.73	GI: 198424255	211	6	14	Cytoskeletal
39	β -tubulin	29	4.6	50	4.73	GI: 198424255	82	5	14	Cytoskeletal
40	CAVP-target protein	27	4.5	25.4	4.78	GI: 198422600	116	4	27	signaling

41	20S Proteasome $\alpha 5$ subunit	29	4.8	30.2	4.88	GI: 198432945	53	3	15	protein degradation
42	20S Proteasome $\alpha 5$ subunit	29	4.9	30.2	4.88	GI: 198432945	52	3	12	protein degradation
43	β -tubulin	29	4.8	50	4.73	GI: 198424255	50	4	11	cytoskeletal
44	CAVPT protein isoform 3	28	4.7	25.4	4.78	GI: 198422600	86	3	20	signaling
45	CAVPT protein isoform 3	29	5	25.4	4.78	GI: 198422600	80	2	12	signaling
46	Peptidase M1	27	5.6	98.6	5.67	GI: 198436182	67	1	7	protein degradation
47	CsMA-1 isoform 1	26	5.6	43.9	5.37	GI: 198433784	35	3	10	cytoskeletal
49	Muscle Actin Isoform 1	25	4.7	42.2	5.18	GI: 198433891	131	4	13	cytoskeletal
50	Muscle Actin Isoform 1	25	5	42.2	5.18	GI: 198433891	93	4	13	cytoskeletal
51	Muscle Actin Isoform 1	25	5	42.2	5.18	GI: 198433891	71	5	15	cytoskeletal
52	Actin	25	5.3	41.7	5.29	GI: 86610891	44	2	7	cytoskeletal
54	cofilin 2 (muscle)	24	5.6	18.5	5.26	GI: 198423127	209	4	37	cytoskeletal
55	CsMA-1 isoform 1	23	5.5	43.9	5.37	GI: 198433784	181	5	15	cytoskeletal
58	Tubulin α -1 chain	22	5.4	50.1	4.94	GI: 198422770	69	3	8	cytoskeletal
59	Tubulin α -1 chain	22	5.4	50.1	4.94	GI: 198422770	66	1	3	cytoskeletal
60	Muscle Actin Isoform 1	22	5.2	42.2	5.18	GI: 198433891	134	4	13	cytoskeletal
61	β -tubulin	22	5.1	50	4.73	GI: 198424254	78	4	13	cytoskeletal
62	Actin	21	5.1	41.7	5.29	GI: 86610891	86	5	17	cytoskeletal
63	Muscle Actin Isoform 1	22	4.9	42.2	5.18	GI: 198433891	138	5	15	cytoskeletal
64	Muscle Actin Isoform 1	21	4.9	42.2	5.18	GI: 198433891	139	5	15	cytoskeletal
66	GJ19221	19	4.4	61	9.32	GI: 198421062	46	1	1	N/A

C. intestinalis Sp. B (Bergen, Norway) significant proteins

A total of 489 spots were detected on the proteome map for *Sp. B*. Of these, 44 (9% of total) were determined to show significant changes in protein abundance across the treatment groups (ANOVA, $p < 0.05$) (**Fig. 11**). We were able to

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identify 23 (52%) of the 44 significant spots (**Table 2**).

Principal component analysis (PCA) was used to elucidate patterns of protein abundance changes between the three treatments for *C. intestinalis* Sp. B (**Fig. 12**). PC1 explained 42.91% of the variation between the samples and contributes to the separation of all three groups from one-another. Proteins with the greatest positive loadings (greater than 1.00) include: two actin isoforms, one α - and one β -tubulin. All of these are components of the cytoskeleton and up-regulated at the 85% treatment relative to the 100% and 70% treatments. Proteins with the greatest negative loadings (lower than -1.00) include: four actins, one α -tubulin, one ATP-synthase β subunit, one HSP90 β , glucanase, the GTPase Rab6a and ADP-ribosylation factor 5 (ARF5). All these proteins are up-regulated in the 70% treatment relative to the 85% treatment.

PC2 explained 18.12% of the variation between the samples and contributes to the separation of the 100% control from the 85 and 70% treatments. Proteins with the greatest positive loadings (greater than 1.00) include: two actin isoforms and a proteasome β subunit. All three of these proteins were down-regulated in the 85 and 70% treatments relative to the control. Proteins with the

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greatest negative loadings (less than -1.00) include: one actin, two α -tubulin isoforms, one calregulin, one proteasome α -2 subunit, and embryonic 1. All of these proteins were down-regulated in the 100% control relative to the 85 and 70% treatments. Hierarchical clustering sorted the identified proteins into three clusters (**Fig. 13**). The first cluster contains 8 proteins, including two actin isoforms, β -tubulin, tubulin α -1 chain, two calreticulin precursor isoforms, embryonic 1, and proteasome α -2 isoform 1. These proteins display increased abundance in the 85% treatment. The second cluster contains 3 proteins, including two actin isoforms and

proteasome β type 3. These proteins display increased abundance in the 100% and 85% treatments. The third cluster contains 12 proteins, including five actin isoforms, two α -tubulin isoforms, HSP90 β , ATP Synthase β subunit, β -1,3(4)-glucanase, Rab-6A and Arf-5. These proteins display increased abundance in the 70% treatment.

Table 2. Protein identifications (using MS/MS) of spots changing with salinity treatment in *C. intestinalis* Sp. B acute hyposalinity stress experiment.

Spot ID	Protein ID	MM Estimated (kDa)	pI Estimated	MM Predicted (kDa)	pI Predicted	GenBank ID	Mascot Score	Peptide Matches	Sequence Coverage (%)	Functional Category
3	β -tubulin	52	6	50	4.73	198424255	117	2	6	cytoskeletal
4	Tubulin α -1 chain	52	5.9	50.1	4.94	198422770	109	3	9	cytoskeletal
5	CsMA-1 isoform 1	45	5.4	43.9	5.37	198433784	442	5	16	cytoskeletal
6	CsMA-1 isoform 1	45	6.2	43.9	5.37	198433784	227	5	16	cytoskeletal
7	Muscle actin isoform 1	45	6.4	42.2	5.18	198433891	316	5	17	cytoskeletal
9	Muscle actin isoform 1	40	4.8	42.2	5.18	198433891	513	6	20	cytoskeletal
11	Tubulin α -1 chain	40	5.7	50.1	4.94	198422770	76	1	3	cytoskeletal
15	Proteasome β subunit type 3	30	5.1	22.9	5.04	198423327	41	1	8	protein degradation
16	Proteasome α -2 subunit-like isoform 1	28	6.5	26	5.98	198429197	289	4	15	protein degradation
18	GTP-binding protein (RAB6A)	25	5.6	23.8	5.42	198430343	76	2	8	vesicular traffic
20	HSP90 β	25	4.4	83.3	4.6	198414289	83	2		molecular chaperones
21	Embryonic-1	25	4.6	21.7	5.06	198423965	85	2	8	protein degradation
24	ADP-ribosylation factor 5	21	6.2	20.5	5.68	198426355	46	1	4	vesicular traffic
27	Actin	45	5.6	41.7	5.29	86610891	264	6	18	cytoskeletal
28	ATP synthase β -subunit	47	5.6	55.9	5.26	198421396	191	6	14	metabolism
29	Actin	47	5.5	41.7	5.29	86610891	152	3	10	cytoskeletal
30	α -tubulin	41	5.6	50.1	4.94	198422770	67	1	2	cytoskeletal
33	Muscle actin isoform 1	40	4.8	42.2	5.18	198433891	288	4	14	cytoskeletal
37	Calregulin	50	4.5	48.5	4.25	198432093	115	1	4	molecular chaperones
38	Calregulin	50	4.6	48.5	4.25	198432093	147	1	4	molecular chaperones
40	CsMA-1 isoform 1	46	4.8	43.9	5.37	198433784	101	3	10	cytoskeletal
41	CsMA-1 isoform 1	41	5.1	43.9	5.37	198433784	55	2	5	cytoskeletal
43	beta-1,3(4)-glucanase LIC1	46	4.3	39.9	4.6	198427967	80	2	10	metabolism

Ciona savignyi significant proteins

A total of 380 spots were detected on the proteome map for *C. savignyi* (**Fig. 14**). Of these, 19 (5% of total) were determined to show significant changes in protein abundance across the treatment groups (ANOVA, $p < 0.05$). We were able to identify 11 (58%) of the 19 significant spots (**Table 3**).

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Principal component analysis (PCA) was used to elucidate patterns of protein abundance between the three treatments for *C. savignyi* (**Fig. 15**). PC1 explained 37.94% of the variation among samples and contributes to the separation of the 85% treatment from both the 100 and 70% treatments. Proteins with the greatest positive loadings (greater than 1.00) include: two actin isoforms, vimentin and fatty acid binding protein 2 (FABP2). The actins and vimentin were all up-regulated in the 70% treatment while FABP2 was down-regulated in the 85% relative to the other treatments. Proteins with the greatest negative loadings (lower than -1.00) include: α -tubulin and myosin light chain 5, both of which were up-regulated in the 85% treatment.

PC2 explained 21.09% of the variation between samples and separates the 100% and 70% treatments from each other. Proteins with the greatest positive loadings (greater than 1.00) include: two CaVPT protein isoforms and FABP2. All three were down-regulated at 85%. Proteins with the greatest negative loadings (lower than -1.00) include: one CaVPT protein and annexin A13. The CaVPT protein was up-regulated at 70% while the annexin was up-regulated at 85%.

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Hierarchical clustering sorted the identified proteins into two clusters (**Fig. 16**). The first cluster contains 5 proteins, including fatty acid binding protein 2, two actin isoforms, CaVP target protein, and vimentin. These proteins display increased abundance in the 70% treatment. The second cluster contains 6 proteins, including myosin light chain 5, α -tubulin, ATP-synthase β -subunit, two CAVP target protein isoforms and annexin A13. These proteins display increased abundance in the 85% treatment.

Ciona savignyi

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Table 3. Protein identifications (using MS/MS) of spots changing with salinity treatment in *C. savignyi* acute hyposalinity stress experiment.

Spot ID	Protein ID	MM Estimated (kDa)	pI Estimated	MM Predicted (kDa)	pI Predicted	GenBank ID	Mascot Score	Peptide Matches	Sequence Coverage (%)	Functional Category
5	α -tubulin	58	6.3	50.1	4.94	GI: 198422770	51	1	3	Cytoskeletal
6	ATP Synthase β -subunit	48	5.8	55.9	5.26	GI: 198421396	46	5	13	Metabolism
7	IF-A (vimentin)	48	4.6	58	5.05	GI: 86610891	193	6	15	Cytoskeletal
9	Cytoskeletal Actin 1	42	5	41.7	5.29	GI: 3036959	182	5	20	Cytoskeletal
10	Actin	42	4.7	41.7	5.29	GI: 198433891	180	5	17	Cytoskeletal
11	Annexin A13	39	4.4	35	4.64	GI: 198433522	121	4	14	Cytoskeletal
13	CaVP-target protein	31	4.8	25	4.78	GI: 198422600	450	5	32	Signaling
14	CaVP-target protein	26	4.7	25	4.78	GI: 198422600	351	5	32	Signaling
15	CaVP-target protein	26	4.5	25	4.78	GI: 198422600	121	4	30	Signaling
18	Myosin Light Chain 5	21.5	4	19.6	4.93	GI: 198429383	35	2	12	Cytoskeletal
19	Fatty Acid Binding Protein 2	16	6.3	15	6.42	GI: 198424924	176	4	45	Signaling

Results - Chronic Exposure

Animal Mortality

Both tanks initially contained 110 individuals of each species, with 12 individuals of each species removed at each time point. Upon

each collection day, animals collected were screened for their vitality by checking for a

squirt response, valve closure, and tissue

integrity. While no individuals of *C. savignyi* were observed to have died in either tank for the length of the experiment, mortality of *C. intestinalis* individuals was observed in the later time points (**Fig. 17**). In the 85% tank, two individuals of *C. intestinalis* were dead on day 4 (4% of remaining individuals), four on day 8 (11%), and 18 individuals on day 16 (69%). As a result, there were not enough individuals of *C. intestinalis* living on day 16, and that time point was discarded from the analysis for that species. Taken across the entire

experiment, 22% of *C. intestinalis* individuals in the 85% salinity treatment perished. Additionally, 8 individuals of *C. intestinalis* were dead on day 16 in the 100% control tank.

C. intestinalis Significant Proteins

Using a proteome map generated from all gels in the experiment, a total of 263 spots were detected (**Fig. 18**). Of these, 25 (10% of total) were determined to show significant changes in protein abundance across the treatment groups for salinity treatment, 107 (41% of total) changed significantly with time, and 62 (24% of total) showed an interaction effect (ANOVA, $p < 0.01$). We were able to identify 21 (84%), 75 (70%), and 48 (77%) of the significantly changing spots in the salinity, time, and interaction effects, respectively (**Table 4**).

In this analysis, we focused on proteins that were significant only under the salinity effect, interaction effect, or both. We disregarded interpreting the proteins deemed significant under the time effect, as changes in abundance were not the result of the cells' acclimation to altered salinity.

Hierarchical clustering sorted the identified proteins significant under the salinity and interaction effects into clusters containing proteins that display similar patterns of abundance across treatments (**Fig. 19; Fig. 20**). Proteins

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identified in the salinity and/or interaction effects of
C. intestinalis fall into three broad functional categories:
protein homeostasis (2 proteins total: protein disulfide
isomerase and proteasome α -subunit), metabolic (7 proteins
total: two ATP synthase β subunits, adenylate kinase, GAPDH,
alcohol DH, triosphosphate isomerase, and Malate DH), and the
largest, cytoskeletal (49 proteins total: 33 actin isoforms, six

myosin isoforms, seven intermediate filament proteins, and three CaVPT protein isoforms).

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Table 4. Protein identifications (using MS/MS) of spots significantly changing abundance under the salinity and interaction effects in *C. intestinalis* chronic hyposalinity stress experiment.

Spot ID	Protein ID	MM Estimated (kDa)	pI Estimated	MM Predicted (kDa)	pI Predicted	GenBank ID	Mascot Score	Peptide Matches	Sequence Coverage (%)	Functional Category
8	ATP synthase, F1 complex, β -subunit	76	5.04	55.9	5.25	GI: 198421396	158	6	21	metabolism
12	actin, α -1, skeletal muscle	67	4.74	32.2	5.08	GI: 198424170	93	2	15	cytoskeletal
14	muscle actin isoform 2	67	5.64	42	5.46	GI: 198433893	215	2	22	cytoskeletal
25	IF-F (desmin)	64	4.67	47.1	5.1	GI: 12329935	266	2	19	cytoskeletal
29	actin, α -1, skeletal muscle	60	4.8	32.2	5.08	GI: 198424170	78	3	16	cytoskeletal
37	IF-F (desmin)	59	4.43	47.1	5.1	GI: 12329935	124	2	6	cytoskeletal
39	Superfast myosin heavy chain	58	6.38	239	5.6	GI: 198414692	458	8	27	cytoskeletal
40	Superfast myosin heavy chain	58	6.42	239	5.6	GI: 198414692	418	7	27	cytoskeletal
48	muscle actin isoform 1	56	4.79	42.2	5.18	GI: 198433891	212	5	22	cytoskeletal
53	Alcohol DH, iron-containing	52	6.45	50.3	6.28	GI: 198417702	59	1	10	metabolism
54	Muscle actin isoform 2	52	5.03	42	5.46	GI: 198433893	224	7	19	cytoskeletal
61	muscle actin isoform 1	52	4.82	42.2	5.18	GI: 198433891	221	4	22	cytoskeletal
63	muscle actin isoform 1	52	5.64	42.2	5.18	GI: 198433891	325	6	22	cytoskeletal
64	actin, α -1, skeletal muscle	51	5.99	32.2	5.08	GI: 198433596	144	3	18	cytoskeletal
65	muscle actin isoform 1	52	5.69	42.2	5.18	GI: 198433891	327	6	26	cytoskeletal
66	muscle actin isoform 2	51	4.72	42	5.46	GI: 198433893	154	3	18	cytoskeletal
68	IF-A (vimentin)	51	4.64	58	5.05	GI: 74096043	90	3	15	cytoskeletal
69	muscle actin isoform 1	51	4.68	42.2	5.18	GI: 198433891	226	5	22	cytoskeletal
74	muscle actin isoform 2	49	5.99	42	5.46	GI: 198433893	214	3	28	cytoskeletal
76	IF-A (vimentin)	48	4.73	58	5.05	GI: 74096043	90	2	15	cytoskeletal
78	muscle actin isoform 2	48	6	42	5.46	GI: 198433893	209	5	24	cytoskeletal
79	IF-A (vimentin)	46	4.63	58	5.05	GI: 74096043	73	1	15	cytoskeletal
80	IF-A (vimentin)	47	4.68	58	5.05	GI: 74096043	161	2	19	cytoskeletal
81	muscle actin isoform 2	45	4.81	42	5.46	GI: 198433893	201	3	24	cytoskeletal
93	Muscle actin isoform 1	41	5.02	42.2	5.18	GI: 198433891	341	6	26	cytoskeletal
96	Malate DH, mitochondrial	40	5.94	36	6.45	GI: 198434598	261	3	20	metabolism
98	muscle actin isoform 1	40	4.78	42.2	5.18	GI: 198433891	136	2	22	cytoskeletal
101	muscle actin isoform 1	36	4.79	42.2	5.18	GI: 198433891	414	8	22	cytoskeletal
103	Muscle actin isoform 1	36	5.05	42.2	5.18	GI: 198433891	308	7	26	cytoskeletal
104	muscle actin isoform 1	35	4.88	42.2	5.18	GI: 198433891	243	3	22	cytoskeletal
107	Muscle actin isoform 1	35	5.01	42.2	5.18	GI: 198433891	298	6	26	cytoskeletal
108	CsMA-1 isoform 2	34	5.73	42	5.3	GI: 198433786	254	3	29	cytoskeletal
110	Muscle actin isoform 2	34	4.97	42	5.46	GI: 198433893	159	4	24	cytoskeletal
113	muscle actin isoform 1	33	4.86	42.2	5.18	GI: 198433891	287	7	22	cytoskeletal

116	CaVP-target protein isoform 3	33	4.8	25.4	4.78	GI: 198422600	230	5	30	signaling
117	muscle actin isoform 1	33	4.93	42.2	5.18	GI:198433891	181	2	22	cytoskeletal
121	actin, α -1, skeletal muscle	32	5.71	32.2	5.08	GI: 198433596	205	4	18	cytoskeletal
122	Muscle actin isoform 2	32	5.01	42	5.46	GI: 198433893	117	2	19	cytoskeletal
125	Muscle actin isoform 1	31	5.08	42.2	5.18	GI: 198433891	148	3	16	cytoskeletal
128	CsMA-1 isoform 2	29	5.65	42	5.3	GI: 198433786	324	6	28	cytoskeletal
131	triosephosphate isomerase 1b	29	5.85	26.8	5.66	GI: 198413792	110	2	9	metabolism
133	CsMA-1 isoform 2	28	5.07	42	5.3	GI: 198433786	57	1	8	cytoskeletal
134	Hypothetical protein	28	5.6	12.6	5.24	GI: 297301442	85	3	5	N/A
141	CaVP-target protein isoform 3	26	4.78	25.4	4.78	GI: 198422600	124	1	14	signaling
144	CaVP-target protein isoform 3	26	4.7	25.4	4.78	GI: 198422600	126	1	22	signaling
149	Adenylate kinase isoenzyme 1	22	5.22	36.9	5.82	GI: 198436210	234	4	27	metabolism
158	Glyceraldehyde-3-phosphate DH	39	6.51	35.6	6.91	GI: 198433006	72	2	18	metabolism
169	IF-A (vimentin)	64	5.22	58	5.05	GI: 74096043	267	7	21	cytoskeletal
170	ATP synthase, F1 complex, β -subunit	60	5.26	55.9	5.25	GI: 198421396	158	6	21	metabolism
175	muscle actin isoform 1	54	5.19	42.2	5.18	GI: 198433891	338	8	22	cytoskeletal
180	muscle actin isoform 2	51	5.35	42	5.46	GI: 198433891	204	2	24	cytoskeletal
200	muscle actin isoform 1	89	5.65	42.2	5.18	GI: 198433891	347	9	26	cytoskeletal
202	Superfast myosin heavy chain	86	5.7	239	5.6	GI: 198414692	144	2	15	cytoskeletal
209	Protein Disulfide Isomerase	72	4.46	63.4	4.84	GI: 198418983	222	7	31	protein
222	Myosin light chain 5 isoform 1	16	4.51	19.2	4.75	GI: 198429379	252	3	26	Cytoskeletal
242	Proteasome subunit, β type, 2	24	6.48	22.7	6.09	GI: 198426046	216	2	24	protein
245	Myosin light chain 1, skeletal muscle	15	4.31	16.9	4.43	GI: 198424414	150	2	26	cytoskeletal
249	Myosin light chain 1, skeletal muscle	12	4.29	16.9	4.43	GI: 198424414	209	4	26	cytoskeletal
255	actin, α -1, skeletal muscle	9	6.97	32.2	5.08	GI: 198424170	124	2	15	cytoskeletal

C. savignyi Significant Proteins

Using a proteome map generated from all gels in the experiment, a total of 250 spots were detected (**Fig. 21**). Of these, 26 (10% of total) were determined to show significant changes in protein abundance across the treatment groups for salinity treatment, 94 (38% of total) changed significantly with time, and 27 (11% of total) showed an interaction effect (ANOVA, $p < 0.01$). We were able to identify 21 (81%), 60 (64%), and 17 (63%) of the significantly changing spots in the salinity, time, and interaction effects, respectively.

Hierarchical clustering of significant proteins under both the salinity and interaction effects sorted them into clusters containing that display similar patterns of protein abundance across treatments (**Fig. 22; Fig. 23**). Proteins identified in the salinity and/or interaction effects of *C. savignyi* fall into four broad functional categories: protein homeostasis (5 proteins total; two HR-29-like protein isoforms, two cathepsin D isoforms, and one proteasome α subunit), signaling (1 protein total; cold-inducible RNA BP), metabolic (2 proteins total; one creatine kinase and malate DH), and the largest, cytoskeletal (22 proteins total; 17 actin isoforms, three myosin isoforms, and two intermediate filament proteins).

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Table 5. Protein identifications (using MS/MS) of spots significantly changing abundance under the salinity and interaction effects in *C. savignyi* chronic hyposalinity stress experiment.

Spot ID	Protein ID	MM Estimated (kDa)	pI Estimated	MM Predicted (kDa)	pI Predicted	GenBank ID	Mascot Score	Peptide Matches	Sequence Coverage (%)	Functional Category
52	actin	74	5.78	41.7	5.29	GI: 86610891	76	2	12	cytoskeletal
56	IF-A (vimentin)	67	5.43	58	5.05	GI: 74096043	169	2	28	cytoskeletal
66	muscle actin isoform 1	65	4.88	42.2	5.18	GI: 198433891	368	6	26	cytoskeletal
88	IF-A (vimentin)	57	4.89	58	5.05	GI: 74096043	175	3	28	cytoskeletal
96	muscle actin isoform 1	54	5.27	42.2	5.18	GI: 198433891	436	9	26	cytoskeletal
118	creatine kinase	52	6.78	42.4	5.89	GI: 74096183	283	7	22	metabolism
124	muscle actin isoform 2	50	6.26	42	5.46	GI: 198433893	188	5	24	cytoskeletal
129	muscle actin isoform 2	47	6.24	42	5.46	GI: 198433893	209	4	24	cytoskeletal
136	cathepsin D	45	6.11	41.9	4.69	GI: 198421979	153	4	20	protein homeostasis
144	Malate DH	41	6.39	36	6.45	GI: 198434598	121	1	13	Metabolism
148	cathepsin D	41	6.1	41.9	4.69	GI: 198421979	157	2	20	Protein homeostasis
163	muscle actin isoform 1	37	4.89	42.2	5.18	GI: 198433891	376	6	26	Cytoskeletal
166	muscle actin isoform 2	37	4.98	42	5.46	GI: 198433893	201	4	22	Cytoskeletal
175	actin, α -1, skeletal muscle	34	5.64	32.2	5.08	GI: 198433596	154	4	18	Cytoskeletal
178	actin, α -1, skeletal muscle	34	5.71	32.2	5.08	GI: 198433596	133	3	18	Cytoskeletal
179	Psma6-prov protein isoform 2	34	6.37	27.9	6.34	GI: 198422472	192	4	18	protein homeostasis
182	actin, α -1, skeletal muscle	34	6.09	32.2	5.08	GI: 198424170	95	2	18	cytoskeletal
183	HR-29-like protein	33	6.43	25.1	5.89	GI: 74096307	197	2	38	extracellular matrix
189	CAVPT protein isoform 3	33	5.03	25.4	4.78	GI: 198422600	414	7	37	signaling
194	actin, non-muscle 6.2	32	5.57	41.7	5.29	GI: 198424630	186	6	19	cytoskeletal
195	HR-29-like protein	32	6.73	25.1	5.89	GI: 74096307	152	1	37	extracellular matrix
202	CsMA-1 isoform 2	29	4.98	42	5.3	GI: 198433786	104	2	13	cytoskeletal
226	myosin light chain 5	17	4.77	19.2	4.75	GI: 198429379	344	5	19	cytoskeletal
228	myosin light chain 5	17	4.68	19.2	4.75	GI: 198429379	348	4	32	cytoskeletal
229	cold-inducible RNA BP	17	5.57	16.5	5.25	GI: 198426742	130	2	65	signaling
230	myosin light chain 5	16	4.6	19.2	4.75	GI: 198429379	258	3	28	cytoskeletal
232	actin	14	4.83	41.7	5.29	GI: 86610891	185	5	34	cytoskeletal
236	myosin light chain 5 isoform 1	13	5.87	19.2	4.75	GI: 198429379	450	7	42	cytoskeletal
241	CsMA-1	12	4.97	42.1	5.23	GI: 198429195	86	2	11	cytoskeletal
242	actin, α -1, skeletal muscle	12	6.74	32.2	5.08	GI: 198424170	246	2	26	cytoskeletal
245	actin, α -1, skeletal muscle	11	6.81	32.2	5.08	GI: 198424170	205	2	26	cytoskeletal
249	CsCA1	10	4.84	41.7	5.29	GI: 3036959	252	5	34	cytoskeletal

Discussion - Acute Exposure

Principal Component Analyses

As both principal components (PCs) from *C. intestinalis* Sp. A (Santa Barbara, CA) contributed to the separation of the 85% treatment from the other two treatments shows that the response of *C. intestinalis* Sp. A is most robust at this level of stress. The overlap between the 100% control and 70% shows that *C. intestinalis* Sp. A is not modifying its proteome under severe hyposaline stress within the 6 hr. exposure period. Indeed the abundance profiles of most proteins identified for *C. intestinalis* Sp. A show a biphasic response to acute hyposaline stress with either an increase or decrease in abundance at 85% from the 100% control with a nearly control levels at 70%.

Additionally, the PCAs of both *C. savignyi* and *C. intestinalis* Sp. B (Bergen, Norway) show that all three treatment groups separate distinctly from one-another. This suggests that these two species are capable of mounting a response at the proteome level to salinities as low as 70% stress while *C. intestinalis* Sp. A is incapable of doing so within the 6 hr. exposure period plus recovery. This is

notable in comparison to *C. intestinalis* Sp. B, where the control salinity was over 200 mOsm less than the control for organisms collected in California (923 mOsm Santa Barbara vs. 704 mOsm Bergen), so individuals of *C. intestinalis* Sp. B are already adapted to salinity levels similar to the 85% treatment for *C. intestinalis* Sp. A.

Translational arrest, the halting of protein synthesis during stress, could explain this lack of change in *C. intestinalis* Sp. A. Translational arrest is used to selectively translate mRNAs encoding repair proteins and enzymes while minimizing energy consumption synthesizing nonessential housekeeping proteins (Yamasaki & Anderson, 2008; Yamasaki et al., 2009). While osmotic stress has been implicating in promoting some of the factors leading to translational arrest, namely in the TOR (target of rapamycin) pathway (Reiling & Sabatini, 2006), we did not identify any molecular chaperones or other repair proteins to be increasing abundance at the 70% treatment as would be expected under translational arrest. This suggests that translational arrest has not exhibited an effect in these cells within the 6 hr exposure period, and the lack of a response at the 70% treatment in *C. intestinalis* Sp. A is

due to the severity of the stress, not necessarily an adaptive mechanism of the cell.

A behavioral response could potentially explain the PCA pattern seen in *C. intestinalis* Sp. A. Siphon closure could limit exposure to a hyposaline medium, but the ascidian tunic is not entirely water-impermeable and does not always protect against osmotic influx (Goodbody, 1961; Sims, 1984).

When looking at the ecology of these animals, we see that *C. savignyi* is capable of expanding its population faster than *C. intestinalis* Sp. A following winter rains (Lambert & Lambert, 2003; Lambert & Lambert, 2008). The PCAs of these species suggest that *C. intestinalis* Sp. A has a limited range of salinity tolerance compared to *C. savignyi*, and such tolerances may play a role in their differing ecology in the bays and estuaries of the California coast.

Proteins by Functional Group

Cytoskeletal & Extracellular Matrix proteins

Actins were the most numerable proteins identified as changing across the three species (**Fig. 24**). For *C.*

intestinalis Sp. A (Santa Barbara, CA) 37 (57%) of the 65 significant spots were identified as cytoskeletal (actins, intermediate filaments, tubulins). To determine that the high percentage of cytoskeletal identifications was due to stress and not random chance associated with high normal abundance, we randomly selected 65 of the 347 detected *C. intestinalis* Sp. A spots (the same number that were significant under the original one-way ANOVA) for identification to test for their abundance within this random set. Of the randomly selected spots 24 (37%) of the 65 spots were identified as cytoskeletal in nature. This much lower ratio suggests that cytoskeletal proteins are preferentially changing in response to hyposaline stress.

Extracellular matrix and cytoskeletal proteins in *C. intestinalis* Sp. A separate into two distinct clusters, the first containing proteins with decreased abundance in the 85% treatment relative to 100% and 70% and the second containing proteins with increased abundance in the 85% treatment relative to the others. The first cluster contains 12 proteins, including 6 actin isoforms, 3 collagen isoforms, 2 α -tubulins and one β -tubulin. The second cluster contains 28 proteins including 17 actin isoforms, 3 α -tubulins and 8 β -tubulins. The majority of

actins (17/23 total) identified in *C. intestinalis* Sp. A are upregulated in the 85% treatment. The majority of both α - and β -tubulins (11/14 total) were upregulated at the 85% treatment along with the aforementioned actins. Additionally, we identified capping and cofilin 2, actin-binding proteins that modulate the rate of actin treadmilling, both with relatively higher abundance in 100% compared to 70%, but with capping exhibiting relatively low abundance compared to cofilin 2 in the 85% treatment (see below).

Cytoskeletal proteins in *C. intestinalis* Sp. B

separate into three clusters of protein abundance. The first cluster exhibits an increase in the 85% treatment and includes two actin isoforms, an α -tubulin and a β -tubulin. The second cluster has decreased

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abundance from 100% down to 70% and includes two actin isoforms. The third cluster shows increased abundance in the 70% treatment and includes five actin isoforms, two α -tubulins and two small G-proteins involved in vesicle transport, Rab-6A and Arf-5. Two of the actins in the first cluster have identical molecular weight counterparts in the second cluster, suggesting a treatment-induced post-translational modification shifting the pI of these proteins. Taking these shifts in MW into account, all actins identified in *C. intestinalis* Sp. B exhibit increased abundance in response to either the 85% or 70% treatments as do the α - and β -tubulins.

C. savignyi had two actin isoforms exhibiting increased abundance in the 70% treatment. We also identified an intermediate filament A (vimentin) with a similar pattern of abundance. Myosin light chain 5 and α -tubulin increased abundance in the 85% treatment. Annexin A13, a protein believed to be the progenitor of other vertebrate annexins (Turnay et al., 2005), was found to have increased in the 85% treatment as well (see below).

The actin cytoskeleton has long been cited as playing a role in sensing and responding to changes in cell volume, but precise mechanisms have not yet been elucidated (for

reviews see Di Ciano-Oliveira et al., 2006; Pedersen et al., 2001). Additionally, the actin cytoskeleton can serve as a structural protective mechanism (Cai et al., 1998), controlling volume regulatory ion transport, and regulating gene transcription following volume fluctuation. Osmotic swelling results in a loss of membrane invaginations modulated by the actin cytoskeleton (Okada, 1997) and a decrease in net cellular content of polymerized actin (Pedersen et al., 1999). Cytochalasin and colchicines have been shown to inhibit RVD by disrupting F-actins and microtubules (Downey et al., 1995) and osmotic stress induces the remodeling of the cortical cytoskeleton to strengthen the cell cortex (Di Ciano et al., 2002).

Our results show an overall increase in the abundance of actin and tubulin isoforms in response to hyposaline exposure in all three species, suggesting that a major restructuring of the cytoskeleton occurs following hypo-osmotic challenge. This is supported by the opposing patterns of abundance in cofilin and cappin in *C. intestinalis* Sp. A (**Fig.**

25). Cofilin (also known as actin depolymerizing factor) and cappin are part of the core set of proteins responsible for regulating the growth of actin filaments (Pollard & Borisys, 2003). Cofilin severs and depolymerizes old actin filaments, freeing barbed ends for new polymerization (DesMarais et al., 2005; Ghosh et al., 2004) while cappins inhibit polymerization by binding to the barbed ends of actin filaments and preventing the association of F-actin monomers (Pollard & Borisys, 2003;

Carlier, 1998). The slight increase in cofilin abundance at the 85% treatment suggests an increase in the rate of actin filament turnover enhanced by a decrease in capping, leaving the ends of actin filaments open to further disassembly. Vimentin, shown to increase at 70% in *C. savignyi* is the primary intermediate filament protein implicated in cell integrity (Goldman et al., 1996), and may play a role in resisting mechanical stress during volume increase caused by severe hyposalinity stress.

Our data also suggests that vesicle recycling may be increased under hyposalinity stress. Both Rab6A and Arf5 exhibited increased abundance at 70% in *C. intestinalis* Sp. B and serve functional roles in vesicular trafficking. Rab6A regulates anterograde and retrograde movement of late endosomes from the Golgi and Arf5 governs assembly of coat protein 1 (COPI) and clathrin coats at Golgi membranes, a process key to initial vesicle formation (Chavrier & Goud, 1999; Kawasaki et al., 2005). The increase in tubulin abundance under treatment across all three species may be providing components for a microtubule network within the cell for vesicle transport (Cole & Lippincott-Schwartz, 1995).

A number of proteins that serve functional roles in vesicle transport were identified across all three species. Annexin A13, identified in *C. savignyi*, has extensive homology with annexin B13, a protein involved in raft-mediated delivery of vesicular transport to the apical plasma membrane of MDCK cells (Fiedler et al., 1995; Astanina et al., 2010). Annexin A13 differs from annexin B13 in that its function is calcium-independent (Lecat et al., 2000). Lastly, myosin light chain 5 was upregulated at 85% in *C. savignyi*, and has been shown to regulate the velocity of vesicle transport within the cell (Langford, 2002). Myosin light chain 5 functions as a processive actin-based motor, concentrated in the cortical actin cytoskeleton where it is hypothesized to interact with the microtubule-based motor kinesin in transferring cargo from the microtubule cytoskeleton to the peripheral actin cytoskeleton (Mehta et al., 1999; Ali et al., 2008). Increased vesicle recycling in response to cell swelling was demonstrated in at least one other study (van der Wijk et al., 2003).

Protein Processing and the Proteasome

Proteins related to protein processing and the proteasome were identified in both *C. intestinalis* Sp. A

and *C. intestinalis* *Sp. B* (Fig. 26). In *C. intestinalis* *Sp. A*, one proteasome α -5 isoform decreased in abundance in the 85% and 70% treatments while the other increased in

the 85% treatment. These two proteins have identical molecular weights, suggesting a role in treatment-induced post-translational modification of this protein. Another proteasome subunit, proteasome α -6 subunit isoform 2 decreased in the 85% treatment while a peptidase M1 increased in the 85% treatment relative to the others. Chaperone proteins HSP70 and FK506 Binding Protein 9 (which aids in folding proline-rich regions of peptide chains) decreased in the 85% treatment. Based on HSP70's location

on the gel (~32 kDa), it is most likely a larger member of the 20-30 kD family of HSPs.

In *C. intestinalis* *Sp. B* proteasome α -2 subunit isoform 1 increased in the 85% treatment while a proteasome β subunit type 3 decreased in the 85% and increased in the 70% treatment relative to the control. HSP90 β decreased in the 85% treatment and increased at 70%, but based on its location on the gel (~32 kDa) it also is most likely a member of the 20-30 kDa family of HSPs, like HSP27. Lastly, two isoforms of the ER-localized chaperone calregulin increased in abundance in the 85% treatment in *C. intestinalis* *Sp. B*. No proteins involved in protein processing were identified in *C. savignyi*.

It has been hypothesized that protein synthesis is increased under hypotonic stress in order to maintain a constant protein concentration in the cytosol (Haussinger, 1996). It would be expected that under this hypothesis, a decrease in proteolytic proteins would be observed in order to enhance net synthesis. Our data instead suggests an increase in proteasome subunits and peptidases, which parallels results observed in mouse embryonic stem cells (Mao et al., 2008), but stands in contrast with results published by Haussinger (1996). A hypothesis put forth by

Mao et al. (2008) suggests that increased protein synthesis during hypo-osmolarity would require increased protein turnover in order to provide the free resources for increased biosynthesis. Within *C. intestinalis* Sp. B, the observed increases in protein chaperones (HSPs and calregulin) due to hypo-osmolarity support the hypothesis that protein synthesis is increased simultaneously with protein degradation. It is less clear that this is the case with *C. intestinalis* Sp. A, where it seems protein synthesis is decreased under hypo-osmolarity, as both identified chaperone proteins, HSP70 and FK506 BP, decreased at 85%. Protein degradation may also be less pronounced in this species as only one of the three proteasome subunits increased under stress along with a peptidase.

Calcium Binding Proteins

Proteins associated with Ca²⁺ were the Calcium Vector Protein Target (CaVPT) proteins in *C. intestinalis* and *C. savignyi* (**Fig. 27**). In *C. intestinalis*, three isoforms of CaVPT protein increased in abundance in the 85% treatment. In *C. savignyi*, two isoforms decreased in abundance as salinity decreased and one increased in abundance in the 70% treatment.

Not very much is known about the function of CaVPT in invertebrates. Based on structural characteristics, it is hypothesized to be

involved in myofibrillar assembly (Takagi & Cox, 1990; Cox, 1986). Our data shows that *C. savignyi* increases the abundance of CaVPT at the more severe 70% stress compared to *C.*

intestinalis Sp. A, which increase CaVPT at the less severe 85% stress.

Metabolism

Proteins involved in energy metabolism and other related functions were identified across all three species (**Fig. 28**). ATP Synthase β subunits were identified between all three species. In *C. intestinalis* Sp. A one isoform decreased in the 85% treatment while another increased in the same treatment. In *C. savignyi* an isoform increased in the 85% treatment. Lastly, in *C. intestinalis* Sp. B one isoform decreased in the 85% treatment and then increased in the 70% treatment relative to the control.

Additionally, a number of enzymes not directly related to ATP production were identified across all three species. Peroxiredoxin 6 (Prx6) in *C. intestinalis* *Sp. A* increased in the 85% treatment.

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Fatty acid binding protein (FABP) 2 in *C. savignyi* decreased in abundance in the 85% treatment. In *C. intestinalis Sp. B* embryonic 1 increased in the 85% treatment and 70% compared to the control while β -1,3(4)-glucanase, an enzyme that degrades cellulose, decreased in the 85% treatment.

The increase in ATP synthase β across all three species supports an increase in energy production. ATP

production is key to fueling the active transport mechanisms in RVD along with the energy intensive processes of cytoskeletal restructuring and protein synthesis. The decrease in FABP2, an intestinal isoforms responsible for assimilation of dietary lipid (Storch & Thumser, 2010; Storch & McDermott, 2009), in *C. savignyi* suggests a decrease in fatty acid metabolism under stress.

The increase in Prx6 in *C. intestinalis* suggests that oxidative stress may be a co-stressor to hyposalinity stress. Peroxiredoxins are an abundant class of antioxidant enzymes that reduce peroxides (Wood et al., 2003). Prx6 exhibits phospholipase A2 activity (Chen et al., 2000) and is hypothesized to repair membrane damage induced by oxidative stress and can shuttle between cytoplasm and the mitochondria (Eismann et al., 2009). Prx6 was also shown to increase under hypo-osmotic conditions in mouse embryonic stem cells (Mao et al., 2008) as well as during oxidative stress in human lung cells (Chowdhury et al., 2008), suggesting that oxidative stress is a co-stressor to hyposalinity stress. While this protein was significantly identified with a single peptide that covered only 4% of the sequence length, its appearance in the dataset by Mao

et al. (2008) enhances our confidence in its identification in our dataset.

Lastly, The glucanase enzyme identified in *C. intestinalis* Sp. B carries homology with the glycoside hydrolase 16 domain (GH16), which has been implicated in the anti-microbial Toll signaling pathway despite the absence of hydrolase functionality (Lee et al., 2009). The decrease in abundance of GH16 at 85% suggests that there may be an suppression of the immune system during stress. The function of embryonic 1 is unknown, according to BLASTp searches, lacks extensive homology with any other protein.

Species-specific responses to acute stress

Based on the PCA results, *C. intestinalis* Sp. A appears incapable of mounting a stress response at the 70% treatment, or is employing a behavioral modification to limit its exposure to the stress. It does have a response at the 85% treatment, which appears tailored toward maintaining cell integrity and reducing energy consumption. Cell integrity is achieved as cofilin and cappin regulate actin turnover dynamics and vimentin is increased while energy is conserved by decreasing protein synthesis and

degradation. Peroxiredoxin 6 exhibited an increase, which suggests oxidative stress may be a challenge in this species, providing a possible explanation as to why this species would minimize protein turnover.

All three species exhibited increases in actin and tubulins isoforms in response to stress along with increases in ATP-synthase subunits. While all three species exhibited responses within the same protein functional classes, the patterns of abundance change within each species which suggests that all three have established different defense mechanisms in response to hyposaline stress.

Both *C. intestinalis Sp. B* and *C. savignyi* exhibited responses at the 70% treatment, but different from their respective responses at 85%. *C. intestinalis Sp. B* exhibited increases in proteins involved in vesicle transport (Rab6A and Arf5) while protein turnover (increases in chaperones and proteasome subunits) is increased along with ATP synthesis. A decrease in the immune capability may be indicative of a trade-off from the energy requirements to maintain cellular integrity during hyposaline stress, as indicated by the decrease in the GH16 domain in *C. intestinalis Sp. B*.

Unlike the other two species, *C. savignyi* did not exhibit alterations in protein turnover. Similar to *C. intestinalis* Sp. B, proteins involved in peripheral vesicle transport were increased. *C. savignyi* had the fewest protein spots exhibit changes in abundance in response to hyposaline stress, indicating a more limited stress response. Perhaps other stress proteins may be constitutively higher in *C. savignyi* compared to the other two *Ciona* congeners (Serafini et al., 2011) (see **Conclusions**).

Discussion – Chronic Exposure

Protein Homeostasis

C. intestinalis

Only one protein involved in protein homeostasis, proteasome α subunit, significantly changed abundance under the salinity effect. This protein decreased in the 85% treatment, with differences greatest at 6 h and the later time points of 4 and 8 d (**Fig. 31**). Only one protein involved in protein homeostasis, protein disulfide isomerase, was significant under the interaction effect. This protein showed a down-regulation at 6 h relative to the control (**Fig. 32**).

The down-regulation of protein disulfide isomerase suggests that protein synthesis is decreased under initial stress but returns to normal levels afterwards while there is a net decrease in protein degradation given by the proteasome α subunit. These results suggest that net protein synthesis is increased under hyposaline stress in order to maintain an adequate protein concentration in the cell or because of an increase in cell volume (Haussinger, 1996).

C. savignyi

Four proteins involved in protein homeostasis were significant under both the salinity and interaction effects. Two isoforms of the ascidian sHSP protein HR-29 show nearly opposite patterns of abundance, either up- or down-regulated in the 85‰ treatment primarily at the later time points, 8 and 4 d. respectively. The other two proteins, proteasome α subunit and the peptidase cathepsin D both showed a net increase in the 85‰ treatment, with the largest increase in abundance at 4 d (**Fig. 33**).

One protein, a cathepsin D isoform, significantly increased abundance under the interaction effect alone. Cathepsin D is a lysosomal cysteine protease responsible for activation of other cathepsins by cleaving their N-terminal protopeptides (Turk et al., 2001). The greatest increase in the 85‰ treatment was observed at 4 days of exposure (**Fig. 34**).

C. savignyi does not appear to modify abundance of proteases and chaperones until much later time points. At 4 d, there appears to be an increase in proteolytic activity (proteasome α subunit, cathepsin D), which is enhanced by a lowering of protein synthesis as indicated by HR-29 protein, a sHSP unique to the body wall muscle of ascidians

(Caspers et al., 1995). Later, at 8 d protein synthesis increases (HR-29). Overall, it seems that *C. intestinalis* modifies protein turnover earlier in response to hyposaline stress than *C. savignyi*.

Signaling

One signaling protein, cold-inducible RNA binding protein (CIRP), was significantly changing under the interaction effect. It displayed up-regulation in the 85% treatment at 4 d (**Fig. 34**).

In mammals and amphibians, CIRP is induced by cold stress and leads to suppression of cell growth (Nishiyama et al., 1997). A similar CIRP has been shown to be upregulated in salmon (*Salmo salar*) in response to hyperosmotic stress, suggesting that CIRP in aquatic animals may play a role in adaptation to osmotic conditions rather than cold stress, though its role in this context has not yet been elucidated (Pan et al., 2004).

Metabolic

C. intestinalis

One protein, an ATP synthase β subunit isoform, changed significantly under the salinity effect, decreased on average in the 85% treatment with greatest decrease in abundance at 6 h. Only one protein, GAPDH, was significant under both salinity and interaction effects, increasing abundance on average in the 85% treatment, with the greatest increase at 12 h (**Fig. 31**).

The remaining five proteins were significantly changing under the interaction effect alone. Another ATP synthase β subunit isoform is down-regulated at 6 h but is then up-regulated at 24 h. Malate dehydrogenase (DH) follows the same pattern. Adenylate kinase shows an increase in abundance at 48 h followed by a down-regulation at 4 and 8 d. Alcohol DH is down-regulated at 4 d. Triosphosphate isomerase 1 increased at 48 h (**Fig. 32**).

Early in the time course at 6 h, ATP production and metabolism appear depressed as ATP synthase β subunits exhibit decreases along with malate DH. This seems to recover with GAPDH increasing at 12 h and remaining elevated for the rest of the experiment, likely playing a

role in energy production but also potentially alleviating oxidative stress (Dastoor & Dreyer, 2001). We also observe increases in another ATP synthase β subunit isoform along with malate DH at 12 h, followed by increases in adenylate kinase and triosphosphate isomerase 1 at 48 h. At the later time points we see alcohol DH decrease at 4 d along with decreases in adenylate kinase at 4 and 8 d.

A number of these proteins also play important roles in the synthesis of glycerol, an osmolyte produced in response to hyper-osmotic challenge (Nevoigt & Stahl, 1997). The glycerol phosphate shuttle utilizes NADH to synthesize glycerol 3-phosphate, which can be converted back to dihydroxyacetone phosphate in the mitochondria to reduce FAD to FADH₂ in order to power oxidative phosphorylation. The increases that we see in GAPDH and triosphosphate isomerase 1 serve to produce the precursors for glycerol 3-phosphate (NADH and dihydroxyacetone phosphate, respectively).

Malate DH seems to be altered in abundance in parallel with ATP synthase subunits. The production of NADH by malate DH would also provide additional protons for the synthesis of glycerol 3-phosphate and to fuel oxidative phosphorylation. The decreases observed in alcohol DH could

be indicative of the cell limiting consumption of NADH in the conversion of acetaldehyde to ethanol, leaving more NADH free in the cell to fuel the aforementioned processes.

We also observe a number of proteins involved in maintaining energy homeostasis, such as adenylate kinase, a protein involved in maintaining a pool of ATP from excesses of ADP and AMP. All of these enzymes serve to maintain a high level of ATP in the cell in order to offset the energy costs of countering stress.

C. savignyi

One protein, creatine kinase, changed significantly under the salinity effect, down-regulated on average in the 85% treatment with its greatest decrease in abundance at 24 h (**Fig. 33**). Only one protein, Malate DH, was significantly changing under the interaction effect, with higher abundance at 6 h and lower at 24 h (**Fig. 34**).

As comparatively fewer metabolic proteins were identified in *C. savignyi* compared to *C. intestinalis*, it suggests that *C. savignyi* does not need to modify its metabolism as much as its sister species in order to manage hypo-osmotic stress. Initially, at 6 h, we see there was an increase in malate DH that may be indicative of

increased energy expenditure. This is followed by a decrease at 24 h, along with creatine kinase, which may suggest that some level of energetic homeostasis is achieved after 24 h of stress.

Cytoskeletal

C. intestinalis

Of all the actins identified in *C. intestinalis*, eight were significantly changing under the salinity effect, three under both the salinity and interaction effects, and 22 under the interaction effect alone (**Fig. 29**). The salinity effect actin isoforms were mostly up-regulated in response to stress, with only two of the eight exhibiting down-regulation across all time points on average. All three actins significant under both the salinity and interaction effect were up-regulated on average in response to stress, with two isoforms primarily up in the later time points (4 or 8 d) and the other up-regulated in the 12h and 24h time points. The patterns for the actin isoforms significant under the interaction effect are less clear, but a majority of them (19 actin isoforms) exhibited initial change in its level of abundance within acute exposure (first 24 h of exposure), with 12 down-regulated

in the acute and the other 7 up-regulated in response to stress. The remaining three isoforms exhibited initial change during chronic exposure (from 2 to 8 d), with two down-regulated and the other up-regulated in response to stress.

Six myosin isoforms were identified in *C. intestinalis*, with three changing abundance significantly under both salinity and interaction effects and three significant under the interaction effect alone. Of the three that were significantly changing under both salinity and interaction effects, two (both myosin heavy chain) were up-regulated on average across time points, with the biggest increase in abundance at the later time points (4 and 8 d). The other myosin (myosin light chain 5) decreased abundance across time points with a notable decrease at 48 h and 4 d. Of the three myosins significantly changing solely under the interaction effect, two (myosin light chain 1) exhibit increases at 24 h and another (myosin heavy chain) exhibits a decrease at 24 h.

Seven intermediate filament proteins were identified in *C. intestinalis*. Two of the proteins, isoforms of vimentin and desmin, were both significantly changing abundance under both the salinity and interaction effects.

Both were down-regulated on average in response to stress, with desmin exhibiting a marked down-regulation at the 12 h time point. The other five proteins were significantly changing under the interaction effect alone. Two of the interaction-significant proteins, vimentin and desmin, are down-regulated at the 12 h time point while the other three, all vimentin isoforms, show increases under stress at 24 h and 4 d.

Lastly, three CaVPT isoform 3 proteins were identified in *C. intestinalis*. One was significantly changing under both salinity and interaction effects and displayed an increase in the 85% treatment at 4 d. The other two isoforms were changed abundance significantly under the interaction effect alone with one exhibiting an increase at 4 d and the other a down-regulation at 6 h.

As mentioned in the discussion of the acute exposure, the actin cytoskeleton is more than a structural unit, but also functions to both sense and respond to changes in cell volume. For *C. intestinalis*, while actin isoforms were split nearly evenly between increasing and decreasing abundance during stress, most that exhibited decreases in abundance did so during the acute time points (within 24 h). Without knowing the abundance of actin binding

proteins like capping or actin depolymerizing factor (cofilin), it is difficult to speculate as to what is happening to the cytoskeleton. It appears that there is a clear discrepancy between actin dynamics at the acute time points and later on in the chronic time points (after 24 h) for *C. intestinalis*.

Myosins are closely associated with the actin cytoskeleton. Two myosins involved in vesicle transport, myosin V and myosin I, exhibited decreases in abundance. Myosin V functions as a processive actin-based motor, where it is hypothesized to interact with the microtubule-based motor kinesin in transferring cargo from the microtubule cytoskeleton to the peripheral actin cytoskeleton (Mehta et al., 1999; Ali et al., 2008). Myosin I is also involved in vesicular traffic, linked primarily to the movement of lysosomes within the cell (Cordonnier, 2001). Decreases in these two proteins suggest that vesicular movement may be inhibited during hypo-osmotic stress, even when some have reported that cell swelling increases vesicular recycling (van der Wijk et al., 2003). Increases in two isoforms of heavy-chain myosin at the later 4 and 8 d time points may be indicative of an adaptation to increase the capacity of

the body wall muscle and enhance siphon closure during osmotic challenge.

This appears to be contrasted by early decreases in desmin at 12 h, an intermediate filament that enhances tensile strength of myofibrils in muscle tissue (Li et al., 2007). Isoforms of another intermediate filament, vimentin show initial decreases at 12 h, but others show increases at later time points. Vimentin is the primary intermediate filament protein implicated in cell integrity (Goldman et al., 1996), and the increases seen at later time points may be an adaptation to chronic hyposaline conditions in order to resist mechanical stress from further changes in cell volume.

As mentioned on p.128, not very much is known about the function of CaVPT in invertebrates. As it is hypothesized to function in myofibrillar assembly (Takagi & Cox, 1990; Cox, 1986), so it may be linked to the changes observed in desmin and heavy chain myosins in our data.

C. savignyi

For the actins identified in *C. savignyi*, 11 were changing significantly under the salinity effect, one under

both the salinity and interaction effects, and five under the interaction effect alone (**Fig. 30**). The salinity effect actin isoforms were mostly up-regulated in response to stress, with only one of the 11 exhibiting down-regulation across all time points on average. The actin isoform significant under both the salinity and interaction effect was up-regulated on average in response to stress, with a major increase in abundance at 16d. The patterns for the actin isoforms significant under the interaction effect are less clear, but two exhibited initial change in abundance within acute exposure (first 24h. of exposure), with one down-regulated and the other up-regulated in response to stress. The remaining three isoforms exhibited initial change during chronic exposure (48h to 16d.), with two increasing and the other decreasing abundance in response to stress.

Four myosin isoforms were identified in *C. savignyi*, with one significant under the salinity effect alone, one significant under both salinity and interaction effects and two significant under the interaction effect alone. Myosin light chain 5, significant under the salinity effect, was down-regulated on average across time points. Another myosin light chain 5, significant under both salinity and

interaction effects, was down-regulated on average across time points with a decrease in the 24h through 4d. Of the two myosin light chain 5 isoforms significant solely under the interaction effect, one decreased at the 6h time point while the other decreased at 4d.

Two intermediate filament proteins, both vimentins, were identified in *C. savignyi*. One was significant under the salinity effect and exhibited down-regulation on average across all time points under stress. The other was significant under the interaction effect and showed an increase at 4d.

Lastly, a CaVPT isoform 3 was identified under the salinity effect in *C. savignyi* and was down-regulated on average in response to stress.

In contrast to *C. intestinalis*, the majority of actins identified in *C. savignyi* increased in abundance in response to hyposalinity, with only two isoforms out of fifteen exhibiting decreases. Once again, it is difficult to determine what this means without knowing the abundances of actin-binding proteins, but increases in F-actin monomers could provide more material for strengthening the actin cytoskeleton following change in cell volume. Gauging fluctuations in actin abundance as an indicator for

acclimation to stress, the increases in actin abundances at 16 d in *C. savignyi* suggests that individuals are still making adjustments to hypo-salinity even so far into the time course. However, any adjustments made at these later time points do not seem to impact the fitness of these individuals, as no mortalities were observed in the tanks housing *C. savignyi* during the length of the exposure.

Myosin V isoforms decreased in *C. savignyi*, similar to what we saw in *C. intestinalis*. This may be indicative of a decrease in vesicular traffic to the periphery of the cell. No other myosin types were identified in *C. savignyi*, such as myosin I or myosin heavy chain. Two intermediate filament proteins, both isoforms of vimentin, were shown to be changing. Like in *C. intestinalis*, there was a mix of increase and decrease in vimentin abundance, which may reflect a restructuring of the cellular scaffold or cell shape, especially given vimentin's role in maintaining cell integrity. The lack of desmin and myosin heavy chain identifications indicates that *C. savignyi* may not need to modify its musculature in response to this level of hyposaline stress. This is supported by the decrease in abundance of CaVPT protein isoforms in *C. savignyi*. If this protein indeed has a role in myofibrillar assembly, a lack

of sarcomere strengthening or modification might be a factor in decreasing its abundance in the cell.

Species-specific Responses to Chronic Stress

As seen in the acute exposure experiment, the PCA results (generated using individuals from the same *C. intestinalis* population used in this experiment) demonstrated that *C. intestinalis* Sp. A appears incapable of mounting a stress response at the 70% treatment, while the other two species, *C. savignyi* and *C. intestinalis* Sp. B (individuals from a Norwegian population in the north Atlantic) were capable of modifying protein abundance at the lowest treatment. The failure of *C. intestinalis* Sp. A to mount a response at the low 70% salinity led us to limit the exposure level for this chronic study to 85%.

Given the mortality data from this experiment it appears that even 85% salinity may be too low for *C. intestinalis* to manage over the long term, given that deceased individuals were discovered just four days into the exposure continuing through to the last day, preventing us from having enough individuals for our final 16 day time point for this species. This is contrasted with the lack

of mortality by *C. savignyi* in either the control or exposure tanks over the 16 day exposure.

While the two species share similar trends in the proteins altered in response to chronic hypo-salinity exposure, there are a few key differences. *C. intestinalis* appears to modify components involved in protein turnover and homeostasis at earlier time points than *C. savignyi*. Only one protein directly involved in transcriptional signaling, CIRP, was shown to increase abundance in *C. savignyi* alone. A smaller number of metabolic proteins were identified in *C. savignyi*, suggesting that *C. savignyi* does not need to alter its metabolism in response to hypo-saline stress to the same degree as *C. intestinalis*. And lastly, *C. savignyi* demonstrated a greater proportion of actin isoforms to be up-regulated in response to stress compared to *C. intestinalis*, while *C. intestinalis* had more proteins involved in sarcomere function increase abundance during stress, suggesting that *C. intestinalis* modifies its muscle structure in favor of its cellular cytoskeleton in response to hypo-salinity.

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Conclusions

The nearly global distribution of *Ciona* congeners across a wide expanse of latitudes provides a unique study system for tracking the responses of marine invertebrates to global and regional impacts of global warming. The availability of sequenced and annotated genome experiments makes them ideal targets for -omics approaches.

In the case of the Pacific coast of North America, where *C. savignyi* and *C. intestinalis* compete, our study indicates that cellular responses to hyposalinity stress may be able to play a role in the future of these communities. The apparent inability of *C. intestinalis* Sp. A to alter protein abundances to the low 70‰ salinity in the acute exposure along with the mortalities observed at 85‰ in the chronic exposure may confer *C. savignyi* an advantage should the frequency of intense rain events increase in this region.

While we saw numerous changes in protein abundance within our 6-hour exposure period, protein responses to stress vary with the time point studied, as illustrated by studies of congeneric marine snail species (genus *Tegula*) under heat stress. (Tomanek and Somero, 2000). The chronic exposure allowed us to look at the long-term acclimation to

this stress. The inclusion of time points within the first 24 h of exposure enabled us to track the immediate changes in protein abundance in response to hyposalinity in addition to the acclimation that occurs later in long-term time points. The long-term design of this experiment allowed us to observe the mortalities in *C. intestinalis* that compliment the data generated from the PCA in the acute exposure.

Hyposaline stress is not the only abiotic factor influencing these communities; heat stress plays a role as well. Another proteomics study in our lab has established *C. intestinalis* Sp. A, not *C. savignyi*, to be more tolerant of acute heat stress, with *C. intestinalis* Sp. A constitutively expressing higher abundances of HSP70 at the temperatures tested (Serafini et al., 2011). Clearly, the myriad of changes and stresses imposed by global warming on coastal marine invertebrate communities will work in concert to influence the biogeographic spread of these organisms, and future studies in marine stress should look at the synergistic effects of multiple stressors on the physiology of these organisms.

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