The proteomic response of *Ciona intestinalis* to ocean acidification and acute heat stress: Impacts of global climate change in coastal marine systems

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The proteomic response of *Ciona intestinalis* to ocean acidification and acute heat stress: Impacts of global climate change in coastal marine systems

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1. Introduction

The oceans cover over two-thirds of the Earth's surface. They play a vital role in global biogeochemical cycles, contribute enormously to the planet's biological ecosystems and provide a moderating effect on global climate (Raven *et al*., 2005). The Earth's radiative heat balance is currently out of equilibrium as increased emissions of atmospheric greenhouse gases (carbon dioxide) have trapped some of the heat energy that would otherwise re-radiate to space thus causing a rise in average global temperatures (IPCC, 2007). As a result, the oceans become a sink for this excess heat and carbon dioxide, thus altering ocean biogeochemistry in the form of increased oceanic temperature and decreased oceanic pH. These factors are increasingly being recognized as important co-stresses that driver drastic change in biological systems, including, but not limited to, coastal and marine systems.

Temperature changes in coastal and marine ecosystems will influence organism metabolism and alter ecological processes such as productivity and species interaction (Lovejoy, 2005). Species are adapted to specific ranges of environmental temperature. As temperatures change, species' geographic distributions will expand or contract, creating new combinations of species that will interact in unpredictable ways. Species' that are unable to migrate or compete with other species for resources may face extinction (Sorte C., Hofmann G., 2004). Quantification of the molecular chaperone Heat Shock Protein 70 (Hsp70) in the intertidal dogwhelk *Nucella canaliculata* demonstrated its abundance decreased and face increased thermal stress on the edge or outside of its natural occurring range (Sorte C., Hofmann G., 2004).

From 1961 to 2003 the oceans have warmed by an estimated 0.1°C (range of 0.0°C to 0.5°C depending on depth) by absorbing 84% of the increased global heat (Levitus *et al.*, 2005). Future projections show the average surface temperature of the Earth is likely to increase by 2 to 11.5°F (1.1-6.4°C) by the end of the 21st century, relative to 1980-1990, with a best estimate of 3.2 to 7.2°F (1.8-4.0°C). The average rate of warming over each inhabited continent is very likely to be at least twice as large as that experienced during the 20th century (IPCC, 2007).

Increased gas emissions have not only contributed to the physical impacts of ocean warming over the last century, but have installed many chemical changes in the seawater. Elevated CO_2 concentrations in the atmosphere will dissolve into ocean surface waters resulting in a decrease in oceanic pH altering ocean carbonate chemistry (also known as Ocean Acidification) (Doney *et al.*, 2009). As the inorganic carbon system is one of the most important chemical equilibria in the ocean, it is largely responsible for controlling the pH of seawater. Dissolved inorganic carbon (DIC) exists in seawater in three major forms: bicarbonate ion (HCO_3^-), carbonate ion (CO_3^{2-}), and aqueous carbon dioxide ($CO_{2(aq)}$), which here also includes carbonic acid (H_2CO_3). At a pH of 8.2, ~88% of the carbon is in the form of HCO_3^-, 11% in the form of CO_3^{2-}, and only ~0.5% of the carbon is in the form of dissolved CO_2 (Millero *et al.*, 2002). When CO_2 dissolves in seawater, H_2CO_3 is formed. Most of the H_2CO_3 quickly dissociates into a hydrogen ion (H^+) and HCO_3^-. A hydrogen ion can then react with a CO_3^{2-} to form bicarbonate. Therefore, the net effect of adding CO_2 to seawater is to increase the concentrations of H_2CO_3, HCO_3^-, and H^+, and decrease the concentration of CO_3^{2-} and lower pH ($pH = -\log[H^+]$)(Millero *et al.*, 2002).

A reduction in oceanic pH will have profound impacts on the physiological processes in marine organisms (Fabry *et al.*, 2009). In particular, decreased calcium carbonate ($CaCO_3$) saturation in seawater can have important repercussions on marine organisms' calcification rates. As the seawater is less saturated with this mineral, it is less readily available for normal production of shells and plates. Additionally, these changes in oceanic chemistry can alter respiratory function (Melzner *et al.*, 2009) and create a disturbance to acid–base (metabolic) physiology (Fabry *et al.*, 2009). Growth rates of marine organisms can be greatly affected, especially in the barnacle *Semibalanus balanoides* where growth was slower in elevated CO_2 (Findlay *et al.*, 2010). Additionally, co-stresses temperature and pH may have potential interactive effects on such physiological processes. Sea urchins (*Strongylocentrotus*

franciscanus) were raised under conditions simulating future atmospheric CO_2 levels of 540 and 970 ppm. When larvae raised under these conditions were exposed to 1-h acute heat stress, their physiological response (measured by expression of the molecular chaperone *hsp70*) was reduced relative to control larvae raised under normal CO_2 conditions (O'Donnell et al., 2009).

Experimentation has shown acute elevation of CO_2 greatly alters subcellular processes through reduction of protein synthesis and ion exchange (Pörtner & Langenbuch 2005). Such profound physiological effects are more prominent in invertebrates as opposed to fish (Pörtner & Langenbuch 2005), suggesting some but not all taxa may be disproportionately affected by changes in CO_2 and pH. Despite the challenging task of experimentation with realistic long term projections of CO_2, several studies have confirmed its potential detrimental effects on marine organisms. A decline in growth and metabolism was observed in mussels over a 3 month time period and 0.7-unit pH reduction (Michaelidis et al. 2005). Additionally, a decrease in the growth and survival rates of gastropods and sea urchins was observed as a result of a 6-month elevation of CO_2 in which the pH was lowered 0.03 units (Shirayama & Thornton 2005). Such findings may be attributed to the impacts on metabolic physiology as well as calcification rates.

The atmospheric CO_2 value today is ~100 ppmv greater than the pre-industrial value (280 ppmv), and the average surface ocean pH has dropped by 0.1 unit, which is about a 30% increase in $[H^+]$ (Feely *et al.* 2004). Under the IPCC emission scenarios (Houghton *et al*., 2001), average surface ocean pH could decrease by 0.3–0.4 pH units from the pre-industrial values by the end of this century (Caldeira and Wickett, 2005). This suggests that marine organisms will have to adapt to altered ranges of pH under ocean acidification conditions.

In spite of promising alternative and complementary techniques, the field of proteomics has provided a methodology for parallel characterization and quantification of patterns of protein expression in response to environmental stresses (Görg A., 2004). Application of two-dimensional gel electrophoresis (2-DE) with immobilized pH gradients (IPGs) followed by protein identification via mass spectrometry (MS) is currently the workhorse for proteomics (Görg A., 2004). The proteomic response of an organism to environmental co-stresses like temperature and acidity can be documented through a map of protein spots, which reflects changes in protein expression level (synthesis or degradation), isoforms or post-translational modifications (Görg A., 2004).

The proteomic response to thermal stress in marine organisms may be a current focus of research; however ocean acidification is in its infancy. Two recent studies have been conducted in which mass-spectrometry based proteomics was used to compare acute and chronic thermal stress across marine invertebrate species (Tomanek and Zuzow, 2010; Serafini *et al*., 2011). Research involving blue mussel congeners (*Mytilus galloprovincialis and Mytilus trossulus*) focused their efforts to determine similarities and differences in the proteome responses between species to thermal stress in a native more heat-sensitive species and an invasive more heat-tolerant species (Tomanek and Zuzow, 2010). Sea squirt congeners (*Ciona intestinalis and Ciona savignyi*) were used in another study to observe differences in distribution ranges as well as analyze molecular adaptations between species with the goal of highlighting changes in cellular responses to thermal stress (Serafini et al, 2011). Results showed changes in a number of protein functional groups (cytoskeletal proteins and molecular chaperones, metabolic proteins) of which cytoskeletal proteins had higher levels of expression in *Ciona intestinalis* than *Ciona savignyi* lending data to such affects of heat stress (Serafini et al, 2011).

Acute pH stress in marine systems has only recently emerged on the forefront of biological research and is not well established. Likewise, very few studies have focused on the proteome response of marine invertebrates such as mussel congeners (*Mytilus*) or sea squirt congeners (*Ciona*) under pH stress. However, recent progress in the genome sequencing of *Ciona intestinalis* has led to the development of a great tool for investigating the gene functions and expressions involved in several biological events in ascidians, thus ushering in extensive protein analysis (Inaba *et al*., 2007). Based on its novelty and potential in the scientific world, *Ciona intestinalis* was chosen as an ideal model invertebrate to study. *Ciona intestinalis* is solitary hermaphroditic species of ascidian, commonly known as sea squirts (Inaba *et* al., 2007). Originating in northern Europe, populations spread to a nearly worldwide coastal distribution through anthropogenic means; e.g. ship hull fouling (Dybern, 1963). In these new environments, *C. intestinalis* is very successful at densely occupying free substrate, whether artificial substrates like ship hulls or natural substrates like rocks and shells. This excludes native species and overall reduces species richness in these new ecosystems (Dybern, 1963).

The goal of the present study begins to address the adaptive capabilities of a marine invertebrate like *C. intestinalis* to changing environmental acidity. In many cases, ocean acidification will be a co-stressor in tandem with a warming ocean. Hence, a focus on the

interaction between oceanic thermal and pH stressors was considered as an organism's response to one stressor may depend on another, or vice-versa. Using two-dimensional gel electrophoresis (2-DE) and statistical analysis via two-way ANOVA ($p<0.02$), I was able to interpret and detect significant changes in protein expression in response to each stressor as well as a combined effect. Hierarchical clustering grouped these significant proteins based on similarities in their expression patterns by generating a heat map. Furthermore, identification of these proteins using mass spectrometry (MS) would perhaps lend insight into expression differences based on cellular function among determined protein clusters within or across a treatment group(s).

2. Materials and Methods

2.1 Animal Collection and Maintenance

Ciona intestinalis sp. B were collected from the Grimstad Harbor near Bergen, Norway by James Koman and Loredana Serafini, affiliates of the Environmental Proteomics Laboratory, with assistance from Birthe Mathiesen (affiliated with Sars Centre for Marine Molecular Biology) on September 9th, 2009. This harbor is the site of *Ciona* collection for the Jiang research group within the Sars Centre. Due to an abundance of sea squirt individuals, collection was accomplished by simply picking *Ciona* off of pulled boat line ropes. Only *Ciona intestinalis* were present in the harbor as *Ciona savignyi* are found only in the Pacific Ocean. Specimens were transported back to the Sars Centre where *Ciona* were separated based on two experimental approaches. *Ciona* were separated into 4 baskets with 12 individuals in each basket (n=12) for the acute temperature experiment and left overnight in the 15^{o}C control tank. The remaining *Ciona* were separated into 2 baskets with 12 individuals in each basket (n=12) for the acute pH experiment and left in the 15^{o}C control tank at a pH of 8.3, representative of normal ocean acidic conditions. Following completion of the experimental design, individuals from each group for each experiment were combined into samples in 50 mL tubes (2 individuals per samples; 6 samples per temperature and pH group). Animals were transported back to San Luis Obispo, California in coolers, on dry ice.

2.2 Experimental Design

2.2.1 Acute Heat Stress

Following an overnight incubation in a 15^{o}C temperature-controlled tank with circulating seawater and aeration, *Ciona intestinalis* were transferred to tanks with sequentially increased temperatures by 6°C h^{-1} from 15°C to 18°C, 20°C or 23°C (n = 12 for all groups). Once target temperature was achieved for each sample group, sea squirts were kept at these temperatures for a 6 h incubation and subsequently brought back to 15°C for a 16 h recovery period. Immediately following recovery, individuals were pooled into samples to obtain sufficient amounts of protein (2 *C. intestinalis* individuals per sample; 6 samples per temperature group), and samples were flash-frozen in liquid nitrogen and stored at -80°C. Note: For my experimental purposes, only the 15°C and 18°C treatment samples were used for analysis.

2.2.2 Acute pH stress

The overall basic experimental design of the acute temperature stress experiment was completed for the acute pH stress experiment with the exception of varying acidities, not temperatures within treatment tanks. *Ciona intestinalis* (n=24) incubated at 15^{o}C for several days were placed in a 15^{o}C temperature-controlled tank at pH 8.3 (normal ocean acidification) with circulating seawater and aeration. Sea squirts were kept at these conditions for a 1 h acclimation. *Ciona intestinalis* were then separated with 12 individuals being transferred to a 15^{o}C pH-controlled tank at pH 7.7 and the remaining 12 individuals being transferred to an 18^{o}C pH-controlled tank at pH 7.7 both controlled via pH meter with filtered sea water and aeration. The incubation (6h) and recovery (16h) times were completed in the same fashion as the acute temperature stress experiment. Immediately following recovery, individuals were pooled into samples to obtain sufficient amounts of protein (2 *C. intestinalis* individuals per sample; 6 samples per temperature and pH group), and samples were flash-frozen in liquid nitrogen and stored at -80°C.

2.3 Dissection and homogenization

The protocols in this experiment follow those in Serafini et al., 2011. Frozen samples were thawed and the outer tunic membrane was dissected off of each individual. The contents of the stomach and intestine were removed, and the remaining tissues (all internal organs and structures) were pooled into ice-cold ground-glass homogenizers. The tissue was lysed in a ratio

of 1:2 of tissue to homogenization buffer (7 mol l^{-1} urea, 2 mol l^{-1} thiourea, 1% ASB(amidosulfobetaine)-14, 40 mmol l^{-1} Tris-base, 0.5% immobilized pH 4-7 gradient (IPG) buffer (GE Healthcare, Piscataway, NJ, USA) and 40 mmol l^{-1} dithiothreitol).
The homogenate was subsequently centrifuged at room temperature for 30 min at 16,100 *g* and the supernatant was used for further processing. Proteins of the supernatant were precipitated by adding four volumes of ice-cold 10% trichloro-acetic acid in acetone and incubating the solution at -20°C overnight. After a 15 min centrifugation at 4°C and 18,000 g, the supernatant was discarded and the remaining pellet was washed with ice-cold acetone, and centrifuged again before being re-suspended in rehydration buffer (7 mol L^{-1} urea, 2 mol L^{-1} thiourea, 3.25 mmol L^{-1} CHAPS (cholamidopropul-dimethylammonio-propanesulfonic acid), 2% NP-40 (nonyl phenoxylpolyethoxylethanol-40), 0.002% bromophenol blue, 0.5% IPG buffer and 100 mmol L^{-1} dithioerythritol) through vortexing. The protein concentration was determined with the 2D Quant kit (GE Healthcare), according to the manufacturer's instructions.

2.4 Two-dimensional gel electrophoresis

Proteins (400 µg) were loaded onto immobilized pH gradient strips (pH 4-7, 11 cm; BioRad, Hercules, CA, USA) for separation according to their isoelectric point (pI). I started the isoelectric focusing protocol with 12 h of active rehydration (50V), using an isoelectric focusing cell (BioRad). The following protocol was used for the remainder of the run (all voltage changes occurred in rapid mode): 250 V for 15 min, 10,000 V for 3 h, 10,000 V for 80,000V h, and a 500 V hold step. Once removed from the IEF cell, the strips were frozen and stored at -80°C.

Frozen strips were thawed and incubated in equilibration buffer (375 mmol L^{-1} Tris-base, 6 mol L^{-1} urea, 30% glycerol, 2% SDS (sodium dodecyl sulfate) and 0.0002% bromophenol blue) for 15 min, first with 65 mmol L^{-1} dithiothreitol and then, second, with 135 mmol L^{-1} iodoacetamide. IPG strips were placed on top of an 11.8% polyacrylamide gel with a 0.8% agarose solution containing Laemmli SDS electrophoresis (or running) buffer (25 mmol L^{-1} Tris-base, 192 mmol L^{-1} glycine, 0.1% SDS). 11 cm IPG strips for each experimental group were run on small-format gels, 13.3 x 8.7 cm, at 220V for 55 minutes (Criterion Dodeca Cell; BioRad). Re-circulating water baths set to 12°C were used to keep running buffer cool. Gels were subsequently stained with colloidal Coomassie Blue (G-250) overnight and destained by washing

repeatedly with Milli-Q water for 48 h. The resulting gel images were scanned with an Epson 1280 transparency scanner.

2.5 Gel image analysis

Digitized images of two-dimensional gels were analyzed using Delta2D (version 4.0; Decodon, Greifswald, Germany). I used the group warping strategy to connect gel images through match vectors. All images within a treatment group represented a mapped out proteome fused together as a single composite image, thus representing average volumes for each spot. I determined the boundaries of the identified spots within the proteome map and transferred them back to all gel images using match vectors. After background subtraction, protein spot volumes were normalized against total spot volume of all proteins in a gel image.

2.6 Statistical analysis

Normalized spot volumes were analyzed within Delta2D by using an analysis of variance (two-way ANOVA) within each species and with temperature as the main effect. For the two-way ANOVA a null distribution was generated using 1000 permutations to account for the unequal variance and non-normal distributions of the response variables and a p-value of 0.02 was used instead of using a multiple-comparison correction, which was deemed too strict given that the number of protein spots is much smaller in comparison to microarray experiments. In my heat maps, I used hierarchical clustering to group proteins based on similarities in their expression patterns. To accomplish this, I used average linking in the statistical tool suite within Delta2D, and used a Pearson's correlation metric.

3. Results

Gels from all 24 *Ciona intestinalis* samples were matched and used to generate a proteome map, which shows the average normalized pixel volume of all proteins detected (Fig. 1). Spot boundary analysis within the composite map gave rise to the detection of 247 individual protein spots. Using a two-way ANOVA based on permutations, I determined that 64 protein spots (or 26% of the total) showed significant expression changes across temperature treatment, pH treatment and interaction between the two ($p<0.02$). Interpretation of the data was carried out via hierarchical clustering to generate heat maps which grouped proteins based on similarities in their expression patterns across treatment groups.

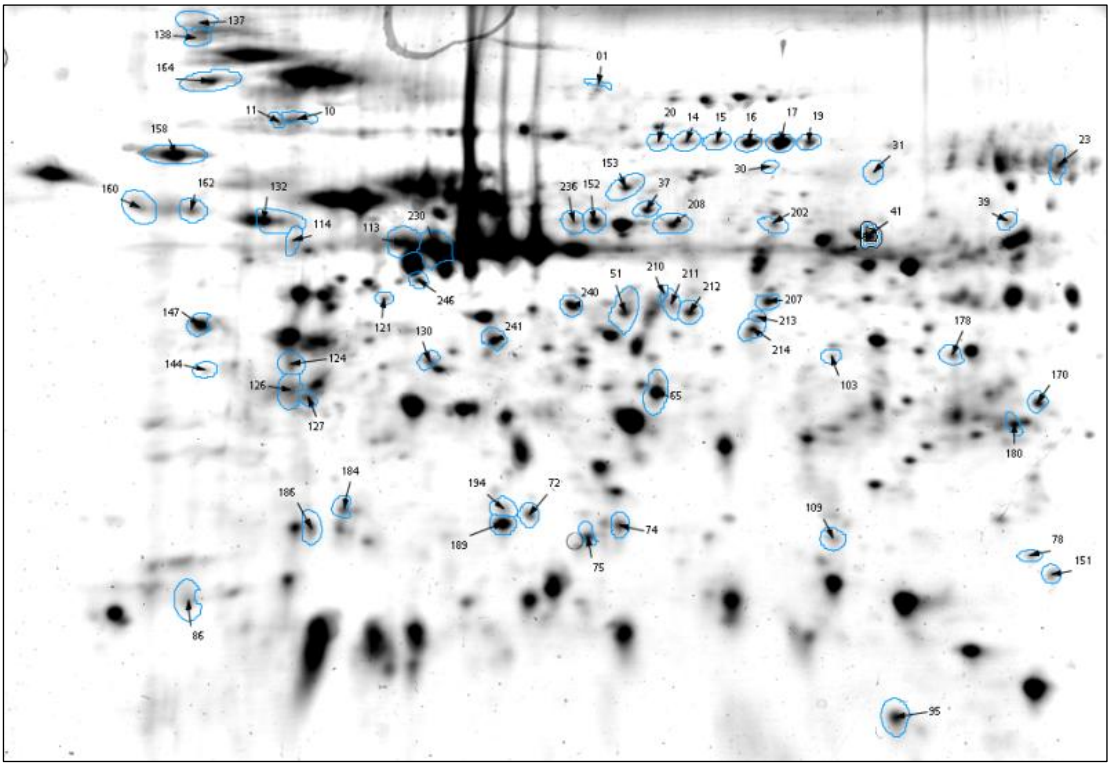

Figure 1. A composite gel image (or proteome map) displaying the 247 individual protein spots detected in the Ciona intestinalis acute heat stress and pH stress experiments. The proteome map represents the average normalized pixel volumes for each protein spot across all 24 sample gels analyzed. Highlighted spots were those that showed significant changes in abundance in response to acute heat stress, pH stress and interaction between the two treatments. (two-way ANOVA, $p < 0.02$).

3.1 Acute temperature stress experiment

Of the total 247 spots detected in *C. intestinalis*, 21 spots (or 8%) showed significant expression changes across both temperature treatments (15°C and 18°C). Hierarchical clustering separated these proteins into three main clusters that show similar expression patterns in response to temperature treatment (Fig. 2).

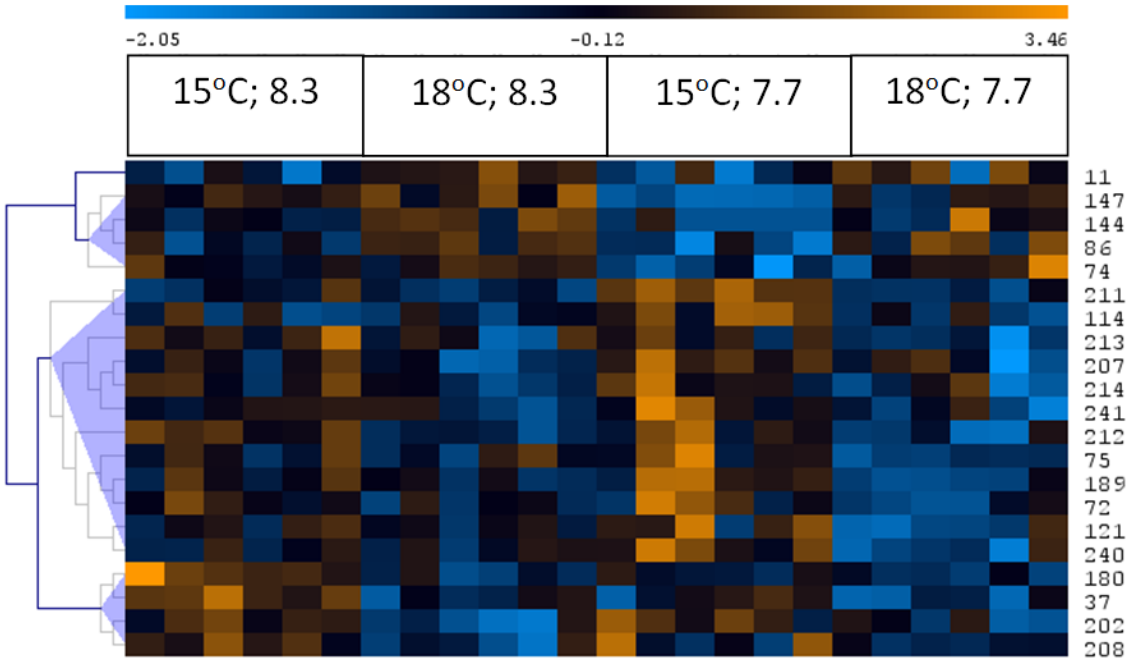

Figure 2. Hierarchical clustering of detected proteins using Pearson's correlation in response to acute heat stress. Blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature treatments are labeled along the upper horizontal axis, while the right vertical axis represents the standardized expression patterns of detected proteins.

3.2 pH stress experiment

In addition to temperature's role as a stressor on the *Ciona* congeners, its correlation to ocean acidification is another key aspect of global warming and its adverse consequences. Therefore, an additional experiment using both experimental temperature groups at a lower pH was completed. Results from the permutation-based ANOVA (two-way; $p<0.02$) showed that 26 of the 247 spots detected or 11% had significant expression changes across treatment groups. The heat map in Figure 3 demonstrates the four main groups these 26 proteins were clustered into based on the similarity of their expression patterns.

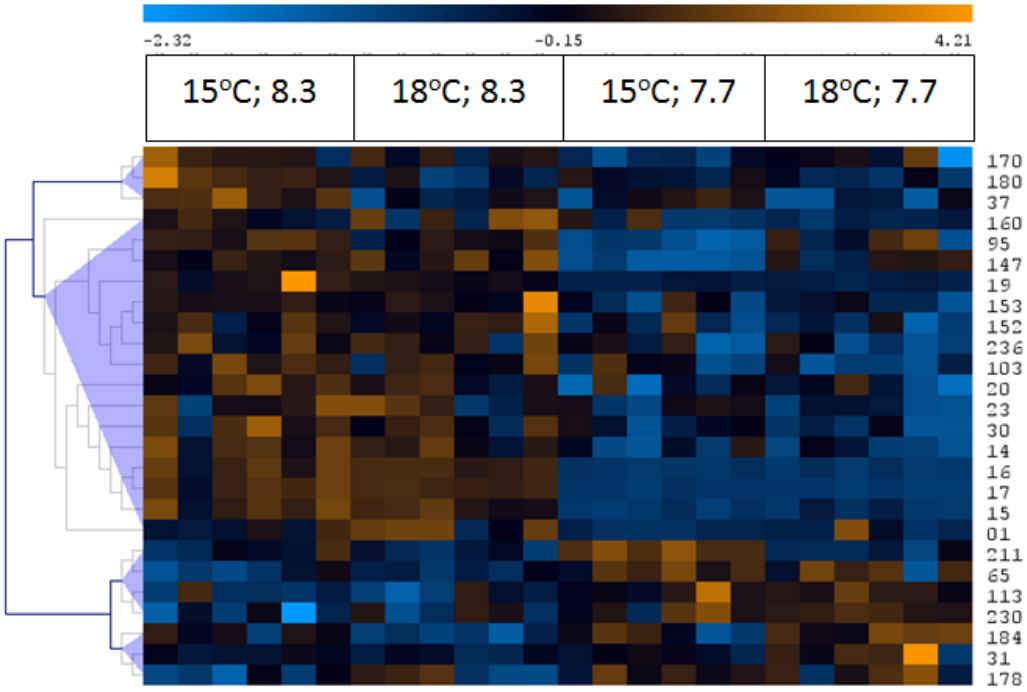

Figure 3. Hierarchical clustering of detected proteins using Pearson's correlation in response to pH stress. Blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature treatments are labeled along the upper horizontal axis, while the right vertical axis represents the standardized expression patterns of detected proteins.

3.3 Interaction: temperature and pH

Comparisons made between two such variables as temperature and pH pose the chance an organism behaves differently when subjected to combinations of stressors. Hence, an organism's response to pH may depend on the temperature, or vice-versa. Statistical analysis of the two-way ANOVA ($p<0.02$) has presented 27 proteins (11%) in which expression changed significantly of the 247 spots detected in *Ciona intestinalis*. The 27 significantly changing proteins are represented in a heat map (Figure 4) in which hierarchical clustering has grouped them into 4 main clusters based on the similarity of their expression patterns.

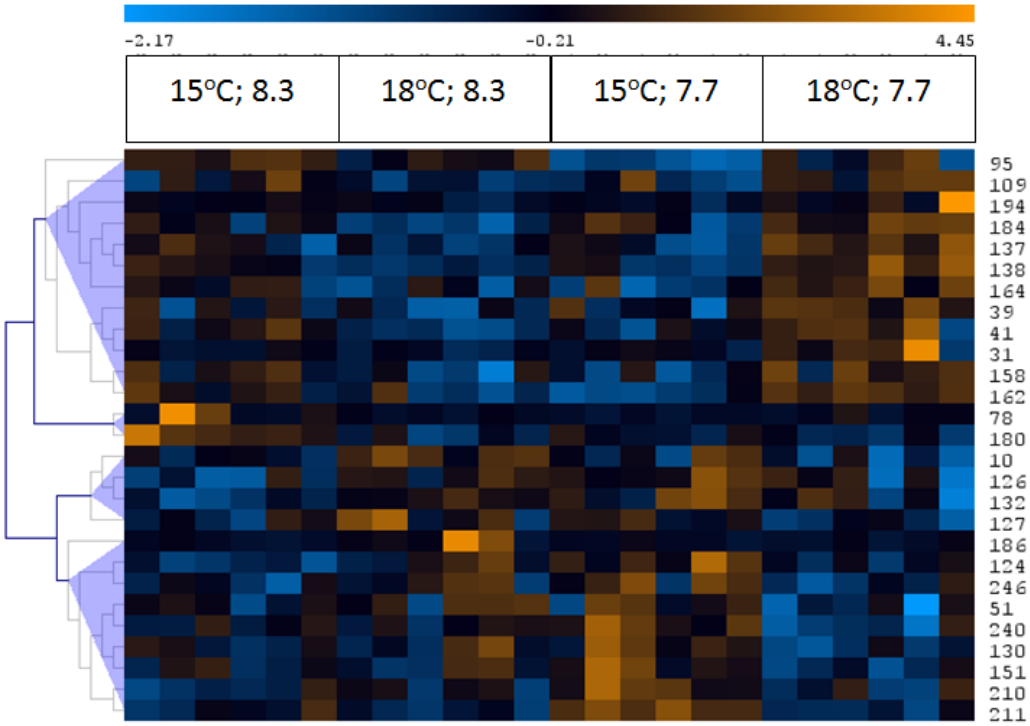

Figure 4. Hierarchical clustering of detected proteins using Pearson's correlation in response to interaction between acute heat stress and pH stress. Blue represents a lower than average standardized spot volume, whereas orange represents a greater than average standardized spot volume. The temperature treatments are labeled along the upper horizontal axis, while the right vertical axis represents the standardized expression patterns of detected proteins.

4. Conclusion

Global warming and increased atmospheric CO_2 are causing the oceans to warm, decrease in pH and become hypercapnic. These stressors have deleterious impacts on marine invertebrates. Increasing temperature has a pervasive stimulatory effect on metabolism until lethal levels are reached, while hypercapnia has a narcotic effect. Ocean acidification is a key threat to calcification because it decreases availability of the carbonate ions required for skeletogenesis and also exerts a direct pH effect on metabolic physiology. The present study has only begun to address these adverse impacts of global climate change through novel approaches in the field of proteomics. Considering the extra baggage climate change stressors pose to an already multi-stressor world for marine invertebrates, the ability of *Ciona intestinalis* to elicit a significant proteomic response under stress from changes in temperature, pH as well as an interactive effect represents the emerging potential for further study. Only mere speculation can be made regarding the thresholds of thermal stress and hypercapnia over time and across generations. Ocean pH, pCO_2 and $CaCO_3$ co-vary and will change simultaneously with temperature, thus challenging our ability to predict future outcomes for marine biota.

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