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Proteome and Growth Responses of the Key Species *Calanus finmarchicus* to future Ocean Acidification and Warming

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Abstract

The zooplankton *Calanus finmarchicus* is a key species in the North Atlantic, by linking the primary production with upper trophic levels and by contributing to vertical export of carbon. Copepod eggs were cultured to a sub-adult stage, under different regimes of temperatures (11 and 14 °C) and pCO_2 concentrations (390 and 2080 ppm) in a full factorial design. Morphological characters were measured and the proteome was analyzed using two-dimensional electrophoresis (2-DE) followed by mass spectrometry. To the author's knowledge, this is the first study to use a 2-DE proteomic approach to examine the combined effect of acidification and warming on any marine organism. Somatic growth (body and lipid sac) was reduced in response to warming, but the effects was somewhat ameliorated by acidification in the animals exposed to both stressors. This has not been reported elsewhere, and emphasizes the challenges with predicting the combined effect of acidification and warming. Fourteen proteins were differentially expressed, whereby warming inflicted a more pronounced effect than acidification also on this level. Only a few proteins were affected by acidification alone, and these showed no difference in expression when the copepods were exposed to both stressors. This imply that the acidified effect was ameliorated by warming, which is opposite from what was found for the antagonistic effect observed with the morphological variables. The identified proteins belongs to the cytoskeleton, metabolism or the stress class. The moderate proteome response and the types of proteins affected reflects the changes seen in somatic growth, e.g. an up-regulation of a glycolytic enzyme points to increased metabolism and less energy to store as lipids. Future proteome studies incorporating posttranslational modifications, protein-protein interactions and multiple generations of an organism, may contribute to further knowledge about the mechanisms that are affected by ocean acidification and warming.

Sammendrag

Dyrealgaen *Calanus finmarchicus* (Raudåte) er en nøkkelart Nord-Atlanteren, ved at den kobler primærproduksjonen med øvre trofiske nivåer og bidrar med vertikal eksport av karbon. Kopepodegg ble dyrket til CV-stadiet under ulike regimer av temperatur (11 og 14 °C) og pCO_2 konsentrasjoner (390 og 2080 ppm) i et full-faktorielt design. Morfologiske karakterer ble målt og proteomet ble analysert med to-dimensjonal elektroforese (2-DE), etterfulgt av massespektrometri. Etter det forfatteren vet, er dette det første studiet som bruker en 2-DE metode til å undersøke den kombinerte effekten av havforsurning og oppvarming på en marin organisme. Økt temperature førte til redusert somatisk vekst (kropp og fettsekk), men effekten ble noe bedret av havforsurning da dyrene ble eksponert for begge stressfaktorene samtidig. Dette har ikke blitt rapportert noe annet sted og understreker utfordringene med å forutse den samlede effekten av havforsurning og oppvarming. Fjorten proteiner ble ulikt uttrykket, hvorav varme påførte en klarere effekt enn havforsurning, også på dette nivået. Kun noen få proteiner ble påvirket av havforsuring alene, og disse visste ingen forskjell i uttrykk da kopepodene ble eksponert for begge stressfaktorer. Dette indikerer at havforurensingseffekten ble bedret av varme, som er motsatt av det som ble funnet for den antagonistiske effekten observert i de morfologiske variablene. De identifisert proteinene tilhører cytoskjelettet, metabolisme eller stressklassen. Den moderate proteomresponsen og proteintypene som ble påvirket speiler endringene i somatisk vekst, for eksempel en oppregulering av et glykolyseenzym viser til økt metabolisme og dermed mindre energi til å lagre som lipider. Fremtidige proteomestuider som inkorporer posttranslasjonelle modifikasjoner, protein-protein interaksjoner og flere generasjoner av en organisme, kan bidra med mer kunnskap om mekanismene som blir påvirket av havforsurning og oppvarming.

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Chapter 1

Introduction

1.1. Background and future prospects

Carbon dioxide (CO_2) can be found naturally in the atmosphere as one of the greenhouse gases. Since the beginning of the industrial revolution (around the 1750s) over 600 billion tons of CO_2 has been emitted into the atmosphere as a result of human activity such as fossil burning, deforestation and cement production (Houghton, 2009, National Research Council, 2010). This has resulted in an increase in the atmospheric partial pressure of CO_2 (pCO_2) from 280 to 384 ppm (Houghton, 2009, Steinacher et al., 2009). This far exceeds the estimated natural range of pCO_2 (172 to 300 ppm) during the last 800 000 years and possibly for the last 20 million years (Houghton et al., 2001, Luethi et al., 2008). The alteration of greenhouse gases (methane and nitrous oxide have also increased) drives changes in the global climate. Among the consequences are snow and ice melt, a rising global sea level, more extreme weather and a rise in average air and ocean temperature. These changes are likely to negatively affect biological systems worldwide (Solomon, 2007, Noyes et al., 2009).

Another effect of the increased CO_2 concentration is ocean acidification. So far the ocean has functioned like a buffer and 25 - 50 % of the anthropogenic CO_2 emission has been absorbed here (Turley et al., 2006, Steinacher et al., 2009). However, the uptake of CO_2 lowers the pH, which is the process referred to as ocean acidification (Børsheim and Golmen, 2010).

Future CO_2 scenarios suggest that the oceanic pH will continue to decline globally, and that the largest estimated pH decrease will occur in the Arctic. Estimates suggest a global decrease with 0.23 - 0.40 pH units within 2100, based on the B1 IPCC SRES and A2 IPCC SRES, respectively (Steinacher et al., 2009). This corresponds to atmospheric pCO_2 of 538 and 840 ppm, respectively. However, according to the IS92a scenario, the pH could decrease with as much 0.77 pH units within 2300, which corresponds to a pCO_2 concentration of 1900 ppm (Caldeira and Wickett, 2003). In addition, the ocean surface temperature is likely to increase, and estimates suggest a 1.1 - 6.6 °C increase within the century, depending on the scenario used (Solomon, 2007).

1.2. Ocean acidification and warming

1.2.1. The carbonate system

The carbonate system consists of some few essential compounds, CO_2, bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), hydrogen ion (H^+) and hydroxide (OH^-). $CO_2(g)$ from the atmosphere dissolves in seawater until equilibrium is reached, which is when the net exchange is zero (Equation 1.1). Then the pCO_2 in the two mediums will be equal, which is linked to the concentration of CO_2 given by Henry's law (Equation 1.2) (Zeebe and Wolf-Gladrow, 2001).

$$CO_2(g) \rightleftharpoons CO_2(aq),\ K_0 \qquad \text{(Equation 1.1)}$$

where K_0 is the solubility constant of CO_2 in seawater.

$$[CO_2] = K_0 \bullet pCO_2 \qquad \text{(Equation 1.2)}$$

where the brackets represent total stoichiometric concentration.

When CO_2 is absorbed in seawater, a fraction hydrates to yield the transient form of true carbonic acid (H_2CO_3). In reality, the concentration of H_2CO_3 in seawater is very low and the sum of $[H_2CO_3]$ and $[CO_2]$ is usually denoted $[CO_2]$. Therefore, when CO_2 dissolves in seawater, HCO_3^-, CO_3^{2-} and H^+ are formed (Equation 1.3 and 1.4). The proportion of the carbonate species are dependent on dissociation constants, which again are functions of salinity, temperature and surface pressure (Zeebe and Wolf-Gladrow, 2001).

$$CO_2(aq) + H_2O \rightleftharpoons HCO_3^- + H^+, K_1 \qquad \text{(Equation 1.3)}$$

$$HCO_3^- \rightleftharpoons CO_3^{2-} + 2H^+, K_2 \quad \text{(Equation 1.4)}$$

Where K_1 and K_2 is the first and second dissociation constant of carbonic acid, respectively.

At surface-seawater pH of 8.1 (the total pH scale), alkalinity of ~2.4 mmol/kg (carbonate alkalinity), temperature of 25 °C and salinity of 35 psu, the speciation between $[CO_2]$, $[HCO_3^-]$ and $[CO_3^{2-}]$ are 0.5, 86.5 and 13 %, respectively. As mentioned earlier, the atmospheric CO_2 concentration has increased substantially since the 1750s. However, this has not caused a proportional increase in the dissolved inorganic carbon concentration (DIC, Equation 1.5). In fact, a doubling of atmospheric CO_2 concentration only leads to a 10 % change in the DIC. This is due to a process called CO_2-buffering, where CO_2 is scavenged by CO_3^{2-} according to Equation 1.6. The effect is quantified by the Revelle factor or buffer factor (Zeebe and Wolf-Gladrow, 2001).

$$DIC = [CO_2] + [HCO_3^-] + [CO_3^{2-}] \qquad \text{(Equation 1.5)}$$

$$CO_2 + H_2O + CO_3^{2-} \rightleftharpoons 2HCO_3^- \qquad \text{(Equation 1.6)}$$

From all this it can be elucidated that an increase in $[CO_2(aq)]$ leads to an increase in $[HCO_3^-]$, $[H^+]$, $[H_2CO_3]$ and a decrease in $[CO_3^{2-}]$ and pH. The carbonate system, together with boron compounds ($B(OH)_3$), $B(OH)_4^-$), are important for the seawaters buffer capacity and the reason for the relative stable pH and moderate climate change effects seen so far (Zeebe and Wolf-Gladrow, 2001, Comeau et al., 2009).

1.2.2. Acid-base regulation in marine organisms

When CO_2 concentration increases in the seawater, it crosses body layers of organisms by diffusion and equilibrates in intra- and extracellular spaces, which can give a hypercapnic environment (excess of CO_2 in body fluids). This could lead to a pH drop (acidosis) if not compensated for (Pörtner et al., 2004, Fabry et al., 2008). Studies have shown that susceptibility to ocean acidification is linked to the organism's ability to compensate for this acid-base imbalance (Melzner et al., 2009, Hofmann and Todgham,

2010). This ability originate from an active mode of life, high metabolic rates or living in environments with high CO_2 fluctuations (Pörtner et al., 2004, Melzner et al., 2009).

In air breathers, an elevation of pCO_2 is often extruded through increased ventilation. However, in water breathers this is made difficult by the low diffusion gradient between body fluids and the ambient water (Melzner et al., 2009). Therefore, the immediate response to elevated H^+ concentration is buffered partly or completely by an increase in bicarbonate or non-bicarbonate buffers (Pörtner et al., 2004). In the intracellular fluid, the level of non-bicarbonate buffers are usually high, and subsequently the pH drop is lower. The pH drop in the extracellular fluid is dependent on the organisms buffer capacity and differ by species (Pörtner et al., 2004). Bicarbonate could either be transported from the outer environment in exchange for chloride (Cameron, 1985, Pörtner et al., 2004, Fabry et al., 2008) or from calcium carbonate storages or dissolution of shells that contains calcium carbonate (Spicer et al., 1988, Michaelidis et al., 2005, Fabry et al., 2008)

In the longer terms (hours to days) more permanent actions is necessary in order to restore the original pH or obtain a new equilibrium. This involve transport of acid-base equivalent ions over cell membranes, where H^+ is exchanged for sodium (Fabry et al., 2008). The ion-transport rely on ion-gradients set up by baso-lateral enzymes like Na^+/K^+-ATPase (Pörtner et al., 1998). However, a metabolic cost follows, and estimates of Na^+/K^+-ATPase activity suggest that 2.8 - 40 % of total energy budget may be allocated to this (Whiteley, 2011). In addition to a metabolic cost, hypercapnia in body fluids can lead to metabolic depression, reduced protein synthesis and decreased affinity to respiratory pigments for oxygen (Whiteley and Taylor, 1992, Langenbuch et al., 2006, Melzner et al., 2009).

1.2.3. Ocean acidification and effects on marine organisms

Current research have focused mostly on calcifying organisms such as species from the echinoderms, mollusks and cnidarians groups (Whiteley, 2011, Pedersen et al., 2014b). These species are likely to be more susceptible due to their dependence on carbonate minerals (aragonite, calcite or magnesium calcite) to form their skeleton or external shell (Whiteley, 2011, Michaelidis et al., 2005, Ries et al., 2009, Kurihara, 2008). The saturation state of carbonate minerals are thought to decrease in response to ocean

acidification (Melzner et al., 2009, Steinacher et al., 2009). Many of the calcifying organisms have poor capacity to counteract acid-base imbalance, in addition to being sessile and hypometabolic (have low metabolic rate) (Pörtner, 2008). Studies have shown that acidified seawater has caused dissolution of the calcified skeleton or reduced calcification rates (Arnold et al., 2009, Comeau et al., 2010, Whiteley, 2011, Lischka and Riebesell, 2012).

Though the main focus has been centered around calcifiers, similar physiological and metabolic functions exists in non-calcifiers, which might be vulnerable to decreased pH (Fitzer et al., 2012). Some of the processes that have been affected are metabolism, growth and reproduction (Pörtner et al., 1998, Michaelidis et al., 2005, Rosa and Seibel, 2008, Whiteley, 2011, Fitzer et al., 2012, Pedersen et al., 2014a). Many of the non-calcifying species are important in a socio-economically way, and have important roles in the ecosystem (Wiacek et al., 2013).

1.2.4. Ocean warming and effects on marine organisms

Global warming alone has already been linked to a shift in geographical distribution, population collapse and local extinction (Perry et al., 2005, Hoegh-Guldberg et al., 2007, Pörtner and Knust, 2007, Edwards et al., 2009). Temperature is a vital environmental factor involved in both growth, reproduction, development, recruitment dynamics and invertebrates' distribution (O'Connor et al., 2007). Temperatures outside a species thermal window (a limited range of body temperatures were the species perform optimally) can therefore be detrimental (Pörtner and Farrell, 2008).

When Forster and Hirst (2012) looked at development rate (increase in life stage per time) and growth rate (increase in weight per time) in the brine shrimp *Artemia franciscana*, they found that elevated temperature increased the developmental rate, to a higher degree than growth rate, so that the adult stage was reached before the largest potential size was achieved. This phenomenon follows the temperature-size-rule (TSR), which states that individuals grown in colder environments reach the adult stage at a larger size when grown in warmer environments (Atkinson, 1994). This has been observed in various types of ectotherms (animals were the body temperature is mainly controlled by the ambient medium) including plants, bacteria, protists, invertebrates and vertebrates, and has been

the case for more than 80 % of the cases studied (Atkinson, 1994). Daufresne et al. (2009) even suggest that the reduced body size (from the individual to community structure) could be one of the universal ecological consequences of global warming, together with a shift of species ranges towards higher altitude and latitudes and the seasonal shifts in life-cycle events. Other observed effects are enhanced growth, abnormalities, metabolic stress and mortality (Byrne and Przeslawski, 2013).

1.2.5. Combined effects from ocean acidification and warming on marine organisms

It is likely that multiple stressors, such as acidification and warming will happen simultaneously during climate change (Solomon, 2007, Pörtner, 2008). However, few studies have so far focused on the combined effect of ocean acidification and warming, especially on species that are less dependent on carbonate minerals (Gooding et al., 2009, Whiteley, 2011).

As mentioned earlier temperature outside the thermal window can be detrimental, and an additional stressor, such as ocean acidification may for example narrow this window and contribute to a synergistic effect (e.g. the sum of the effect is greater than the sum of the individual stressors) (Pörtner and Farrell, 2008). It is not certain how a response to one stressor will limit the response to another, or even if one stressor could offset the effect of the other stressor (Roff and Fairbairn, 2007). However, in 16 of 23 species reviewed by Byrne and Przeslawski (2013), the most common interaction effects of acidification and warming were negative additive- or antagonistic (the sum of the effect is similar or lower than the sum of individual stressors, respectively). In cases where antagonistic effects have been observed, warming has ameliorated some of the negative effects caused by acidification (McCulloch et al., 2012, Byrne and Przeslawski, 2013). Predicting the combined effect is difficult, and is made further problematic due to the difference in sensitivity between life stages, life histories and species (Byrne and Przeslawski, 2013).

1.3. Using proteomics to assess the effect of ocean acidification and warming

The proteome is the entire complement of proteins produced by an organ or organism, and proteomics is the study of this system. The proteome can also include protein variants and posttranslational modifications (PTMs) (Garcia-Reyero and Perkins, 2011). At a biochemical level, fitness can be defined as a result of the ability of proteins to function in their intracellular and extracellular milieu (Feder and Walser, 2005). The proteome can respond differently depending on environmental conditions, which is one of the reasons that proteomics is considered a good method to identify possible adverse effects caused by stressors such as ocean acidification and warming. Previously demonstrated effects, but also new and essential information can be discovered by studying the proteomic response (Garcia-Reyero and Perkins, 2011). Other –omics technologies also exist, such as transcriptomics (the study of all RNA transcripts). However, studies have found that less than half of all gene transcript is translated into proteins (Feder and Walser, 2005).

Currently, few studies have used proteomics to study the global effect of ocean acidification or warming. The organisms studied so far include mussels, oysters, fish, barnacle and a tubeworm. Collectively, the studies suggest that acidification affect proteins that are involved in the cytoskeleton, metabolism, stress and calcification. However, the system affected and whether it is suppressed or induced vary between species. Overall, the proteome of fish species and the barnacle was less affected than that of the oysters and tubeworm in the listed studies (Gunnarsson, 2010, Tomanek et al., 2011, Wong et al., 2011, Dineshram et al., 2012, Dineshram et al., 2013, Mukherjee et al., 2013, Maneja et al., 2014). In acute and long-term heat studies, it was found that proteins involved in the cytoskeleton, metabolism, proteolysis, stress, calcium-binding and protein folding was affected (Tomanek and Zuzow, 2010, Fields et al., 2012, Serafini et al., 2011). To the author's knowledge, no study have investigated the combined effect of future ocean acidification and warming on the proteome of any marine organism. However, one study has examined the proteomic response of acidification (pH 7.6) and hypoxia (reduced oxygen concentration, 4.8 mg O_2/l in ambient and 2.8 mg O_2/l in test treatment) in tubeworm larvae (*Hydroides elegans*). In this study, the expression level of calcification-related proteins were closer to the control in the group treated with both

stressors than with acidification alone. It was hypnotized that mild hypoxia stress could offset the negative effect of acidification (Mukherjee et al., 2013). In another study, the proteome of two natural populations of *C. sinicus*, living under different environmental regimes in nature (different temperature and oxygen levels), were investigated (Wiacek et al., 2013). It was found that 56 proteins were differentially expressed in the two populations, of which 45 could be successfully identified. These proteins belonged to different classes, but it was difficult to place them in perfectly defined groups. However, 12 proteins were cytoskeleton and myofibrillar proteins. The study revealed fine adaption of molecular machinery in response to different environmental conditions (Wiacek et al., 2013).

1.3.1. Method and techniques

Different approaches have been adopted to study the proteome and its response to environmental stressors. One of them involves protein expression signatures (PES), which are obtained through two-dimensional electrophoresis (2-DE). In the 2-DE approach, proteins are separated in a gel based on differences in their physico-chemical properties (molecular weight and isoelectric point, pI). Differences in the protein patterns between the treatments can be used as a biomarker of exposure. This approach has been further improved using surface-enhanced laser desorption/ionization mass spectrometry (SELDI-MS), which obtains the mass fingerprints of the proteins instead of only protein spots in gels (Tomanek, 2011).

Furthermore, it is possible to annotate proteins with the use of mass spectrometry (MS), which can provide more information about which biological components/system that have been effected by the treatment (Reece, 2004). Prior to MS, the proteins are digested into proteolytic peptides, most often by the use of the enzyme trypsin. In tandem mass spectrometry (one type of MS, also known as MS/MS), the peptides are isolated and fragmented. Both the mass of the peptide and the fragmented ions are measured, and more information of each peptide is obtained. This makes it possible to identify a protein, even on the basis of a single peptide (Eriksson and Fenyö, 2011).

The peptide sequences from the MS/MS can be run through a database to search for known proteins with matching sequences. The database could contain either protein

sequences from actual proteins or sequences that are generated from gene transcripts. The list of possible matching candidates produced by a program (e.g. Proteome Discoverer) has a false discovery rate (FDR) < 0.05, which is equal to a 95 % confidence level. The fragmented ions of the unknown peptide can be observed in fragment spectrum, and good coverage is based on how many of the fragmented ions that also exist in the known peptide. Based on this, the peptide is given a confidence level of high, medium or low (Proteome Discoverer User Guide).

1.4. The model species: *Calanus finmarchicus*

The calanoid copepod *Calanus finmarchicus* (Gunnerus, 1770) is a zooplankton in the crustacean subphylum and a key species in the marine food web (Mauchline, 1998). It can be found in high abundance, and is widely distributed in the Arctic, Atlantic and Coastal waters (Broms et al., 2009). The copepod is a filter feeder and feeds mainly on phytoplankton (Nejstgaard et al., 1997). It has six nauplii (larva) stages (NI-NVI) and six adult stages (CI-CV and adult male/female, Figure 1.1). In the stage CIII-CV during phytoplankton bloom, the copepods store energy as lipids (purely as wax esters) in membrane-bound compartments called oil or lipid sacs (Miller et al., 1998, Irigoien, 2004). Between July and September, when adequate lipid levels are stored, and possibly at certain temperatures or photoperiods (Miller et al., 1991, Jónasdóttir, 1999, Irigoien, 2004), the copepod (CIV- or CV-stage) descend to 600 - 1500 m for overwintering (Edvardsen et al., 2006). They here spends up to nine months of diapause, where they use 5 - 50 % of the lipid storage (Jónasdóttir, 1999, Heath et al., 2008). From January to March the copepod ascend to the surface where they molt into the adult stage and develop gonads (Jónasdóttir, 1999, Edvardsen et al., 2006). The female produce eggs preferably before or early in the spring phytoplankton bloom (Niehoff et al., 1999). During vertical migration in the water column the animals may experience temperature fluctuations between 0 - 16 °C (Bonnet et al., 2005). However, optima temperature in nature is thought to be around 6 - 10.6 °C (Helaouët and Beaugrand, 2007).

The exoskeleton of *C. finmarchicus* consists of mineralized chitin, and they are therefore thought to be less vulnerable for dissolution of their shell than calcifying organisms in response to ocean acidification (Fitzer et al., 2012, Whiteley, 2011). Copepods do not have gills, so the oxygen uptake and CO_2 excretion is thought to happen over the epithelia of the integument and hindgut (Mauchline, 1998).

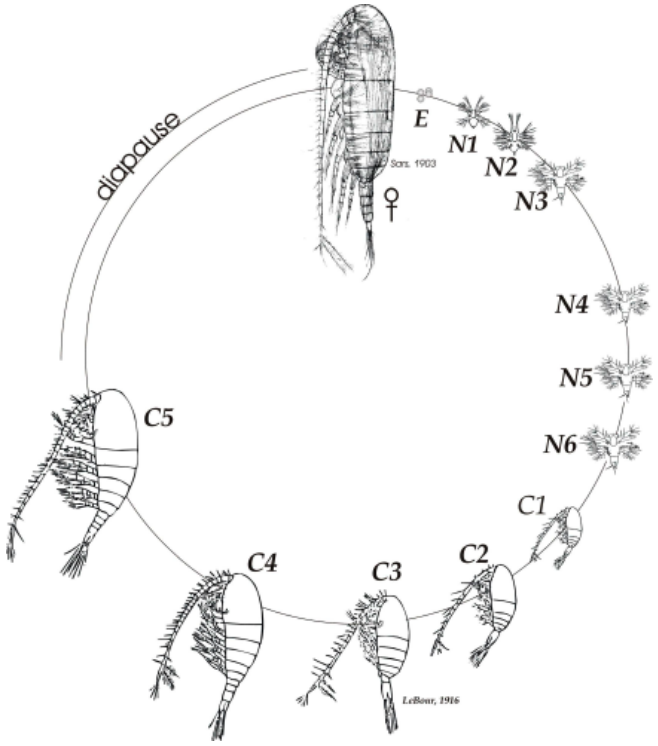

Figure 1.1. Life cycle of *Calanus finmarchicus* (Baumgartner, 2009)

Copepod larvae and early juvenile stages represent important food sources for commercially important fish, such as herring (*Clupea harengus*), mackerel (*Scomber scombrus*) and cod larvae (*Gadus morhua*) (Runge, 1988, Kaartvedt, 2000, Beaugrand et al., 2003, Børsheim and Golmen, 2010). In addition, *Calanus* species contribute to vertical export of carbon through fecal pellets, which helps reducing the CO_2 concentration in the atmosphere (Mayor et al., 2012, Edwards et al., 2009).

1.4.1. Effects on *C. finmarchicus* and other copepods

Ocean acidification and effects

Most studies on copepods have been short-term studies (limited time of a life cycle) which have used CO_2 concentrations far above near future levels. These have found effects on

survival, egg production, hatching success and/or development rate (Kurihara et al., 2004a, Kurihara et al., 2004b, Zhang et al., 2011, Weydmann et al., 2012, Kita et al., 2013, Lewis et al., 2013, McConville et al., 2013, Pedersen et al., 2014b). However, the effects were present only at pCO_2 concentrations of ≥ 5000 ppm. Only a few short-term studies, have observed effects at more moderate pCO_2 levels. *Centropages tenuiremis* and *Ascartia spinicauda* showed reduced survival and hatching success at 2000 ppm (Zhang et al., 2011). In addition, *C. tenuiremis* displayed reduced egg production (Zhang et al., 2011) and changes in biochemical variables (glutathione peroxidase, superoxide dismutase and glutathione) in response to the same pCO_2 concentration (Zhang et al., 2012), as well as increased respiration and feeding rate at 1000 ppm (Li and Gao, 2012). The latter suggest that *C. tenuiremis* have tried to balance the energy cost of CO_2-induced stress. A study of copepods from below the Arctic ice also showed reduced survival of adult and nauplii *Oithona similis* and nauplii *Calanus* spp. (mainly *C. glacialis* and *C. hyperboreus*) at 700 and 1000 ppm (Lewis et al., 2013). In addition, a study of *A. clausi* found decreased excretion rate in response to pH 7.83 (Zervoudaki et al., 2013). Studies of *C. finmarchicus* have found reduced hatching success in wild-caught *C. finmarchicus* when females and then eggs were cultured at a pCO_2 concentration of 8000 ppm (pH 6.95) (Mayor et al., 2007). However, this was not found when *C. finmarchicus* eggs from a lab culture reared under optimal conditions were exposed for 8800 ppm (Pedersen et al., 2014b). Increased mortality of nauplii and reduced ontogenetic development rate have been found at pCO_2 concentration of 8800 ppm (Pedersen et al., 2014b).

Few effects have also been observed in medium-term studies (~ a full life cycle), e.g. no significant effects on survival, body size, developmental rate, respiration rate, abundance or developmental stage composition in response to pCO_2 between 185 - 3300 ppm have been demonstrated (Niehoff et al., 2013, Pedersen et al., 2013, Hildebrandt et al., 2014). However, a significant higher mortality was observed for *C. glacialis* exposed for pCO_2 of 3000 ppm and *C. finmarchicus* exposed to pCO_2 of 7300 and 9700 ppm (Pedersen et al., 2013, Hildebrandt et al., 2014). In the latter experiment, ontogenetic development was also likely delayed in response to the same pCO_2 concentrations (Pedersen et al., 2013). Body nitrogen content was reduced in *C. hyperboreus* when they were exposed to pCO_2 of 3000 ppm. Developmental delay has also been observed for *Tigriopus japonicas* as well as reduced hatching success (Kita et al., 2013). However, this was only observed at

pCO_2 concentration of 37 000 µatm and not 5800 µatm which was the other test concentration used (Kita et al., 2013).

A long-term study (multiple generations) using *A. tsuensis* also failed to discover any effects on survival, body size, development and reproductive outcome when the copepods were exposed to pCO_2 of 2380 ppm over three generations (Kurihara and Ishimatsu, 2008). When *C. finmarchicus* was exposed to pCO_2 of 1080, 2080 and 3080 ppm during food limited conditions, negative effects on development, growth and fecundity were linked to an increased energy cost, which was seen through an increased metabolic rate and decreased scope for growth (Pedersen et al., 2014a). However, the delay in development observed in the parental generation exposed to pCO_2 of 2080 ppm was not observed in the following generation, suggesting that *C. finmarchicus* may be able to adapt to pCO_2 conditions predicted for the year 2300 (Pedersen et al., 2014a). Another long-term study showed reduced body length and a decline in nauplii production over three generations of *Tisbe battagliai* exposed to pH 7.82 and pH 7.67, indicating that this species may be more sensitive than other copepod species (Fitzer et al., 2012).

Ocean warming and effects

Between the 1960s and post 1990s the total biomass of *Calanus* spp. declined by 70 % in the North Sea, which is thought to be caused by global warming (Beaugrand et al., 2003, Edwards et al., 2009). In addition, there has been a shift in community structure with warm-water species increasing and cold-water species decreasing. For copepods this is apparent with the warm-temperate species *C. helgolandicus* replacing the cold-temperate *C. finmarchicus* in the North Sea (Edwards et al., 2009).

In short-term studies, temperature (+ 2 - 4 °C) have been found to decrease oxidative status and nauplii development, and increase respiration and excretion (Vehmaa et al., 2013, Zervoudaki et al., 2013). Opposite effects of temperature have also been seen in regard to egg viability and reproductive outcome, depending on the temperature increase and the copepod species (Mayor et al., 2012, Vehmaa et al., 2012, Vehmaa et al., 2013, Zervoudaki et al., 2013). In a medium-term study, respiration was found to increase with temperature (+ 5 and 10 °C) (Hildebrandt et al., 2014). In the same study gonad development was found to increase in response to 5 °C, but decrease in response to 10

°C, suggesting that the thermal upper boundary for *C. hyperboreus*, lies somewhere between 5 and 10 °C (Hildebrandt et al., 2014).

An increase in temperature can also decrease the mean body size as mentioned earlier with the TSR (Atkinson, 1994). This has been observed in both medium and long-term experiments on copepods both under laboratory conditions (e.g. Breteler and Gonzalez, 1988, Uye, 1988, Campbell et al., 2001, Hildebrandt et al., 2014) and in the field (e.g. Durbin and Durbin, 1992, Rice et al., 2015). Development time was also found to decrease with increasing temperature (+ 4 - 15 °C), while growth rate was increased (Breteler and Gonzalez, 1988, Uye, 1988, Ban, 1994, Campbell et al., 2001). In an analysis of 181 publications, which included 33 species of copepods, it was found that temperature could explain more than 90 % of the variance seen in growth rate (Huntley and Lopez, 1992).

Combined effects from ocean acidification and warming

Of the studies looking at the combined effects of acidification and warming, there are four short-term studies and one medium-term study (Mayor et al., 2012, Vehmaa et al., 2012, Vehmaa et al., 2013, Zervoudaki et al., 2013, Hildebrandt et al., 2014). The single effects of the two stressors found in the these studies have already been described earlier in the text. Interaction effects were observed in four of the five studies. For *A. clausi* excretion rate was highest, while for *C. hyperboreus* body carbon content was lowest in the acidified and warming treatment combined, both indicating a synergistic effect (Zervoudaki et al., 2013, Hildebrandt et al., 2014). For *Ascartia* spp. the total egg and nauplii production were lower in the warming treatment than in the combined treatment, suggesting that acidification has ameliorated the effect of warming in the combined treatment (Vehmaa et al., 2012). For *A. bifilosa* the antioxidant capacity was lower in the acidified treatment, implying that warming has worked antagonistic on the acidification effect (Vehmaa et al., 2013).

1.5. Aim of the present study

The main objective of this study is to investigate how morphological characters and the proteome of the copepod *C. finmarchicus* will respond to climate change conditions (ocean acidification and warming) projected within the year 2300. Earlier analysis have shown that the most common interaction effect of acidification and warming in invertebrates are either additive or antagonistic (Byrne and Przeslawski, 2013). It is therefore hypothesized that the combined effect of the two stressors would be additive or antagonistic.

To test the hypothesis, collected *C. finmarchicus* eggs will be cultured under different regimes (control, acidified, warming or warming and acidified) until they reach the sub-adult stage (CV-stage). The sub-adults will be photographed, to collect morphological characters (length and volume of the body and the lipid sac), before extracting and separating whole body proteins on DIGE gels. Differentially expressed proteins will be identified using tandem mass spectrometry. Morphological characters will be compared against the proteome. To the author's knowledge, this is the first experiment where a 2-DE proteomics approach is used to study the combined effect of acidification and warming on any marine organism, as well as the first time the proteome of *C. finmarchicus* is analyzed.

Chapter 2

Material and methods

In 2004, *C. finmarchicus* was sampled in the Trondheimsfjord (Norway) and has since been cultivated at NTNU Centre of Fisheries and Aquaculture (SeaLab) (Hansen et al., 2007). The present experiment was performed at SeaLab, but the protein analysis part was done at Statoil (Rotvoll) and St. Olavs University Hospital.

2.1. Exposure groups

Different levels of CO_2 concentration (390 or 2080 ppm) and temperatures (11 or 14 °C) were combined into a full factorial model (2 x 2, Table 2.1). Each combination was replicated using three parallels. The groups receiving a high CO_2 concentration (2080 ppm) will consequently have a lower pH, and will sometimes be referred to as low pH/acidified treatment.

Table 2.1. Animals were exposed to two different temperatures and CO_2 concentrations, in a full factorial model. Each combination was replicated using three parallels. The temperature (T), pCO_2 concentration and pH shown represent target values.

Treatment	**Tank**	**T (°C)**	***p*CO₂ (ppm)**	**$pH_{T,11/14\,°C}$**
Ambient	A1, B1, C1	11	390	8.03
Acidified	A4, B4, C4	11	2080	7.35
Warming	A2, B2, C2	14	390	7.99
Acidified + warming	A3, B3, C3	14	2080	7.32

2.2. Experimental set-up

Twelve cylinder-conically shaped polyester tanks (90 L) were installed in a temperature-controlled room (Figure 2.1). Parallels from each of the four treatments were placed together in blocks (A, B and C), to minimize temperature gradients within the room. Natural seawater from the Trondheimsfjord was pumped from ~70 m depth and filtered before being stored in reservoir tanks. After temperature regulation and maturation, the water entered one of four equilibration columns (EC). These columns consisted of two vertical tubes, one inner and one outer. In the columns, the water was mixed with CO_2 enriched air to appropriate levels regulated by a custom built CO_2 mixture system (HTK Hamburg®GmbH). The gas was introduced through an air stone (lime wood, Aqua medic) as small bubbles at the bottom of the inner tube.

Figure 2.1. A picture of the experimental set up. The copepods were reared in 90 L tanks in three parallels. The temperature was regulated with a temperature controller (mounted on the wall). A temperature sensor and a heat element placed in the middle of the tanks were connected to the temperature controller. The lamps hanging over the tanks simulated sunlight in a 12:12 hour cycle, with light and dark. The CO_2-enriched air was introduced into the tanks via air stones and distributed from an equilibrium column in the middle. The copepods were continuously feed a mixture of live algae, which was added to the surface.

A submersible aquarium pump (Micro-Jet MC 450, Aquarium systems) transferred water from the outer to the inner tube. The counter-current created by the downward movement of the water and CO_2-enriched air in the opposite direction, ensured effective dissolution of CO_2. From the EC, water was distributed to the experimental tanks through an inlet at the top and bottom.

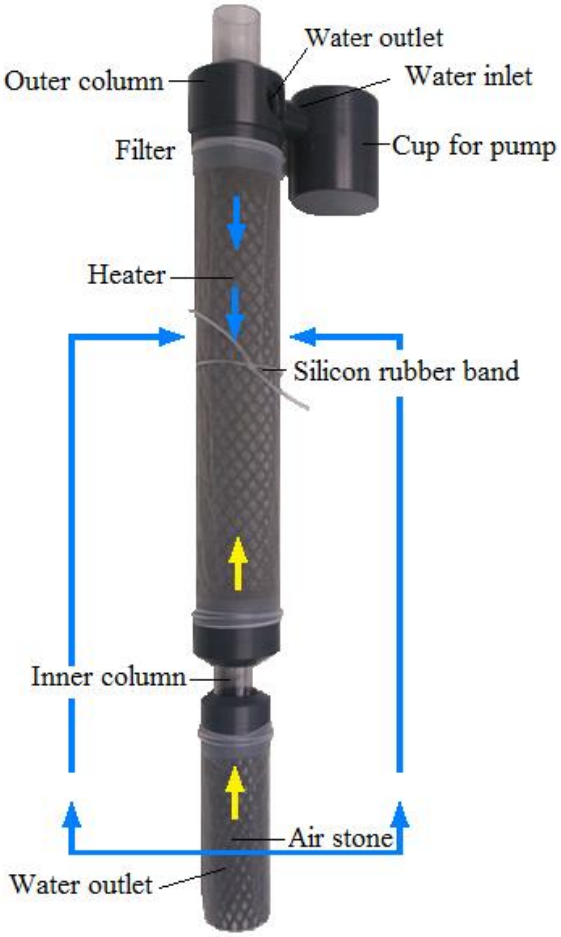

Figure 2.2. A picture of the secondary equilibrium column, coated with a nylon mesh to keep copepods out of the system. In the middle of the column, a silicon rubber band was attached to keep the temperature sensor in place close to middle of the tank. Water was pumped in a downward direction with the help of a submersible pump (blue arrows), while CO_2 was released from an air stone in the bottom of the column (yellow arrows), thus creating a counter-current system similar to the primary equilibrium column.

In addition, CO_2-enriched air was mixed directly in the experiment tank through a secondary ECs, in a similar manner as with the primary EC (Figure 2.2). Around the secondary EC a nylon filtering mesh (pore size 120 µm) was attached to avoid loss of animals and animals entering the system. The filtering mesh was changed once during the

experimental period due to alga and particle coating. The flow rate was set to 3.75 L/h, which corresponds to a full turnover of the tank volume.

A light regime of 12:12 hour light and dark was maintained using a light system. To avoid potential stress caused by bright light, the lamps were covered with black textile.

The temperatures were regulated using custom built proportional-integral-derivative (PID) control boxes. The boxes consisted of four SYL-2372 PID temperature controllers (Auber instruments, Alpharetta, Georgia, USA), four temperature sensors (Platinum RTD. pt 100-element) and four heat elements (Newatt Eco Therm Aquarium Heater, 50W). The temperature sensor was placed with a silicon rubber band around the secondary EC, while the heat element was placed ~10 cm vertically down the inner tube (Figure 2.2).

2.3. Measurements of water parameters and food levels

Prior to the start of the experiment, all water parameters were stable. Alga concentrations were measured twice a week, while all other water parameters were measured every day. To measure alkalinity, salinity and pH, a 50 - 100 ml seawater sample was collected in airtight borosilicate glass bottles inside a filtering cup, which kept the copepods out of the sample. The glass bottles were placed in a thermostat water bath (25 °C).

2.3.1. Alkalinity

Alkalinity was measured as total alkalinity. A sample was taken from one of the replicate tanks, and analyzed using the method described by Xiaowan et al. (2009). A volume of approximately 25 ml sample was weighted and transferred to a titration cell, before analyzed using an automatic potentiometric titrator with PHC2001-8 pH electrode and water-jacked titration cell with a magnet stirrer (TitraLab TIM860 Titration Manager, Radiometer Analytical SAS; Villeurbanne, France). In contrast to Xiaowan et al. (2009), who used artificial seawater, NaCl (0.68 M) was used to adjust the ionic strength of the titrant. The accuracy of the titrator was examined by measuring the alkalinity of certified seawater (Scripps Institution of Oceanography, La Jolla, USA).

2.3.2. Salinity

Salinity was measured from one of the replicate tanks, with a refractometer (H_2Ocean ATC salinity refractometer). This was done according to the manufactures instructions. The instrument was calibrated using certified seawater prior to use.

2.3.3. The pH

Measurement of pH was based on the method described by Dickson et al. (2007), which uses the total hydrogen ion concentration pH (pH_T) scale. However, for the sake of simplicity, pH_T will be referred to as just pH. The pH was measured at 25 °C using a Cary 50 Bio UV-visible Spectrophotometer (Varian, Inc., Mulgrave, Australia) with a Cary Single cell peltier accessory (Varian) and a Micro-Jet MC450 multi-use micro-pump (Aquarium Systems, Sarrebourg, France).

The absorbance of the water samples was measured at wavelengths 434, 578 and 730 nm at the RNA-DNA submeny of the Cary WinUV software (Varian). About 3.7 ml of the sample was applied until a positive meniscus was reached in a gas-tight optical glass cuvette with a Teflon® cap. A blanc was measured first, then 50 µl of *m*-cresol purple was added to the sample.

2.3.4. Temperature

Temperature was measured with a glass rod thermometer (VWR®Precicion Thermometer, accuracy ± 0.3 °C), which was placed on the end of the filtering cup. The thermometer and filtering cup were rinsed before transfer to the next tank.

2.3.5. Food

On the start day of the experiment, 5 dl of alga stock was added to each of the tanks to reach target concentration. Three types of unicellular algae were used as food for the copepods, *Rhodomonas baltica*, *Dunaliella tertiolecta* and *Isochrysis galbana*. A 225 µg/L carbon equivalents were given of *R. baltica* and *D. tertiolecta*, and additionally, 25 C µg/L were given of *I. galbana*. Out of the 225 C µg/L, 91.73 % came from *R. baltica*, and 8.27 % from *D. tertiolecta*. This mixture has shown to work well in earlier experiments (Hansen et al., 2007). The level of alga carbon was expected not to mask

effects from the treatments, and was below optimum food concentration for *C. finmarchicus*.

The alga solution was distributed through an outlet placed above the surface using a twelve channel peristaltic dosing pump (Watson-Marlow 520S). Water samples (~20 ml) from each tank were collected in a filtering cup with pore size 64 µm. The concentration was measured with a coulter counter (Multisizer^{TM} 3 Coulter Counter® Beckman coulter Inc., USA) and the software program Multisizer^{TM} 3. The program calculates the cell density (cells/ml) by integrating an area of number of particles counted and particle diameter (µm). *R. baltica* and *D. tertiolecta* was measured in the area 5.333 to 9.561 µm. The actual amount of carbon equivalents provided to the copepods was calculated based on cell counts and assuming a carbon content of 54.6 ng and 73.2 ng per cell to *R. baltica* and *D. tertiolecta*, respectively (values determined on the in house cultures).

2.3.6. Calculations of carbonate parameters

The pH, alkalinity and salinity, were all measured at 25 °C, and were together with temperature used to calculate the *in situ* pH at 11 and 14 °C, partial pressure of carbon dioxide (pCO_2), calcite (Ω Ca) and aragonite (Ω Ar) in the program pCO_2- SYS-v2.1.xls (Pelletier et al., 2007). Constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987), and KSO_4 from Dickson (1990) were used.

2.4. Sampling of *C. finmarchicus*

2.4.1. Collection of eggs

Adult copepods from the SeaLab culture (35th generation) were distributed into eight 50 L polyethylene tanks (500 in each) filled with seawater (10 °C). After 24 h, fertilized eggs were collected with a filtering cup (46 µm cut off) and pooled. The number of eggs per volume was determined from counting the number of eggs in sub samples under a Leica M205 C stereomicroscope (Leica Microsystem, Wetzlar, Germany). According to our estimate a total of 58 000 eggs were obtained and these were distributed between 12 glass beakers (166 ml in each). The eggs were acclimated to the CO_2 concentration they would later be exposed to by submerging them into three consecutive glass bowls containing the

respective CO_2 concentration. Before releasing the eggs into the tanks, they were acclimated to either 11 or 14 °C inside 500 mL glass bottles that were submerged into their respective tanks.

2.4.2. Collection of sub-adults

When the copepods reached the CIV-stage, random subsamples (preferably 200 animals) were transferred to 5 L polyethylene buckets for incubation. Daily, animals that had molted into the CV-stage were sorted out under a dissection microscope (Leica MZ 125, Leica Microsystem, Wetzlar, Germany) and transferred to 1 L buckets for 48 h cultivation prior to sampling. This ensured that all sampled animals had a comparative stage and age (CV + 48 h). All water parameters were measured daily. The water was also changed on a daily basis.

A total of, 3 x 5 copepods (from each treatment) were sedated with tricaine methanesulfonate (1.5 g/L FinQuel in seawater, Argent Laboratories, Redmond WA, USA) and photographed with a digital still-video camera (Sony DWF-sx900, Sony Corporation, Tokyo, Japan) and the program Fire-i-software (Unibrain Inc., San Ramon CA, USA). After taking pictures, the copepods were transferred to 1.5 ml eppendorf tubes with nitrogen (g) and frozen at -80 °C.

2.5. Analysis

2.5.1. Biometry

The pictures of the CV animals were analyzed with the software program ImageJ (National Institutes of Health, Bethesda MD, USA), on a pen-on-screen graphical tablet (Wacom Intous3, Wacom CO.,LTD., Saitama, Japan). By also photographing a glass scale ruler (310 345, Wild Heerbrugg, Switzerland), the program was able to convert pixels to millimeters. Length and volume of the body were measured based on the method described by Miller et al. (1998). The length of the body and lipid sac were measured by superimposing lines over the compartments. Length of the body was measured from the anterior part of the head to the posterior part of the fifth thoracic segment, while the lipid sac length was measured from the visible anterior to the posterior end (Figure 2.3). Area

was measured by tracing a line around the body and lipid sac, and calculated by the program. In some of the copepods, the stomach covered parts of the lipid sac, which made it difficult to obtain accurate measurements. These were therefore excluded from the lipid sac analysis.

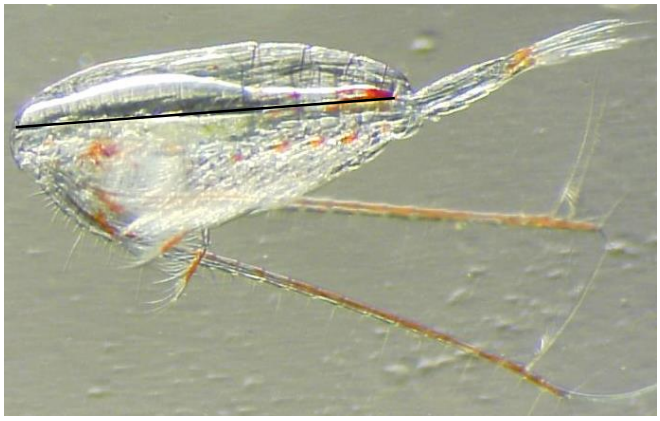

Figure 2.3. Picture of 48 h old *Calanus finmarchicus* (CV-stage). A line was superimposed over the lipid sac and the body to measure length, while a line was traced around them to calculate area and with that volume.

2.5.2. Protein extraction

Before extracting the proteins, frozen copepods were transferred to a tissue grinder (VWR International, Radnor, Pennsylvania, USA) and homogenized in 150 µl (50 mM) Tris-HCl (Tris(hydroxymethyl)aminomethane- hydrochloride, pH 8.2) with 4 % IGEPAL (octylphenoxy] polyethoxyethanol) and protease inhibitor cocktail (P9599 Sigma). An additional 250 µl Tris-HCl, was added to the homogenate, and the mixture was transferred to a 1.5 ml eppendorf tube and placed on dry ice.

The extraction method is based on a method described by Lane (2013), with some adjustment after suggestions from Trygve Devold Kjellsen. The entire method, with the modifications is described here. Extraction of proteins was performed by adding 200 µl 40 % sucrose, 20 µl 2-mercaptoethanol, 10 µl 10 % sodium dodecyl sulphate (SDS) and 500 µl phenol (equilibrated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to each tube. The tubes were shaken vigorously for 30 seconds before centrifugation for 3 min at 13 000 x g. Following centrifugation and phase separation, the upper layer containing the phenol phase was divided into 100 µl aliquots in three 2 ml eppendorf tubes (total 300 µl). Precipitation of proteins from the phenol phase was done by addition of 400 µl ice-cold

(-20 °C) 80 % methanol with 0.1 M sodium acetate. Tubes were placed at -20 °C overnight to allow precipitation.

Following precipitation the tubes were centrifuged at 13 000 x g for 10 min, at 4 °C. The pellets were carefully washed with acetone by shaking of the tubes for 30 seconds. The procedure was repeated twice before pellets were allowed to air-dry briefly to remove residual acetone. The pellet was then dissolved in 30 µl rehydration buffer (7M Urea, 2M Thiourea, 4 % CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate), 30 mM Tris, pH 8.8. The extracts were pooled and the empty tubes were washed with an additional 20 µl rehydration solution making each extract a total of 50 µl.

2.5.3. Protein quantification

Protein concentration was quantified using the Bradford method (Bradford, 1976). Equal amount of Coomassie Serva Blue G was mixed with 17 % phosphoric acid. The sample (5 µl) was diluted with a factor of 20. To make the standard curve, 0.2 mg/ml of albumin bovine serum (BSA) was diluted into concentrations of 6.9, 17.3, 26.0, 43.3, 52.0, 69.3, 86.7 and 104.0 µg/ml.

The BSA solution was measured at wavelength 280 nm using a Cary 50 Bio UV-visible spectrophotometer (Varian). In a 96 well plate (TPP®, Techno Plastic Product AG, Trasadingen, Switzerland), 29 µl sample and 171 µl coomassie blue solution were added. After 5 min, absorbance was measured at 595 nm with a Cary 50 Microplate Reader (Varian), and concentrations were calculated based on the calibration curve (Figure B.1, Appendix B).

2.5.4. Gel electrophoresis and mass spectrometry

Proteins were separated based on pI (first dimension) and size (second dimension) using gel electrophoresis. Differential in gel electrophoresis (DIGE) was used to detect differentially expressed proteins. The differentially expressed proteins were picked from a preparative gel and annotated with mass spectrometry. The DIGE gels was stained with CyDye DIGE Fluor minimal dyes (1 µl/50 µg protein, GE Healthcare) before first and second dimensions of gel electrophoresis, according to protocol from manufacture. Six

DIGE gels were made, each containing one pooled standard and two treatment replicates, which were marked with the fluorescent dyes Cy2, Cy3 and Cy5. It was random which dye and gel the different treatment replicates were given (set-up in Appendix D, Table D.2). The preparative gel was stained after running of the first and second dimension with 1 µM Ruthenium (II) tris (4,7-diphenyl-1,10-phenantrolin disulfonate) (RuBPS), after method (protocol 1) described by Lamanda (2010).

Isoelectric focusing

Immobiline DryStrips (Immobile pH gradient (IPG) gel, 24 cm, nonlinear, pH 3 - 11, GE Healthcare Life Science, CT, USA) were soaked in rehydration buffer (450 µl), and covered with coating fluid (GE Healthcare), overnight. The 24 cm strip was chosen to gain maximum resolution and loading capacity. The broad pH range gives an overview over the total protein distribution, and work with most protein extract from both prokaryotic and eukaryotic cells (GE Healthcare, principles and methods). Following rehydration, the strips were placed in ceramic strip holders (GE Healthcare). Paper wicks were applied to absorb excess ions and buffers. Protein extracts (150 µl) were placed either directly on the gel (DIGE gel) or in sample cups (preparative gels) together with 1 µl IPG buffer (GE Healthcare). The gels were run overnight on the following program at the Ettan IPGphor unit (GE Healthcare), 0.01 h at 500 V, 3.0 h at 2000 V, 5.0 h gradient to 8000 V, 5.0 h at 8000 V and 2.0 h at 500 V.

SDS-PAGE

Before the second dimension, the IPG gels were placed 15 min in equilibration buffer (EB, 6 M urea, 50 mM Tris-HCl pH 8.8, 30 % glycerol, 2 % SDS, 0.01 % bromophenol blue) with 65 mM dithiothreitol (DTT), and 15 min in EB with 135 mM iodoacetamide (IAA). An Ettan DALTsix Large Vertical system (GE Healthcare) was used for the second dimension gel electrophoresis (Figure 2.4).

The anode assembly was placed in the tank chamber, and 37.5 ml anode buffer (from DALT buffer kit, GE Healthcare) in 4.5 L dH_2O was poured in. Two milliliters of dH_2O was applied on the DALT gels and placed in the gel casting cassette. Sealing solution (from the DALT buffer kit) was placed in the corners. The IPG gels were placed on top of the DALT gels, facing away from the glass and with no air bubbles in between.

Approximately 2 ml of the sealing solution was added in a 1 - 2 cm thick line on top of the IPG gels. The gels were run for 30 min at 3 W and 4.5 h at 51 W. The DALT gels were placed in a container with 30 % ethanol and 10 % acetic acid overnight. A digital image of the gel was taken by scanning the gel in a Typhoon trio with Typhoon Trio Scanner control software (GE Healthcare). The software SameSpot (TotalLab, New Castle, UK) was used to identify spots that were significantly differentially expressed between the treatments. Spots from the preparative gel were picked with an Ettan Spot Picker (GE Healthcare), and the program DeCyder (GE Healthcare).

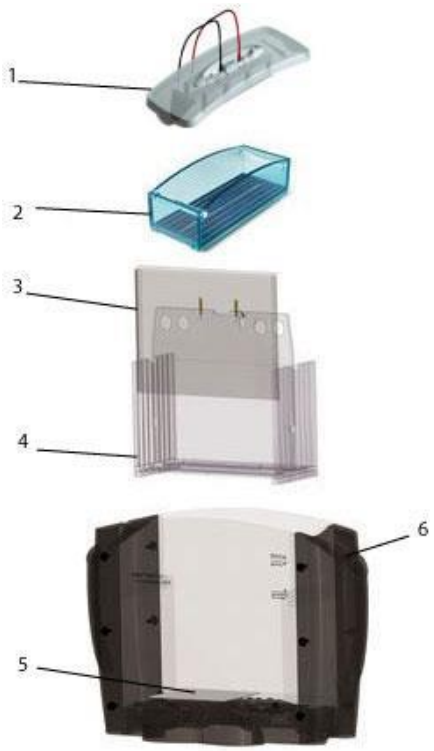

Figure 2.4. Components of the Ettan DALTsix Large Vertical system, 1: Lid assembly, 2: Upper buffer chamber, 3: Gel Casting Cassette 1.0mm, 4: Anode assembly/cassette carrier, 5: Pump 220 V, 6: Tank chamber (GE Healtcare Life Science)

Tandem mass spectrometry

The picked spots were desalted, before peptides in the spots were dried in a centrifuge and resuspended in 0.1 % formic acid. The peptides were analyzed on a liquid chromatography-MS/MS (LC-MS/MS) platform consisting of an Easy-nLC 1000 UHPLC (ultra high performance LC) system (Thermo Scientific/Proxeon) interfaced with a QExactive mass spectrometer (Thermo Scientific) via a nano spray ESI (electrospray ionization) ion source (Proxeon, Odense). Peptides were injected onto a C-18 trap column

(Acclaim PepMap100, 75 μm i.d. x 2 cm, C18, 5 μm, 100 Å, Thermo Scientific) and further separated on a C-18 analytical column (Acclaim PepMap100, 75 μm i.d. x 50 cm, C18, 3 μm, 100 Å, Thermo Scientific) using a 37 min gradient from 10 - 30 % CH_3CN, 0.1 % formic acid at a flow rate of 250 nl/min.

Eluted peptides were analyzed on the Qexactive mass spectrometer operating in a positive ion mode under data dependent acquisition (DDA) using the following parameters: Electrospray voltage 1.9 kV, HCD (higher-energy C-trap dissociation) fragmentation with normalized collision energy 30, automatic gain control (AGC) target value of 3e6 for Orbitrap MS and 1e5 for MS/MS scans. Each MS scan (m/z 400–1600) was acquired at a resolution of 70 000 FWHM (full with a half maximum), followed by 10 MS/MS scans triggered for intensities above 1.7e4, at a maximum ion injection time of 100 ms for MS and 60 ms for MS/MS scans.

Peptide sequences from the MS/MS analysis were run in the database UniprotKB (*Calanus*) and Mascot (metazoa) with the use of the software program Xcalibur Proteome Discoverer Version 1.1. If no candidates from the *Calanus* database were detected, the search was expanded to the metazoan group. All candidates found by the program had a FDR < 0.05. The identity of the proteins were determined on the number of unique peptides and their coverage on the fragment spectra (more information in section 1.3). Coverage in this software is given by color, where high, medium and low levels are designated by green, yellow and red, respectively.

2.6. Data analysis

Permutational multivariate analysis of variance (PERMANOVA) analysis of 2-DE gel electrophoresis (spot volume) and biometry data were performed using *R version 3.0.2* (R Development Core Team, 2014), *post hoc* and test of homogeneity of variance were performed using *SPSS statistics version 21* (IBM® SPSS®). Package used in *R* was VEGAN, with the function "adonis*"*. Significance level was set to 0.05 in all tests.

The test-statistic in PERMANOVA is similar to Fisher's F-ratio and can be calculated from a symmetric distance or dissimilarity matrix (in this case Bray-Curtis dissimilarity).

The *p*-values are acquired using permutations. Since PERMANOVA is a non-parametric test, the assumption about normality is not required (Anderson, 2001). PERMANOVA was used to test treatment effects and to assess whether there had been any interaction between the two factors (temperature and pH). Further comparisons were done using the *post hoc* test Fisher's least significant difference test (LSD). Before running *post hoc* analysis, Levene's test for homogeneity of variance was performed, and data were log-transformed when necessary.

Chapter 3

Results

3.1. Seawater chemistry and feeding conditions

3.1.1. Water parameters

Water parameters were measured from the start of the experiment until the copepod reached the CIV-stage (Table 3.1. first period), and further on when incubated for 48 hours after molting into the CV-stage (Table 3.2, second period). During the first period, temperature, calculated pH and pCO_2 concentration varied only slightly over time (± 0.02 - 0.22 °C for temperature, ± 0.01-0.04 units for pH and ± 22.60 - 224.42 ppm for pCO_2). Temperature was also close to target values (± 0 - 0.2 °C), while pH (± 0.01 - 0.14 units) and pCO_2 varied slightly more from target (± 8 - 235 ppm) during the second period.

Table 3.1. Measured and calculated water parameters in the different exposure treatments during the first period (development from egg to the CIV-stage). The data are shown as mean ± standard deviation (SD: n = 3). Temperature (T), $pH_{25\,°C}$, total alkalinity (A_T) and salinity (S) were used to calculate the *in situ* $pH_{11/14\,°C}$, partial pressure of carbon dioxide (pCO_2) calcite (Ω Ca) and aragonite (Ω Ar) in the program *p*CO2-sys.

	Measured				Calculated			
Treatment	**T (ºC)**	**$pH_{T,25\,°C}$**	**A_T (µmol/kg)**	**S (PSU)**	**$pH_{T,11/14\,°C}$**	***p*CO_2 (ppm)**	**Ω Ca**	**Ω Ar**
Ambient	11.1±0.07	7.78±0.02	2245.13±11.96	33.6±0.44	7.99±0.02	459.29±22.60	2.82±0.10	1.76±0.06
Acidified	11.1±0.22	7.20±0.03			7.36±0.03	2136.56±173.20	0.75±0.06	0.48±0.04
Warming	14.0±0.17	7.80±0.02			7.96±0.03	492.33±33.86	1.90±0.09	7.36±0.03
Acidified + warming	14.0±0.10	7.26±0.02			7.39±0.02	2056.88±115.65	0.88±0.05	0.56±0.03

Table 3.2. Measured and calculated water parameters in the different exposure treatments during the second period (CV-stage). Measured and calculated water parameters in the different exposure treatments. The data are shown as mean ± standard deviation (SD: n = 3). Temperature (T), $pH_{25\,°C}$, total alkalinity (A_T) and salinity (S) were used to calculate the *in situ* $pH_{11/14\,°C}$, partial pressure of carbon dioxide (pCO_2) calcite (Ω Ca) and aragonite (Ω Ar) in the program pCO2-sys.

	Measured				Calculated			
Treatment	**T (ºC)**	**$pH_{T,25\,°C}$**	**A_T (µmol/kg)**	**S (PSU)**	**$pH_{T,11/14\,°C}$**	***p*CO_2 (ppm)**	**Ω Ca**	**Ω Ar**
Ambient	11.1±0.02	7.69±0.00	2240±9.95	33.5±0.27	7.89±0.01	582.05±17.94	2.32±0.06	1.48±0.04
Acidified	11.1±0.03	7.26±0.02			7.42±0.03	1845.16±126.56	0.85±0.06	0.54±0.04
Warming	13.9±0.08	7.71±0.02			7.88±0.02	616±33.03	2.48±0.11	1.58±0.07
Acidified + warming	13.8±0.07	7.23±0.04			7.38±0.04	2088.03±224.42	0.86±0.08	0.55±0.05

3.1.2. Food concentration

The animals were fed with three types of live micro algae. Carbon equivalents from *R. baltica* and *D. tertiolecta* are presented in Figure 3.1 (first period) and Figure 3.2 (second period). As the copepods were not expected to prey of the third alga, *I. galbana*, contribution of carbon equivalents from this species was not included in the figure. Measurements from day 4 to 11, show an increase in the alga concentration. After day 11 the alga concentration started to decline in all treatments. A larger decline was present in both high temperature treatments *versus* the ambient and acidified treatment.

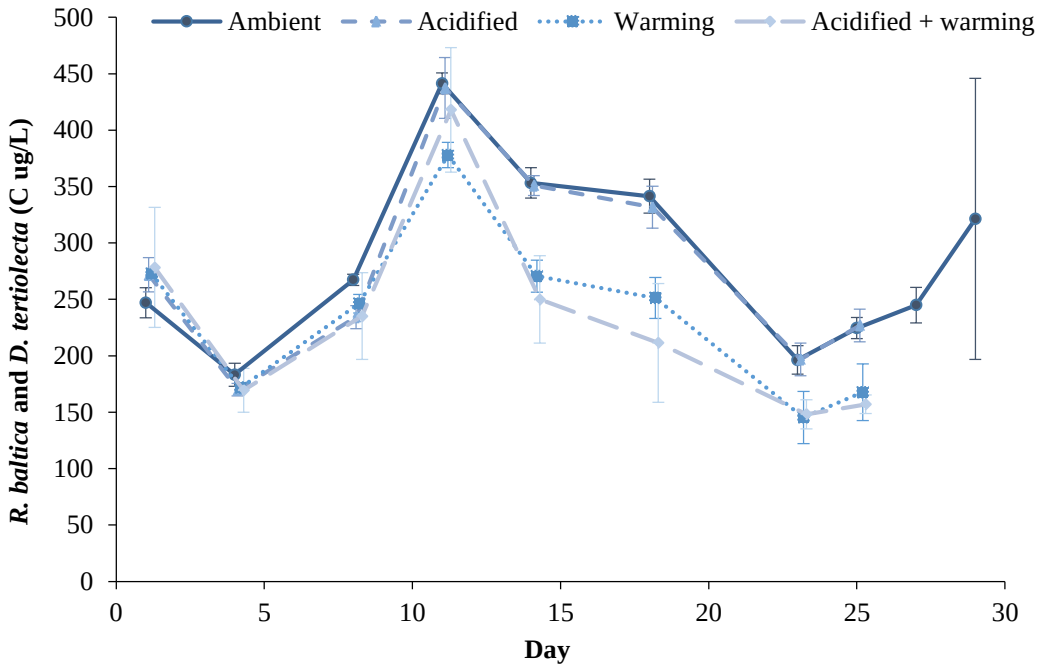

Figure 3.1. Carbon equivalents (µg/L) of *Rhodomonas baltica* and *Dunaliella tertiolecta* during the first period development from egg to CIV-stage). The data are shown as mean ± standard error (SE: n = 3).

Lower alga concentrations were observed in all treatments during the second period. The falling concentration was probably caused by a combination of alga sedimentation and grazing rates of the copepods surpassing the addition of new algae during semi static water changes. Some measurements are missing due to technical problems with the alga counter apparatus.

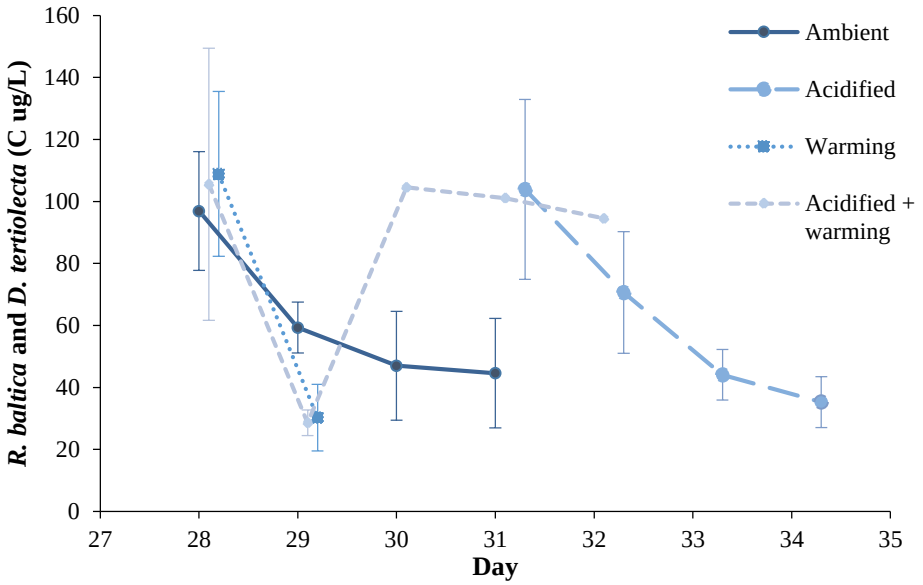

Figure 3.2. Carbon equivalents (µ/L) of *Rhodomonas baltica* and *Dunaliella tertiolecta* during the second period (CV-stage). The data are shown as mean ± standard error (SE: n = 3).

3.2. Somatic growth

All biometric measurements can be seen in Table A.1 (Appendix A). The morphological variables were all affected by warming, but not by pH (Figure 3.3 – 3.5, Table 3.3). It was also found that animals exposed to warming, regardless of pH level, developed into the CV-stage approximately five days earlier than the animals from the ambient temperature treatments (29 *versus* 34 days). Homogeneity of variance and LSD tests are listed in Appendix F (Table F.1 and F.3, respectively).

Table 3.3. PERMANOVA results on biometric data of *Calanus finmarchicus* (CV-stage) exposed for acidification and warming, separately and in combination. Significant treatment effects or interactions are marked in bold ($p < 0.05$). Listed are the degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-statistic (F), coefficient of determination (R^2) and probability value (p).

	DF	SS	MS	F	R^2	*p*
Body length						
Temperature	**1**	**0.002**	**0.002**	**58.097**	**0.816**	**0.001**
pH	1	>0.000	>0.000	0.261	0.004	0.621
Temperature * pH	1	>0.000	>0.000	4.835	0.068	0.075
Residuals	8	>0.000	>0.000		0.112	

Table continued on next page

Table 3.3. Continued

	DF	SS	MS	F	R^2	*p*
Oil sac length						
Temperature	**1**	**0.024**	**0.024**	**42.990**	**0.814**	**0.001**
pH	1	0.001	0.001	1.295	0.025	0.267
Temperature * pH	1	>0.000	>0.000	0.550	0.010	0.491
Residuals	8	0.004	0.001		0.151	
Oil sac volume (% of body)						
Temperature	**1**	**0.128**	**0.128**	**19.577**	**0.578**	**0.005**
pH	1	0.014	0.014	2.175	0.064	0.189
Temperature * pH	1	0.027	0.027	4.141	0.122	0.071
Residuals	8	0.052	0.007		0.236	

3.2.1. Body length

Body length of *C. finmarchicus* was significantly affected by warming ($F_{(1,8)} = 58.097$, $p = 0.001$), but not by pH ($F_{(1,8)} = 0.261$, $p = 0.621$). No significant interaction was found between the two stressors ($F_{(1,8)} = 4.835$, $p = 0.075$).

Further *post hoc* analysis showed that the body length was significantly reduced in animals exposed to warming alone ($p < 0.001$) and acidified- and warming conditions combined ($p = 0.001$), respectively 6.64 and 4.88 % *versus* the ambient treatment.

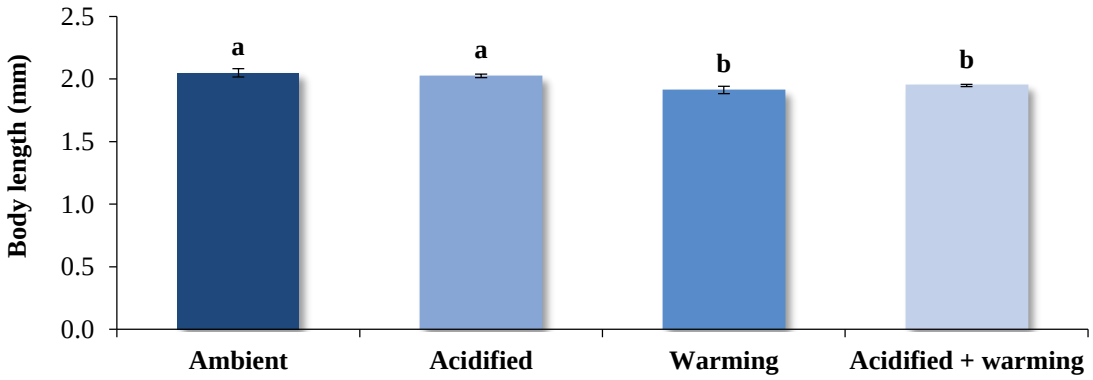

Figure 3.3. Body length of *Calanus finmarchicus* (CV-stage) after acidified and warming treatment, separately and in combination. Ambient (11 °C, 390 ppm), acidified (11 °C, 2080 ppm), warming (14 °C, 390 ppm) and acidified + warming (14 °C, 2080 ppm). The data are shown as mean ± standard deviation (SD: n = 3). Different letters denote significant differences ($p < 0.05$) according to Fisher's least significant difference test (LSD).

3.2.2. Oil sac length

Oil sac length of *C. finmarchicus* was significantly affected by warming ($F_{(1,8)} = 42.990$, $p = 0.001$), but not by pH ($F_{(1,8)} = 1.295$, $p = 0.267$). No significant interaction was found between the two stressors ($F_{(1,8)} = 0.550$, $p = 0.491$).

Further *post hoc* analysis showed that the oil sac length was significantly reduced in animals exposed to warming alone ($p = 0.001$) and acidified- and warming conditions combined ($p = 0.005$), respectively 18.12 and 13.71 % *versus* the ambient treatment.

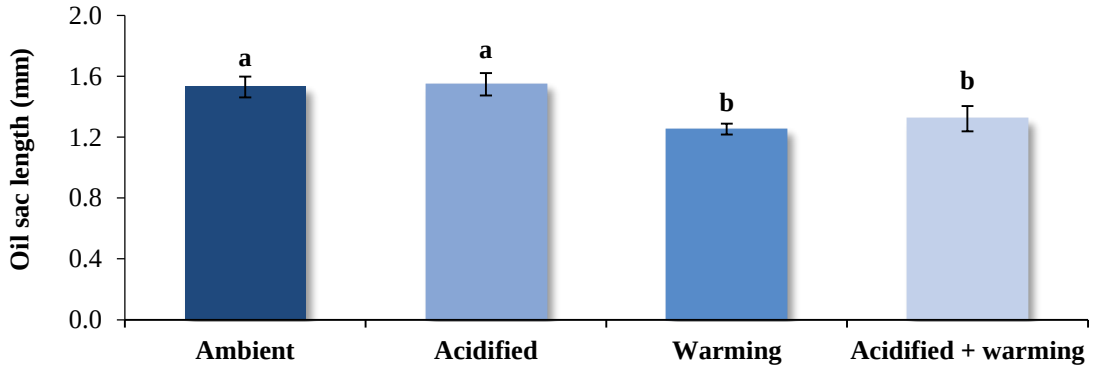

Figure 3.4. Oil sac length of *Calanus finmarchicus* (CV-stage) after acidified and warming treatment, separately and in combination. Ambient (11 °C, 390 ppm), acidified (11 °C, 2080 ppm), warming (14 °C, 390 ppm) and acidified + warming (14 °C, 2080 ppm). The data are shown as mean ± standard deviation (SD: n = 3). Different letters denote significant differences ($p < 0.05$) according to Fisher's least significant difference test (LSD).

3.2.3. Oil sac volume (as percentage of body volume)

Oil sac volume (as percentage of body volume) of *C. finmarchicus* was significantly affected by warming ($F_{(1,8)} = 19.577$, $p = 0.005$) but not by pH ($F_{(1,8)} = 2.175$, $p = 0.189$). No significant interaction was found between the two stressors ($F_{(1,8)} = 4.141$, $p = 0.071$).

Further *post hoc* analysis showed that the oil sac volume was significantly reduced with 46.33 % in animals exposed to warming alone ($p = 0.003$), *versus* the ambient treatment. Although, not significant ($p = 0.077$), the oil sac volume showed a 22.89 % reduction in the warming and acidified treated animals *versus* the ambient treatment.

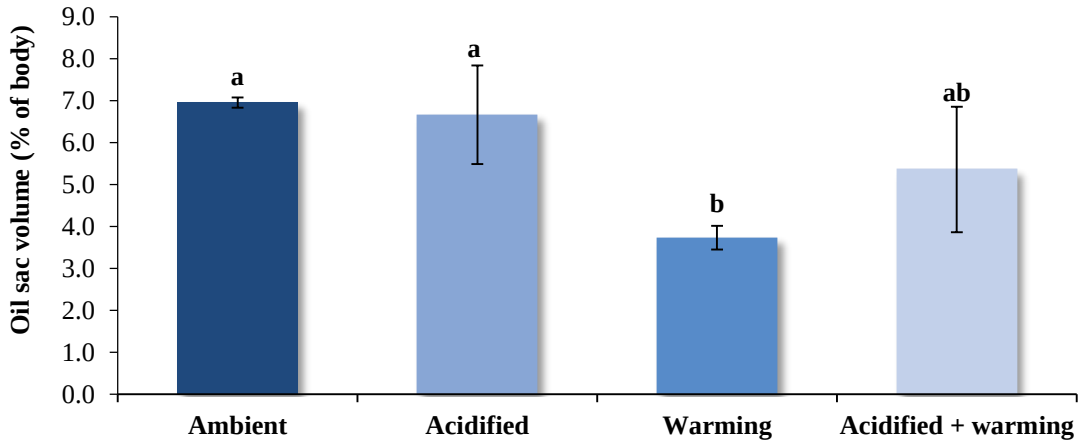

Figure 3.5. Oil sac volume (as percentage of body volume) of *Calanus finmarchicus* (CV-stage) after acidified and warming treatment, separately and in combination. Ambient (11 °C, 390 ppm), acidified (11 °C, 2080 ppm), warming (14 °C, 390 ppm) and acidified + warming (14 °C, 2080 ppm). The data are shown as mean ± standard deviation (SD: n = 3). Different letters denote significant differences ($p < 0.05$) according to Fisher's least significant difference test (LSD).

3.3. Proteome analysis

Figure 3.6 shows one of the DIGE gels with protein extracts from *C. finmarchicus* (whole body). The arrows shows the proteins that were differentially expressed in response to the treatments. A picture of the preparative gel and the measured spot volumes can be seen in Appendix D and C, respectively (Figure D.1 and Table C.1, respectively). Approximately 1300 proteins were found. The majority of these proteins were found in a pH range between 4.6 to 8.8.

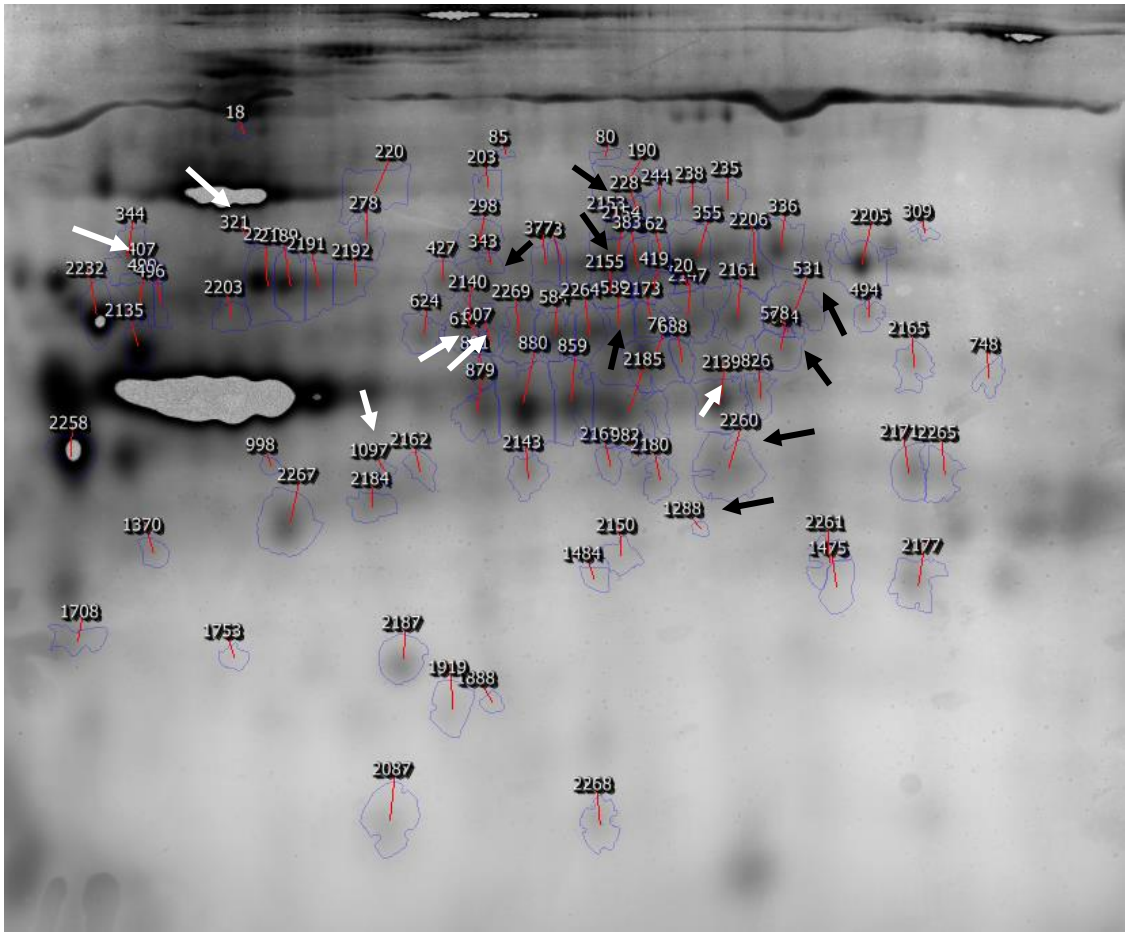

Figure 3.6. DIGE gel with CyDye stained proteins from *Calanus finmarchicus* (CV-stage) in pH range 3 - 11. Arrows show significant differentially expressed proteins, white shows proteins that were tentatively identified and black shows non-identified proteins. Comparison between the different treatments were performed using PERMANOVA ($p < 0.05$), and significant differences was further analysed using Fisher's least significant difference *post hoc* test (LSD).

3.3.1. Protein identification

Database search lead to the tentatively identification of six proteins that were significantly expressed (Table 3.4). All proteins had FRD < 0.05. Two spots were identified as the same protein (spot 321 and 407). Since tandem mass spectrometry was used, it was possible to identify some of the proteins based on a single peptide (Eriksson and Fenyö, 2011).

Table 3.4. List of successfully identified proteins that were differentially expressed in *Calanus finmarchicus* (CV-stage) after warming and acidified treatment, separately and in combination. Proteins were identified with MS/MS and database search in UniprotKB (most spots) and NCBI (spot 607). All proteins had FDR < 0.05.

Spot no.	Unique peptides	Accession no.	Tentative identification	Species
321	8	D2CL59	Mitochondrial heat shock protein 60	*C. glacialis*
407	9	D2CL59	Mitochondrial heat shock protein 60	*C. glacialis*
607	1	68272073	Glyceraldehyde-3-phosphate dehydrogenase	*Tigriopus japonicus*
614	1	F6KHK5	Histone H3	*C. helgolandicus*
1097	2	R9TIJ9	Actin	*C. sinicus*
2139	1	R9TDW4	Glucose-6-phospate isomerase	*C. sinicus*

3.3.2. Protein expression

The identified proteins

All of the identified proteins were negatively influenced by warming, and two were also influenced by pH. A significant interaction was present for one of the proteins (Figure 3.7 – 3.12, PERMANOVA, Table 3.5). Homogeneity of variance and LSD tests can be seen in Appendix F (Table F.2 and F.4, respectively).

Table 3.5. PERMANOVA result of protein expression in *Calanus finmarchicus* (CV-stage) exposed for acidification and warming, separately and combined. Significant treatment effects or interactions are marked in bold ($p < 0.05$). Listed are the degrees of freedom (DF), sum of squares (SS), mean squares (MS), F-statistic (F), coefficient of determination (R^2) and probability value (*p*).

	DF	SS	MS	F	R^2	*p*
Mitochondrial heat shock protein 60						
Temperature	**1**	**0.249**	**0.249**	**9.146**	**0.397**	**0.019**
pH	1	0.032	0.032	1.190	0.0517	0.303
Temperature * pH	**1**	**0.128**	**0.128**	**4.705**	**0.204**	**0.039**
Residuals	8	0.217	0.027		0.347	
Mitochondrial heat shock protein 60						
Temperature	**1**	**0.178**	**0.178**	**11.222**	**0.536**	**0.003**
*p*H	1	0.010	0.010	0.658	0.031	0.464
Temperature * pH	1	0.017	0.017	1.063	0.051	0.321
Residuals	8	0.127	0.016		0.382	
Glyceraldehyde-3-phosphate dehydrogenase						
Temperature	**1**	**0.046**	**0.046**	**5.032**	**0.234**	**0.043**
pH	**1**	**0.053**	**0.053**	**5.750**	**0.268**	**0.033**
Temperature * pH	1	0.025	0.025	2.691	0.125	0.143
Residuals	8	0.074	0.009		0.373	
Histone H3						
Temperature	**1**	**0.035**	**0.035**	**6.986**	**0.297**	**0.039**
pH	**1**	**0.034**	**0.034**	**6.844**	**0.291**	**0.030**
Temperature * pH	1	0.009	0.009	1.705	0.072	0.212
Residuals	8	0.040	0.005		0.340	
Actin						
Temperature	**1**	**0.058**	**0.058**	**7.447**	**0.372**	**0.023**
pH	1	0.033	0.033	4.168	0.208	0.086
Temperature * pH	1	0.003	0.003	0.430	0.021	0.532
Residuals	8	0.063	0.008		0.399	
Glucose-6-phosphate isomerase						
Temperature	**1**	**0.122**	**0.122**	**8.331**	**0.406**	**0.012**
pH	1	0.049	0.049	3.315	0.162	0.080
Temperature * pH	1	0.013	0.013	0.858	0.042	0.412
Residuals	8	0.117	0.015		0.390	

Mitochondrial heat shock protein 60 (spot 321)

By MS/MS analysis spot 321 was identified to be mitochondrial heat shock protein 60 (mHSP60, Figure 3.7). Spot 321 had eight unique peptides that matched with mHSP60, all with good coverage on the fragmentation spectra (Figure E.1, Appendix E).

Mitochondrial HSP60 expression level was significantly affected by warming ($F_{(1,8)}$ = 9.146, p = 0.019), but not by pH alone ($F_{(1,8)}$ = 1.190, p = 0.303). However, a significant interaction between the two stressors was observed ($F_{(1,8)}$ = 4.705, p = 0.039).

Further *post hoc* analysis showed a near significant up-regulation of mHSP60 in animals exposed to the acidified treatment (p = 0.051), 1.96 fold *versus* the ambient treatment. This increase was not observed when the copepods also were exposed to elevated temperature. If a pure additive effect had been apparent, the fold change in the acidified and warming treatment combined would have been 1.84 as indicated on the figure (dashed line). This implies that warming had an antagonistic effect when combined with acidified conditions.

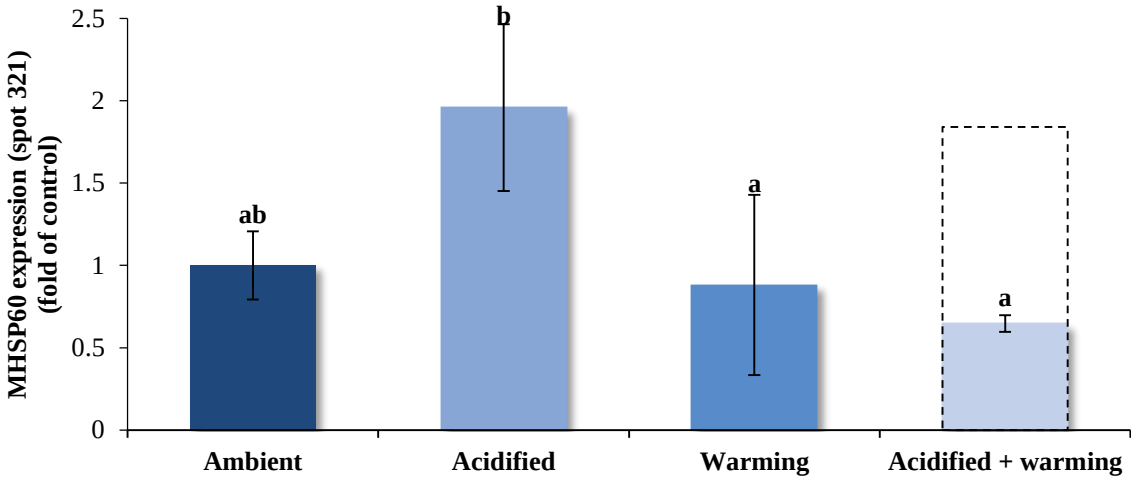

Figure 3.7. Expression of mitochondrial heat shock protein 60 in *Calanus finmarchicus* (CV-stage) after acidified and warming treatment, separately and combined. Ambient (11 °C, 390 ppm), acidified (11 °C, 2080 ppm), warming (14 °C, 390 ppm) and acidified + warming (14 °C, 2080 ppm). The dashed line shows how a pure additive effect would have looked like. The data are shown as mean fold of the control ± standard deviation (SD: n = 3). Different letters denote significant difference (p < 0.05) according to Fisher's least significant difference test (LSD).

Mitochondrial heat shock protein 60 (spot 407)

By MS/MS analysis, spot 407 was also identified to be mitochondrial heat shock protein 60 (mHSP60, Figure 3.8). Spot 407 had nine unique peptides that matched with mHSP60, all with good coverage on the fragmentation spectra (Figure E.2, Appendix E).

MHSP60 expression level was significantly affected by warming ($F_{(1,8)} = 11.222$, $p = 0.003$), but not by pH ($F_{(1,8)} = 0.658$, $p = 0.464$). No significant interaction between the two stressors was found ($F_{(1,8)} = 1.063$, $p = 0.321$), though the overall response pattern was similar to that of other the mHSP60 (i.e. spot 321).

Further *post hoc* analysis showed no significant difference between ambient and treatment exposures.

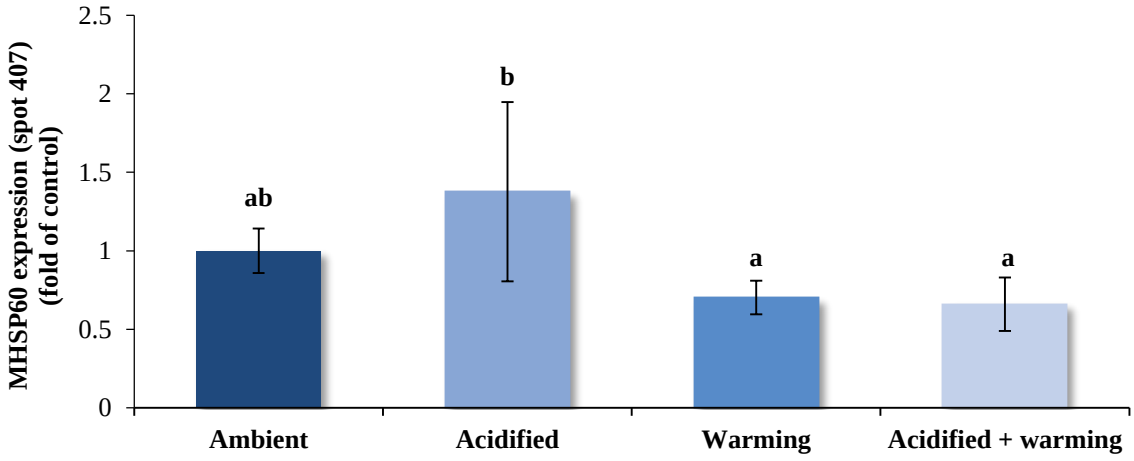

Figure 3.8. Expression of mitochondrial heat shock protein 60 in *Calanus finmarchicus* (CV-stage) after acidification and warming treatment, separately and combined. Ambient (11 °C, 390 ppm), acidified (11 °C, 2080 ppm), warming (14 °C, 390 ppm) and acidified + warming (14 °C, 2080 ppm). The data are shown as mean fold of the control ± standard deviation (SD: n = 3). Different letters denote significant differences ($p < 0.05$) according to Fisher's least significant difference test (LSD).

Glyceraldehyde-3-phosphate dehydrogenase

No good protein candidates were found in the UniprotKB database (*calanus*) for spot 607, so the search was extended to the metazoa group in the NCBI database. The organism closest to *C. finmarchicus*, with a high identity score, was another copepod, *T. japonicas*. The best protein candidate was glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Figure 3.9). Spot 607 had one unique peptide that matched with GAPDH, with good coverage on the fragmentation spectrum (Figure E.3, Appendix E).

GAPDH expression level was significantly affected by both warming ($F_{(1,8)} = 5.032$, $p = 0.043$) and pH ($F_{(1,8)} = 5.750$, $p = 0.033$). No significant interaction between the two stressors was found ($F_{(1,8)} = 2.691$, $p = 0.143$).

Further *post hoc* analysis showed a significant down-regulation of GAPDH in animals exposed to the acidified treatment ($p = 0.027$), 0.60 fold *versus* the ambient treatment.

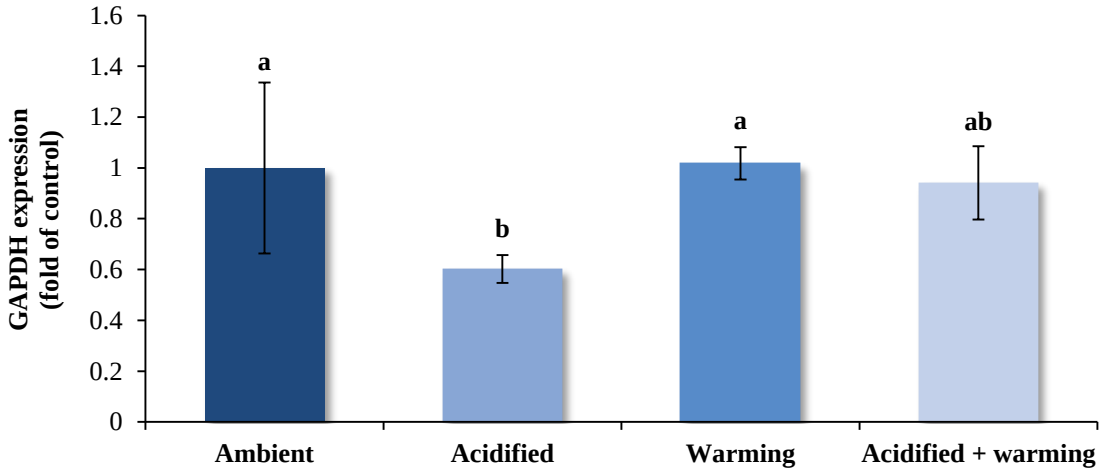

Figure 3.9. Expression of glyceraldehyde-3-phosphate dehydrogenase in *Calanus finmarchicus* (CV-stage) after acidified and warming treatment, separately and combined. Ambient (11 °C, 390 ppm), acidified (11 °C, 2080 ppm), warming (14 °C, 390 ppm) and acidified + warming (14 °C, 2080 ppm). The data are shown as mean fold of the control ± standard deviation (SD: n = 3). Different letters denote significant differences ($p < 0.05$) according to Fisher's least significant difference test (LSD).

Histone H3

By MS/MS analysis spot 614 was identified to be Histone H3 (Figure 3.10). Spot 614 had one unique peptide that matched with Histone H3, with good coverage on the fragmentation spectrum (Figure E.4, Appendix E).

Histone H3 expression level was significantly affected by both warming ($F_{(1,8)} = 6.986$, $p = 0.039$) and pH ($F_{(1,8)} = 6.844$, $p = 0.030$). No significant interaction between the two stressors was found ($F_{(1,8)} = 1.701$, $p = 0.212$).

Further *post hoc* analysis showed a significant down-regulation of Histone H3 in animals exposed to the acidified treatment ($p = 0.031$), 0.72 fold *versus* the ambient treatment.

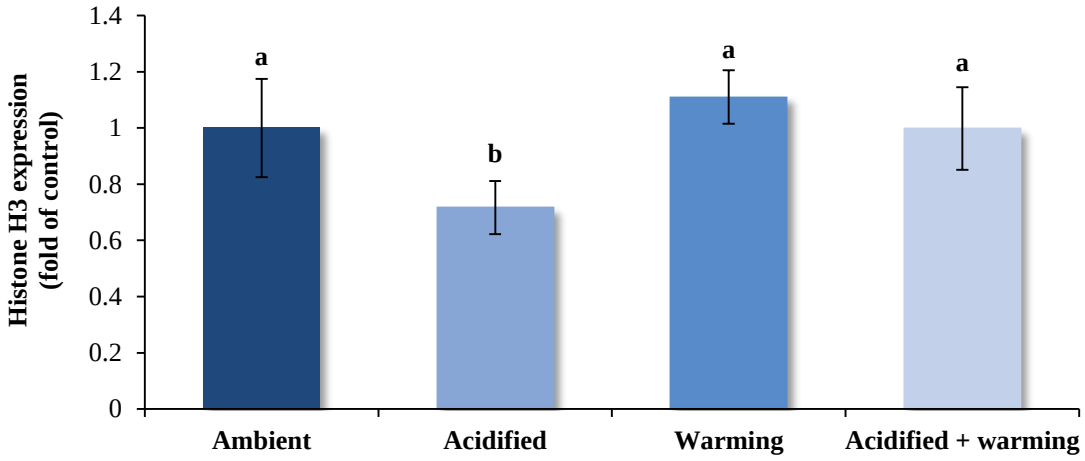

Figure 3.10. Expression of protein Histone H3 in *Calanus finmarchicus* (CV-stage) after acidified and warming treatment, separately and combined. Ambient (11 °C, 390 ppm), acidified (11 °C, 2080 ppm), warming (14 °C, 390 ppm) and acidified + warming (14 °C, 2080 ppm). The data are shown as mean fold of the control ± standard deviation (SD: n = 3). Different letters denote significant differences ($p < 0.05$) according to Fisher's least significant difference test (LSD).

Actin

The best protein candidate by MS/MS analysis for spot 1097 was actin (Figure 3.11). Spot 1097 had two unique peptides that matched with actin, both with good coverage on the fragmentation spectra (Figure E.5, Appendix E).

Actin expression level was significantly affected by warming ($F_{(1,8)} = 7.447$, $p = 0.023$), but not by pH ($F_{(1,8)} = 4.168$, $p = 0.086$). No significant interaction between the two stressors was found ($F_{(1,8)} = 0.430$, $p = 0.532$).

Further *post hoc* analysis showed a significant down-regulation of actin in animals exposed to both acidified and warming conditions combined ($p = 0.012$), 0.61 fold *versus* the ambient treatment.

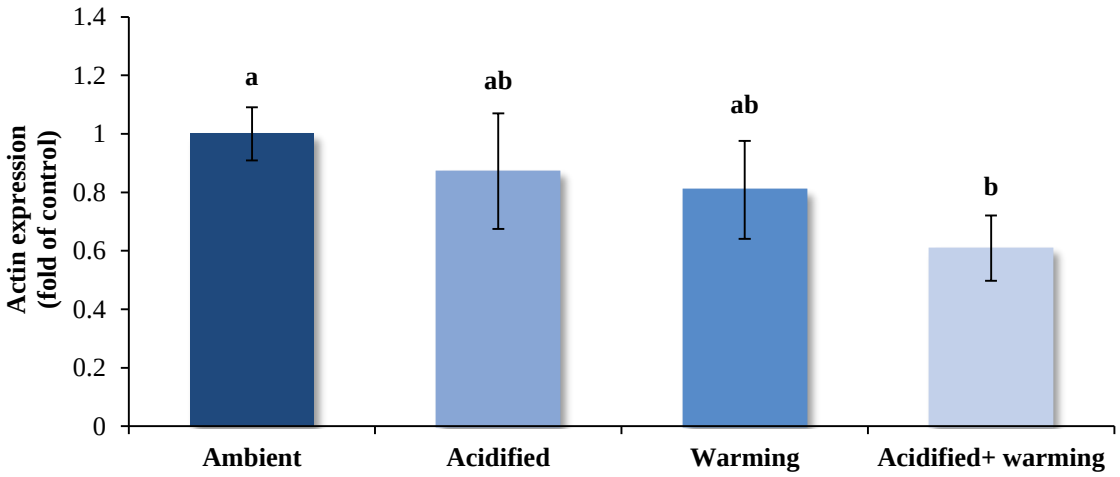

Figure 3.11. Expression of actin in CV *Calanus finmarchicus* (CV-stage) after acidified and warming treatment, separately and combined. Ambient (11 °C, 390 ppm), acidified (11 °C, 2080 ppm), warming (14 °C, 390 ppm) and acidified + warming (14 °C, 2080 ppm). The data are shown as mean fold of the control ± standard deviation (SD: n = 3). Different letters denote significant differences ($p < 0.05$) according to Fisher's least significant difference test (LSD).

Glucose-6-phospate isomerase

The best protein candidate by MS/MS analysis for spot 2139 was glucose-6-phospate isomerase (G6P isomerase, Figure 3.12). Spot 2139 had one unique peptide that matched with G6P isomerase. This peptide had good coverage on the fragmentation spectrum (Figure E.6, Appendix E).

G6P isomerase expression was significantly affected by warming ($F_{(1,8)} = 8.331$, $p = 0.012$), but not by pH ($F_{(1,8)} = 3.315$, $p = 0.080$). No significant interaction between the two stressors was found ($F_{(1,8)} = 0.858$, $p = 0.412$).

Further *post hoc* analysis showed a significant up-regulation of G6P isomerase in animals exposed to warming alone ($p = 0.033$) and acidified- and warming conditions combined ($p = 0.009$), respectively 1.72 and 1.95 fold *versus* the ambient treatment.

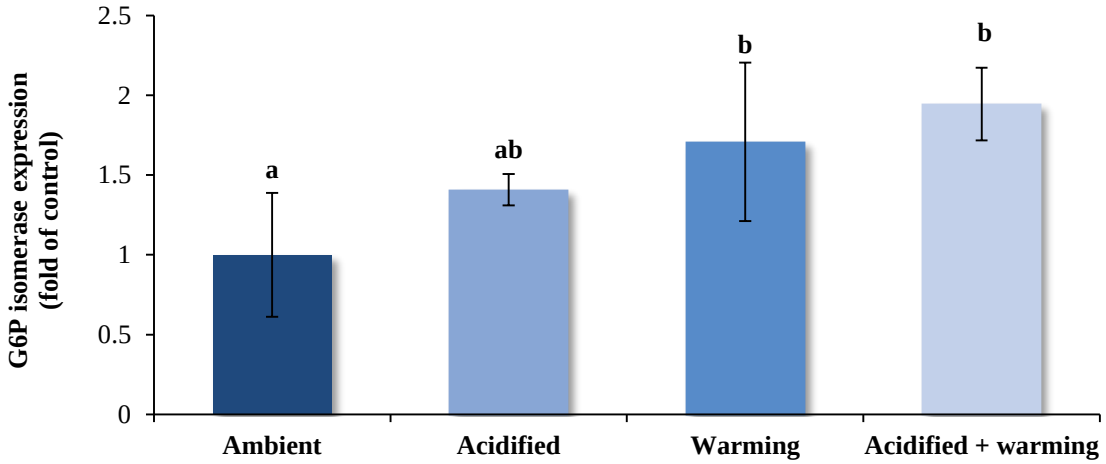

Figure 3.12. Expression of glucose-6-phosphate isomerase in *Calanus finmarchicus* (CV-stage) after acidified and warming treatment, separately and combined. Ambient (11 °C, 390 ppm), acidified (11 °C, 2080 ppm), warming (14 °C, 390 ppm) and acidified + warming (14 °C, 2080 ppm). The data are shown as mean fold of the control ± standard deviation (SD: n = 3). Different letters denote significant differences ($p < 0.05$) according to Fisher's least significant difference test (LSD).

The unidentified proteins

Seven of the unidentified proteins were significantly affected by warming, one by pH and one by both stressors (Figure 3.13, PERMANOVA, Table 3.6). Failure to positively identify these proteins were either due to lack of matching proteins in the database (see fragment spectra in Appendix E, Figure E.7 and E.8) or because it was not possible to retrieve the same protein in the DIGE gels and the preparative gel.

Table 3.6. PERMANOVA result on unidentified protein expression in *Calanus finmarchicus* (CV-stage) exposed for acidification and warming, separately and combined. Significant treatment effects or interactions are marked in bold ($p < 0.05$). Listed are the degrees of freedom (DF), sums of squares (SS), mean squares (MS), F-statistic (F), coefficient of determination (R^2) and probability value (p).

	DF	SS	MS	F	R^2	*p*
Spot 531						
Temperature	**1**	**0.066**	**0.066**	**12.860**	**0.564**	**0.013**
pH	1	>0.000	>0.000	0.037	0.002	0.853
Temperature * pH	1	0.010	0.010	1.891	0.083	0.193
Residuals	8	0.041	0.005		0.351	
Spot 589						
Temperature	**1**	**0.178**	**0.178**	**11.065**	**0.505**	**0.005**
pH	1	0.037	0.037	2.324	0.106	0.124
Temperature * pH	1	0.009	0.009	0.540	0.025	0.500
Residuals	8	0.129	0.016		0.365	
Spot 684						
Temperature	**1**	**0.096**	**0.096**	**10.866**	**0.461**	**0.010**
pH	1	0.026	0.026	2.959	0.126	0.125
Temperature * pH	1	0.015	0.015	1.724	0.073	0.205
Residuals	8	0.071	0.009		0.340	
Spot 1288						
Temperature	**1**	**0.168**	**0.168**	**13.025**	**0.548**	**0.007**
pH	1	0.010	0.010	0.737	0.031	0.393
Temperature * pH	1	0.026	0.026	2.005	0.084	0.199
Residuals	8	0.103	0.013		0.337	
Spot 2140						
Temperature	1	0.004	0.004	1.264	0.060	0.312
pH	**1**	**0.029**	**0.029**	**9.442**	**0.451**	**0.010**
Temperature + pH	1	0.007	0.007	2.242	0.107	0.157
Residuals	8	0.024	0.003		0.382	

Table continued on next page

Table 3.6. Continued

	DF	SS	MS	F	R^2	*p*
Spot 2153						
Temperature	**1**	**0.065**	**0.065**	**8.510**	**0.329**	**0.009**
pH	**1**	**0.063**	**0.063**	**8.355**	**0.323**	**0.010**
Temperature * pH	1	0.008	0.008	1.034	0.040	0.359
Residuals	8	0.061	0.008		0.309	
Spot 2155						
Temperature	**1**	**0.293**	**0.293**	**7.579**	**0.391**	**0.024**
pH	1	0.109	0.109	2.834	0.146	0.136
Temperature * pH	1	0.038	0.038	0.976	0.050	0.317
Residuals	8	0.309	0.037		0.413	
Spot 2260						
Temperature	**1**	**0.177**	**0.177**	**15.193**	**0.574**	**0.007**
pH	1	0.036	0.036	3.071	0.116	0.122
Temperature * pH	1	0.002	0.002	0.183	0.007	0.709
Residuals	8	0.093	0.012		0.302	

All the unknown proteins were significantly affected by warming, except protein 2140 (PERMANOVA, Table 3.6). In general, the unknown proteins were not affected by pH, with the exception of protein 2140 and 2153 (PERMANOVA, Table 3.6). No significant interaction between the two stressors was found for any of the proteins (PERMANOVA, Table 3.6).

Further *post hoc* analysis showed a significant up-regulation of protein 589, 684, 2155 and 2260 in animals exposed to warming alone ($p = 0.020$, $p = 0.015$, $p = 0.031$ and $p = 0.029$, respectively), respectively 1.86, 1.69, 2.74 and 1.75 fold *versus* the ambient treatment. Protein 589, 684, 2155 and 2260 were also significantly up-regulated in animals exposed to acidified- and warming conditions combined ($p = 0.007$, $p = 0.009$, $p = 0.012$ and $p = 0.005$, respectively), respectively 2.08, 1.77, 3.17 and 2.09 fold *versus* the ambient treatment. Protein 531 and 2153 were significantly up-regulated in animals exposed to acidified- and warming conditions combined ($p = 0.024$ and $p = 0.006$, respectively), respectively 1.35 and 1.90 fold *versus* the ambient treatment. Protein 2140 was significantly down-regulated in animals exposed to the acidified treatment ($p = 0.016$), respectively 0.88 fold *versus* the ambient treatment. No significant differences

were found for protein 1288, though it could seem like it was up-regulated in response to warming alone and combined with acidification.

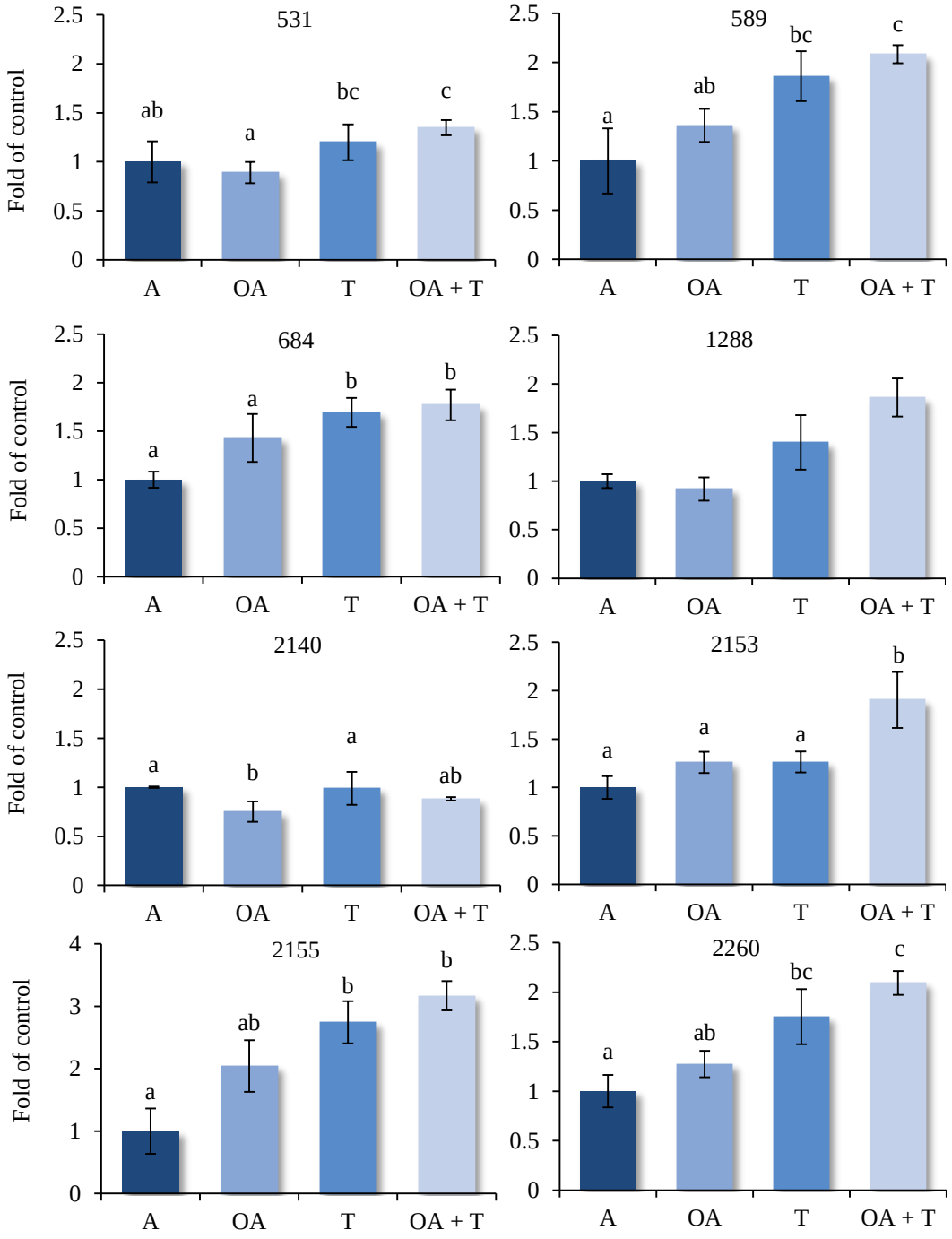

Figure 3.13. Expression of unknown proteins in *Calanus finmarchicus* (CV-stage) after acidified and warming treatment, separately and combined. Ambient (A: 11 °C, 390 ppm), acidified (OA: 11 °C, 2080 ppm), warming (T: 14 °C, 390 ppm) and acidified + warming (OA + T: 14 °C, 2080 ppm). The data are shown as mean fold of the control ± standard deviation (SD: n = 3). Different letters denote significant differences ($p < 0.05$) according to Fisher's least significant difference test (LSD).

Chapter 4

Discussion

In the present study, it was investigated how the copepod *C. finmarchicus* responded to climate change conditions (ocean acidification and warming) projected within the year 2300. The response of the two individual stressors and combined stressors were analyzed using a 2-DE proteomics approach and biometric measurements. To the author's knowledge, this is the first study to conduct a 2-DE proteomics analysis on the combined effect of acidification and warming on any marine organism, as well as the first to look at the proteome of *C. finmarchicus.* It was hypothesized that the combined effect of the two stressors would be additive or antagonistic.

4.1. Water parameters and food concentration

Temperature, calculated pH and pCO_2 concentration varied only slightly over time and were all close to target concentrations during the first period (egg to CIV-stage) (Table 3.1). During the last 48 h (second period, CV-stage) the measured values deviated somewhat more, which is likely due to semi-static conditions (Table 3.2).

Targeted alga concentration were set to 250 C µg/L. During the first 11 days, the concentrations increased in all treatments (Figure 3.1). The first two nauplii stages (NI and NII) are non-feeding stages (Campbell et al., 2001, Ohman and Hirche, 2001, Våge, 2011), and this could explain the increase. After day 11, the concentrations decreased, with the largest decline being present in the two warming treatments. Indication of an increased development in both warming treatments were observed (i.e. the CIV-stage was

reached five days earlier) and one possible explanation for the larger decline, could therefore be that the copepods reached the NIII stage earlier and subsequently started feeding before the copepods in the ambient temperature treatments. Shorter development time in response to elevated temperature has been reported earlier in *C. finmarchicus* (Diel and Breteler, 1986, Campbell et al., 2001). Another explanation is related to metabolism and food uptake. In most cases, elevated temperature leads to an increased metabolism over all life stages as well as an increased food uptake (Podolsky and Emlet, 1993, Byrne and Przeslawski, 2013). Increased metabolism and/or food uptake have also been observed for copepods (Mauchline, 1998, Frangoulis et al., 2005, Almeda et al., 2010, Zervoudaki et al., 2013, Hildebrandt et al., 2014).

Lower alga concentration was observed in all treatments during the second period (CV-stage, Figure 3.2). The falling concentration was probably caused by a combination of alga sedimentation and grazing rates surpassing the addition of new algae during semi static water changes.

4.2. The effect of ocean acidification and warming on somatic growth

4.2.1. Body length

It was not detected any significant effect of pCO_2 on body length (Figure 3.3). This is consisted with earlier studies on *C. finmarchicus* and *A. tsuensis* when food was not limited ($pCO_2 \geq 2300$ ppm) (Kurihara and Ishimatsu, 2008, Pedersen et al., 2013). In a study where *C. finmarchicus* was cultured under food-limited conditions and pCO_2 of 3080 ppm, body length was reduced with ~5 %, however this was not found at pCO_2 of 2080 ppm (Pedersen et al., 2014a). Still, this suggest that copepods may have the ability to compensate for CO_2-induced stress, as longest food is present in excess of demand (Pedersen et al., 2014a). In the copepod *C. tenuiremis*, an increase in respiration and feeding rates were observed in response to low pH. This has also been observed for mussels (Melzner et al., 2011, Thomsen et al., 2013). Contradictory to these results, a reduction of body size was observed for the copepod *T. battagliai* exposed to pH 7.67 and 7.82 over two generations (Fitzer et al., 2012). *T. battagliai* is a small non-vertically

migrating species, and it has been suggested that its small size and life history makes it less resilient against acidification (Fitzer et al., 2012, Lewis et al., 2013).

The body length was significantly reduced in the animals exposed to with warming separately and combined with acidification. A small difference was seen between two warming treated groups (respectively 6.64 and 4.88 % smaller in the warming and warming and acidified treatment combined, *versus* the ambient treatment), though this was not found to be significant. The inverse relationship with temperature and somatic growth/size is in agreement with previously reported findings for copepods (Breteler and Gonzalez, 1988, Uye, 1988, Durbin and Durbin, 1992, Campbell et al., 2001, Hildebrandt et al., 2014, Rice et al., 2015), and the temperature-size-rule explained in the theory section (1.2.4, Atkinson, 1994). The relationship has been reported in several species and may be one of the universal ecological consequences of global warming (Daufresne et al., 2009). In the present experiment, the copepod's development rate was increased in both warming treatments. Forster and Hirst (2012) have proposed that the inverse relationship can be explained with development being increased in a higher degree than growth rate, so that the adult stage is reached before the largest possible size is achieved. This assumes that development and growth are governed by different cellular processes, with have different temperature coefficients (Van der Have and De Jong, 1996, Miller et al., 1977, Forster et al., 2011). The mechanism is not clear, but Van der Have and de Jong (1996) attempted to explain it by assuming that growth is primarily linked to protein synthesis, while development is linked to cellular differentiation or DNA replication. The limiting factor of protein synthesis is the time taken for the large ribosomal subunits to diffuse into the cytoplasm and assemble into a translation unit, while for DNA replication it is the time taken for DNA polymerase to find a DNA template (enzymatic process). The process of diffusion is less temperature sensitive ($Q_{10} \approx 1$), than enzymatic processes ($Q_{10} \approx 2$), and could explain why development is more influenced by temperature than growth (Van der Have and De Jong, 1996). In copepods, development have been found to be more dependent upon temperature than growth in various species of copepods (15 species, among others *C. finmarchicus*) across all life stages (Forster et al., 2011).

A reduction of size may reduce the number of spawned eggs and the total biomass of *C. finmarchicus* (Sommer et al., 2012). The former can reduce the population size of *C.*

finmarchicus, and both may have implications for marine species that feed on this species (Beaugrand et al., 2003).

4.2.2. Oil sac length and volume

As with body length, neither oil sac length nor volume were affected significantly by pCO_2 (Figure 3.4 and 3.5). A reduction of fat content was found in CIV *C. finmarchicus* in response to pCO_2 of 3300 ppm under *ad libitum* food conditions, however no difference was observed at the CV-stage. The authors suggested that the reduction was attributed to a CO_2-induced developmental delay of the CIV-stage (Pedersen et al., 2013).

The oil sac length and volume was significantly decreased (18.12 and 46.33 %, respectively) in animals exposed to warming alone, *versus* the ambient treatment. Depletion of lipids is reported earlier for *C. finmarchicus* in response to elevated temperature (Pierson et al., 2013). As mentioned earlier, studies on copepods have found increased metabolism, and subsequent energy demand, due to elevated temperature (references in section 4.1). Seemingly, the energy demand has not been met with increased ingestion, which might be attributed to food limiting conditions. It may therefore not have been enough energy to store as lipids. It is also possible that the oil sac volume was smaller due to lack of time to accumulate lipids as a result of the increased development rate (Campbell et al., 2001).

The oil sac length was significantly reduced in the group treated with both acidification and warming, compared to the ambient treatment, but the two warming treated groups were not significantly different from each other. This indicates that acidification have had little or no effect on the oil sac length. The oil sac volume was also smaller in the group treated with both acidification and warming (22.89 % smaller vs. the ambient treatment), but not significantly different from the group reared under ambient conditions. As no effect was ascribed to the acidification treatment, this suggest an interaction with acidification ameliorating the warming effect. However, the interaction effect was not found to be statistically significant ($p = 0.071$). Antagonistic effects between these two stressors have been reported earlier both in copepods and other (Vehmaa et al., 2012, Vehmaa et al., 2013, McCulloch et al., 2012) marine invertebrates (review by Byrne and Przeslawski, 2013). However, in all these studies warming has ameliorated the negative

effect of acidification (Byrne and Przeslawski, 2013), and not opposite as in the present study. A concurrent study on the same lab culture of *C. finmarchicus* also found an antagonistic effect with regards to egg production and hatching success (Hanssen, 2014). It was here found a strong correlation with the female oil sac volume and the amount of egg produced (Hanssen, 2014), which suggest that the effects on reproduction may be attributed to the reduced oil sac. This demonstrates the importance of lipids in order for the female to successfully reproduce (Mauchline, 1998, Irigoien, 2004).

Earlier reports have suggested that the antagonistic effect seen on lipid content could be explained by pCO_2 inflicting a developmental delay, which could provide more time for the copepods to accumulate lipids (Johannessen, 2013, Hanssen, 2014). Development delay in copepods have been reported earlier in response to increased pCO_2 (Håkedal, 2013, Pedersen et al., 2013). However, this was at a lower food concentration or at higher pCO_2 concentrations than what was used in the present study. In fact, in a concurrent study on the same *C. finmarchicus* lab culture it was found that the development time was shortest in the animals (NIII- and CI-stage) exposed to both stressors, hence a synergistic effect and not antagonistic as previously suggested (Wæhre, 2014). However, regardless of the mechanism, it is apparent that acidification has a positive effect on either development or growth under elevated temperatures, which results in a less affected oil sac volume.

Lipids are energy molecules, and are used when food is scarce, to survive diapause and after diapause to ascend to the surface, molt into adults and to produce gonads and eggs (Mauchline, 1998, Irigoien, 2004, Pepin and Head, 2009). A reduction of the energy storage might impair any of these processes, and ultimately reduce the population size through diminished reproduction or increased mortality. The lipid storage also have an important role in determining diapause depth and duration (Visser and Jónasdóttir, 1999, Pierson et al., 2013). With a reduced oil sac, the copepods may not manage to descend below the convective mixed layer; and may be transported to the surface frequently (Irigoien, 2004). Copepods remaining at the surface over winter have a higher risk of predation, starvation and may produce offspring that will not survive at the low winter food concentration (Kaartvedt, 1996, Irigoien, 2004). Increased temperature, and subsequent size reduction may also shorten the diapause duration with as much as 40

days, which could influence the finely tuned match with the spring phytoplankton bloom (Pierson et al., 2013). A phenological shift may influence the copepod itself, but as copepods link the primary production with upper trophic levels, this may reduce the energy transfer to commercially important fish species (Daufresne et al., 2009, Rice et al., 2015).

4.3. The effect of ocean acidification and warming on the proteome

A total of ~1300 proteins were found, most within a pH range of 4.6 - 8.8 (Figure 3.6 and D.1, Appendix D). The number of protein spots and pH range observed in the present study is comparable to a proteome study on *C. sinicus*, where 1290 and 1429 proteins were found (at 18 and 5 °C, respectively) within a pH range of 4 - 7 (Wiacek et al., 2013).

In the 2-DE analysis, 14 proteins were differentially expressed compared to ambient conditions. According to the PERMANOVA analysis, ten proteins were significantly affected by temperature, one by pH alone, and three by both stressors (Table 3.5 and 3.6). Six proteins were tentatively identified using MS/MS, and two of them were the same protein (Table 3.4). Failure to positively identify these proteins were either due to lack of matching proteins in the database or because it was not possible to retrieve the same protein in the DIGE and preparative gel. The low number affected by pH is consistent with observations on the proteome response to ocean acidification in cod, herring, stickleback and barnacle exposed to pH 7.6 - 7.7 (Gunnarsson, 2010, Wong et al., 2011, Maneja et al., 2014). This also reflects the low response seen in physiological variables in response to $pCO_2 \leq 2080$ ppm in the *Calanus* genus (Mayor et al., 2007, Pedersen et al., 2013, Pedersen et al., 2014b).

4.3.1. The identified proteins

The five identified proteins can be placed in three groups, stress (mHSP60), metabolism (G6P isomerase and GAPDH) and cytoskeleton/structural proteins (histone and actin). The *post hoc* analysis showed that GAPDH and histone H3 were significantly affected by the acidified treatment alone, while no effect was seen in response to warming alone or

warming and acidified conditions combined. G6P isomerase and actin were both affected by warming, but were more differentially expressed in the combined treatment than in the warming treatment alone (additive effect). Concerning mHSP60, an antagonistic effect was seen with warming ameliorating the negative effect of acidification. All of these proteins have been found to be differentially expressed in response to both acidification (Gunnarsson, 2010, Wong et al., 2011, Tomanek et al., 2011, Dineshram et al., 2012, Dineshram et al., 2013, Mukherjee et al., 2013, Maneja et al., 2014), and/or warming (Tomanek and Zuzow, 2010, Serafini et al., 2011, Fields et al., 2012), in other proteome studies.

Cellular stress

Members of the heat shock protein family, also known as molecular chaperonins, are involved in the cellular defense, and have important roles in mediating correct protein folding, degradation and to prevent protein aggregation under cellular stress (Lindquist and Craig, 1988, Xu and Qin, 2012, Kim et al., 2008). Several environmental stressors have been shown to induce the expression of HSPs, such as heat, salinity and pollutants (Pedersen and Lundebye, 1996, Werner and Nagel, 1997, Spees et al., 2002, Xu and Qin, 2012). HSP60 is an ATP-dependent protein, which is particularly important in assisting of native folding of proteins. Mammalian studies have found indications of HSP60's involvement in germ differentiation, reproduction, development and thermo protection, among others (Kozlova et al., 1997, Choresh et al., 2001, Timakov and Zhang, 2001, Xu and Qin, 2012). HSP60 can be found in all compartments of the cells, but is especially associated with the mitochondria (Kim et al., 2007).

Two of the proteins were identified to be mHSP60 (Figure 3.7 and 3.8). The two proteins are located at different places in the gel, which might be due to posttranslational modifications. A significant interaction and a near significant acidification effect was only observed for one of the two proteins, but the response pattern was similar for both.

MHSP60 was up-regulated in copepods exposed to the acidified treatment (p = 0.051, Figure 3.7). This may suggest that the cells are under acidified-induced stress and need mHSP60 facilitating in either of the earlier mentioned cellular processes. This is contradictory to what others have found, e.g. a proteome study of a tubeworm (*Hydroides*

elegans) observed a significant down-regulation of HSP60 in response to acidification (pH 7.6) and hypoxia (2.8 mg O_2 l^{-1}), separately and combined (Mukherjee et al., 2013). A down-regulation (larvae of barnacle and sea urchins) or no significant difference (reef coral larvae), have also been reported for other HSPs in both genomics and proteomics studies in response to acidification (O'Donnell et al., 2009, Todgham and Hofmann, 2009, Nakamura et al., 2011, Wong et al., 2011). Barnacles and sea urchins are calcifying organisms, which have shown susceptibility towards acidification at lower levels than copepods (review by Kroeker et al., 2010). In addition, early life stages (which is the case for the examined species) are thought to be more vulnerable than the adult stage (Kurihara, 2008, Melzner et al., 2009, Ries et al., 2009, Hofmann and Todgham, 2010, Whiteley, 2011). It has been suggested that acidification have a suppressive effect on HSP expression (Wong et al., 2011), but this does not seem to apply for mHSP60 in *C. finmarchicus*.

MHSP60 expression was not significantly affected by warming alone. In other studies, several HSPs were found to be up-regulated in two species of blue mussels at different elevated temperatures (Tomanek and Zuzow, 2010). The same was seen in the sea squirt *C. savagnyi*, but no difference was seen for the congener species *C. intestinalis* exposed for the same temperatures (Serafini et al., 2011). No up-regulation of HSP may imply that the species is not under any severe stress. The *C. finmarchicus* animals migrate in the water column and may experience temperature fluctuations between 0 - 16 °C (Bonnet et al., 2005). The wide natural temperature range in this species might explain the absence of a significant up-regulation of mHSP60 in the animals that were exposed to at 3 °C warming.

The increase in mHSP60 expression in response to acidification was not observed when the copepods also were exposed to elevated temperature. This implies that warming have had an antagonistic effect when combined with acidified conditions. Constant production of HSPs is energy demanding, and could be at the expense of the synthesis of other proteins that are important for growth and development (Krebs and Feder, 1997, Viant et al., 2003). It may therefore be speculated that the extra cost of thermal stress, have done that the copepods have not had a not energy to use for mHSP60 expression.

Metabolism

Two of the identified proteins are members of the glycolysis, which is a cellular pathway where glucose is broken down and energy is produced as ATP and NADH. G6P isomerase is involved in the second step where it catalyzes the transformation of glucose-6-phosphate into fructose 6-phosphate, while GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate to glycerate-1,3-bisphosphate in the sixth step of the glycolysis (Campbell et al., 2008a, Hauton et al., 2009).

GAPDH was found to be significantly down-regulated under low pH condition (Figure 3.9). Metabolism related genes involved in the tricarboxylic acid cycle and the electron transport chain was found down-regulated under pCO_2 concentrations of 540 ppm and 1010 - 1350 ppm in a sea urchin larva (*Strongylocentrotus purpuratus*) and coral reef (*Acropora millepora*), respectively (Todgham and Hofmann, 2009, Kaniewska et al., 2012). The same was found for two isoforms of ATP synthase beta subunit proteins in tubeworm larvae exposed for pH 7.6 (Mukherjee et al., 2013). A reduction in metabolism can suggest that there is reduced need for energy or that metabolism have been suppressed and that the organism is waiting for more favorable conditions (Todgham and Hofmann, 2009). Metabolic suppression in response to acidification has been reported in many marine species (Pörtner, 2008, Todgham and Hofmann, 2009) and could have negative fitness consequences over longer time (Kaniewska et al., 2012). A protein study of cod and a gene study of an amphipod (*Gammarus locusta*) found that GAPDH was up-regulated in response to pH 7 and 7.47, respectively (Hauton et al., 2009, Gunnarsson, 2010). In the sea squirt *Ciona savignyi* GAPDH was up regulated in response to warming (Serafini et al., 2011). No difference was seen when the copepods were treated with both warming and acidification in this study. This suggests that warming may have ameliorated the effect of acidification so that the expression level was similar to that of the control.

The expression of the other glycolytic enzyme in this study, G6P isomerase, was induced in all treatments compared to control, though a significant difference was only seen in the warming alone and warming and acidified treatment combined (Figure 3.12). An increase in the expression of two proteins involved in metabolism (ATP synthase and mitochondrial malate dehydrogenase) was seen in herring larvae (pCO_2 of 1900 ppm) and

adult oyster (*Crassostrea virginica*, pH 7.5), respectively (Tomanek et al., 2011, Maneja et al., 2014). Warming have also shown to enhance the expression of metabolism related proteins in sea squirts (C. *savignyi* and *C. intestinalis*), while both up- and down-regulation was seen in two species of blue mussels (*Mytilus trossulus* and *M. galloprovincialis*) (Tomanek and Zuzow, 2010, Serafini et al., 2011). An increase in energy related proteins suggests an increased demand for ATP and NADH, in order to maintain cellular homeostasis (Todgham and Hofmann, 2009). Increased metabolism have been reported in copepods as followed by both increased temperature and pCO_2 concentration (Mauchline, 1998, Frangoulis et al., 2005, Almeda et al., 2010, Li and Gao, 2012, Zervoudaki et al., 2013, Hildebrandt et al., 2014, Pedersen et al., 2014a).

Structural proteins

Actin is a globular protein that forms microfilaments, one of three components of the cytoskeleton (Campbell et al., 2008b). Microfilaments forms a structural network in cells, and are involved in processes such cell motility, contractibility, mitosis and cytokinesis, intracellular transport, endocytosis and secretion (Kaniewska et al., 2012).

In the present study, actin was down-regulated in all treatments compared to the control, though a significant difference was only seen in the warming and acidified treatment combined (Figure 3.11). This implies that there has been an additive effect, as no significant difference was seen as followed by warming or acidification alone. Other studies have also reported a down-regulation of cytoskeleton proteins (e.g. action and tubulin isoforms) in both larvae of oysters, herring and tubeworm larvae in response to low pH (pH 7.5, pCO_2 of 1900 ppm and pH 7.6, respectively) (Dineshram et al., 2012, Mukherjee et al., 2013, Maneja et al., 2014). This have also been observed at the gene level for coral reef larvae (Kaniewska et al., 2012). In contrast, several actin isoforms were up-regulated in adult oysters (Dineshram et al., 2013). Heat was found to differentially express cytoskeleton proteins in two species of blue mussels and sea squirts, but the pattern was diverse and varied both with species and temperature (Tomanek and Zuzow, 2010, Serafini et al., 2011). A down-regulation of cytoskeleton elements may have implications for intracellular transport, plasma membrane interactions and cell shape/integrity (Kaniewska et al., 2012).

Histone H3 is a small and highly positively protein belonging to the histone family. The DNA is tightly packed around histones, and forms the structures called chromatin. Modification of histones are important in order for gene regulation to happen (Bhasin et al., 2006). In the present study, histone H3 was significantly down-regulated under low pH condition, but no difference was seen in response to any of the other treatments (Figure 3.10). Altered histone expression in response to acidification is only reported in one other study. In adult oyster, histone H3 was up-regulated in response to pH 7.6 and 7.9 (Dineshram et al., 2013). A down-regulation of histone may have consequences for cell division.

4.3.2. The unidentified proteins

Four proteins were significantly up-regulated in response to both warming alone and warming and acidification combined (protein 589, 684, 2155 and 2260, Figure 3.13). Additionally two proteins were significantly up-regulated (protein 531 and 2153, Figure 3.13) only in response to warming and acidification combined. For all the six proteins, the expression was highest in response to combined exposure to both stressors. This implies that even though the pH effect was not significant (except for protein 2153) an additive effect is still apparent when the two stressors are combined. One protein (protein 2140, Figure 3.13) was down-regulated in the group treated with acidification alone. No difference was seen in the combination treatment, implying that warming may have counteracted the effect of low pH.

4.4. Somatic growth and the differentially expressed proteins

Previous proteome studies that have investigated the effect of ocean acidification or warming have identified four groups that are affected by both stressors; the cytoskeleton, calcification, metabolism and stress proteins (references in section 1.3). The calcification process is of minor importance in copepods (Whiteley, 2011, Fitzer et al., 2012). Proteins from the other three groups were also affected in the present study. As expected, warming had a more pronounced effect than acidification, which is consisted with earlier studies on marine invertebrates (Byrne and Przeslawski, 2013).

Ocean acidification had no significant effect on somatic growth or lipid accumulation. This is also reflected in the little change seen in the proteome of *C. finmarchicus*. This also reflects the low response seen in general for physiological variables in response to $pCO_2 \leq 2080$ ppm in the *Calanus* genus (Mayor et al., 2007, Pedersen et al., 2013, Pedersen et al., 2014b). Only four proteins were significantly differentially expressed in the acidified treatment. Neither of the proteins were differentially expressed in the combined treatment, suggesting that warming has ameliorated the effect of acidification on these proteins. Warming alone inflicted a more pronounced effect on growth and lipid accumulation than warming and acidification combined, implying an antagonistic effect induced by acidification. This was not observed on the proteome level, where it was found that five proteins were significantly up-regulated by warming alone, while eight proteins were significantly up-regulated by the warming and acidified treatment, combined. This suggest an additive effect of warming and acidification.

The induction of G6P isomerase as followed by warming, imply increased production of energy, hence increased metabolism. If the increased energy demand is not met by a corresponding increase in energy intake, less energy can be stored as lipids. This is consistent with findings of the reduced oil sac and body length in the groups treated with warming alone, and warming combined with acidification. Another glycolytic enzyme, GAPDH, was down-regulated by acidification. It is tempting to speculate that less energy is used in the combined treatment due to a suppression of GAPDH. However, a significant suppression was only found in response to the acidified treatment alone.

A reduction of adult size can be a result of either a reduced cell number or volume (Angilletta et al., 2004). A down-regulation of the important cytoskeleton protein actin may indicate a smaller cell volume as the microfilament is important in order to maintain the cell's shape (Kaniewska et al., 2012). It is therefore speculated that the reduction of size is caused by a negative temperature effect on the cytoskeleton. However, further evidence is needed to support this. The down-regulation of histone H3 suggests impairment of cellular division and points to a reduced cell number, but this was only significant in the acidified treatment. In addition, assuming that development is linked to cellular division (Van der Have and De Jong, 1996), a decrease in development would be expected for this to hold true, which was not the case here.

Regardless, caution should be taken when linking physiological responses to a few proteins as metabolism and other cellular processes are functions of multiple enzyme reactions (Hauton et al., 2009).

4.5. Assessing the method and suggestions for further work

The proteome represent the molecular phenotype of cells, and is highly correlated to the fitness of an organism (Feder and Walser, 2005). The use of proteomics can contribute to understand cellular mechanisms behind previously demonstrated effects caused by environmental stressors (Garcia-Reyero and Perkins, 2011, Tomanek and Zuzow, 2010). The 2-DE proteomic approach have shown to be simple to use, highly reproducible and robust (Chich et al., 2007). It is especially useful to include physiological measurements in order to correlate this with the changes seen in the proteome. Some limitation still exist, such as lack of identified proteins in the database, low solubility of membrane and hydrophobic proteins and low detection of low-abundance proteins (Beranova-Giorgianni, 2003). Low-abundance proteins may be detected by enrichment processes, such ubiquitin-tagged protein affinity columns (Tomanek, 2011). Numerous proteins are also subjected to PTMs or are involved in protein-protein interactions (Tomanek, 2014). By the use of antibodies, interacting proteins can be revealed by co-immunoprecipitation. Both interacting proteins and PTMs may be identified by the use of MS-based proteomics. MS-based proteomics are likely to completely replace 2-DE in the future (Walther and Mann, 2010).

If a 2-DE proteomic approach should be used again on the proteome of *C. finmarchicus*, a more narrow pH range with the same strip length (24 cm) would provide higher resolution, which might reveal more differentially expressed proteins. In addition, it could be beneficial to include at least four parallels as this will improve the power in the statistically analysis. It could also be interesting to look at changes in the proteome both over multiple generations as well as under different time points of a life cycle.

The pCO_2 concentration used in the present study is a pessimistic scenario for year 2300, while the temperature increase is within what might be expected within the year 2100. It could be useful to examine the effect of pCO_2 concentrations and temperature changes

that are predicted for the same century. Particularly to see whether acidification have the same antagonistic effect on warming, which was observed on the oil sac volume.

Chapter 5

Conclusion

Morphological variables and the proteome of *C. finmarchicus* have been investigated in copepods exposed to climate change conditions (ocean acidification and warming) projected within the year 2300. It is clear that warming has a more adverse effect than acidification on the morphological variables investigated. However, for both body length and oil sac volume the effect inflicted by warming was somewhat ameliorated by acidification. This has not been reported elsewhere, and emphasizes the challenges with predicting the combined effect of warming and acidification. A reduced body size and oil sac may directly or indirectly have consequences for the timing of the phytoplankton bloom, impair reproduction and reduce the population size, which again could influence fish stock populations and the entire food web.

Only a small fraction of the proteome was found to be affected by the two stressors. Warming had a more pronounced effect than acidification also on this level. All the proteins affected by acidification alone showed no difference in expression in the combined treatment (*versus* control), suggesting an antagonistic effect were warming ameliorate the acidification effect. Also contrary to the morphological results, an additive effect was found for many of the proteins. Up-regulation of a glycolytic enzyme points to increased metabolism, which supports the finding of the reduced body size and oil sac. A down-regulation of a cytoskeleton protein may also contribute to explain the decrease in size as actin is important for cell shape. However, care should be taken when linking physiological responses to a limited number of proteins since cellular processes are functions of multiple enzyme reactions.

Future proteome studies incorporating posttranslational modifications, protein-protein interactions and multiple generations of an organism, may contribute to further knowledge about the mechanisms that are affected by ocean acidification and warming.

References

ALMEDA, R., AUGUSTIN, C. B., ALCARAZ, M., CALBET, A. & SAIZ, E. 2010. Feeding rates and gross growth efficiencies of larval developmental stages of *Oithona davisae* (Copepoda, Cyclopoida). *Journal of Experimental Marine Biology and Ecology,* **387** (1), 24 - 35.

ANDERSON, M. J. 2001. A new method for non-parametric multivariate analysis of variance. *Austral Ecology,* **26** (1), 32 - 46.

ANGILLETTA, M. J., STEURY, T. D. & SEARS, M. W. 2004. Temperature, growth rate, and body size in ectotherms: fitting pieces of a life-history puzzle. *Integrative and Comparative Biology,* **44** (6), 498 - 509.

ARNOLD, K. E., FINDLAY, H. S., SPICER, J. I., DANIELS, C. L. & BOOTHROYD, D. 2009. Effect of CO_2-related acidification on aspects of the larval development of the European lobster, *Homarus gammarus* (L.). *Biogeosciences,* **6** (8), 1747 - 1754.

ATKINSON, D. 1994. Temperature and organism size: a biological law for ectotherms? *Advances in Ecological Research,* **25**, 1 - 58.

BAN, S. 1994. Effect of temperature and food concentration on post-embryonic development, egg production and adult body size of calanoid copepod *Eurytemora affinis*. *Journal of Plankton Research,* **16** (6), 721 - 735.

BAUMGARTNER, M. F. 2009. *Calanus finmarchicus* life cycle [Online]. Available: http://www.gulfofmaine.org/gomt/?p=32 [Accessed 21. April 2014].

BEAUGRAND, G., BRANDER, K. M., LINDLEY, J. A., SOUISSI, S. & REID, P. C. 2003. Plankton effect on cod recruitment in the North Sea. *Nature,* **426** (6967), 661 - 664.

BERANOVA-GIORGIANNI, S. 2003. Proteome analysis by two-dimensional gel electrophoresis and mass spectrometry: strengths and limitations. *Trends in Analytical Chemistry,* **22** (5), 273 - 281.

BHASIN, M., REINHERZ, E. L. & RECHE, P. A. 2006. Recognition and classification of histones using support vector machine. *Journal of Computational Biology,* **13** (1), 102 - 112.

BONNET, D., RICHARDSON, A., HARRIS, R., HIRST, A., BEAUGRAND, G., EDWARDS, M., CEBALLOS, S., DIEKMAN, R., LÓPEZ-URRUTIA, A., VALDES, L., CARLOTTI, F., MOLINERO, J. C., WEIKERT, H., GREVE, W., LUCIC, D., ALBAINA, A., YAHIA, N. D., UMANI, S. F., MIRANDA, A., SANTOS, A. D., COOK, K., ROBINSON, S. & PUELLES, M. L. F. D. 2005. An overview of *Calanus helgolandicus* ecology in European waters. *Progress in Oceanography,* **65** (1), 1 - 53.

BRADFORD, M. M. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry,* **72** (1), 248 - 254.

BRETELER, W. C. M. K. & GONZALEZ, S. R. 1988. Influence of temperature and food concentration on body size, weight and lipid content of two Calanoid copepod species. *Hydrobiologia,* **167/168**, 201 - 210.

BROMS, C., MELLE, W. & KAARTVEDT, S. 2009. Oceanic distribution and life cycle of *Calanus* species in the Norwegian Sea and adjacent waters. *Deep-Sea Research II,* **56**, 1910 - 1921.

BYRNE, M. & PRZESLAWSKI, R. 2013. Multistressor impacts of warming and acidification of the ocean on marine invertebrates' life histories. *Integrative and Comparative Biology,* **53** (4), 582 - 596.

BØRSHEIM, K. Y. & GOLMEN, L. 2010. Forsurning av havet. Kunnskapsstatus for norske farvann. Norwegian Pollution Control Authority

CALDEIRA, K. & WICKETT, M. E. 2003. Anthropogenic carbon and ocean pH. *Nature,* **425** (6956), 365 - 365.

CAMERON, J. N. 1985. Compensation of hypercapnic acidosis in the aquatic blue crab, *Callinectes sapidus*: the predominance of external sea water over carapace carbonate as the proton sink. *Journal of Experimental Biology,* **114**, 197 - 206.

CAMPBELL, N., REECE, J., URRY, L., CAIN, M., WASSERMAN, S., MINORSKY, P. & JACKSON, R. 2008a. Cellular Respiration: Harvesting Chemical Energy. *Biology.* San Francisco, CA, USA: Pearson - Benjamin Cummings.

CAMPBELL, N., REECE, J., URRY, L., CAIN, M., WASSERMAN, S., MINORSKY, P. & JACKSON, R. 2008b. A tour of the Cell. *Biology.* San Francisco, CA, USA: Pearson - Benjamin Cummings.

CAMPBELL, R. G., WAGNER, M. M., TEEGARDEN, G. J., BOUDREAU, C. A. & DURBIN, E. G. 2001. Growth and development rates of the copepod *Calanus finmarchicus* reared in the laboratory. *Marine Ecology Progress Series,* **221**, 161 - 183.

CHICH, J.-F., DAVID, O., VILLERS, F., SCHAEFFER, B., LUTOMSKI, D. & HUET, S. 2007. Statistics for proteomics: experimental design and 2-DE differential analysis. *Journal of Chromatography B,* **849** (1), 261 - 272.

CHORESH, O., RON, E. & LOYA, Y. 2001. The 60-kDa heat shock protein (HSP60) of the sea anemone *Anemonia viridis*: a potential early warning system for environmental changes. *Marine Biotechnology,* **3** (5), 501 - 508.

COMEAU, S., GORSKY, G., JEFFREE, R., TEYSSIE, J.-L. & GATTUSO, J.-P. 2009. Impact of ocean acidification on a key Arctic pelagic mollusc (*Limacina helicina*). *Biogeosciences,* **6**, 1877 - 1882.

COMEAU, S., JEFFREE, R., TEYSSIE, J.-L. & GATTUSO, J.-P. 2010. Response of the Arctic pteropod *Limacina helicina* to projected future environmental conditions. *PLoS ONE,* **5** (6), 1 - 7.

DAUFRESNE, M., LENGFELLNER, K. & SOMMER, U. 2009. Global warming benefits the small in aquatic ecosystems. *Proceedings of the National Academy of Sciences,* **106** (31), 12788 - 12793.

DICKSON, A. G., SABINE, C. L. & CHRISTIAN, J. R. 2007. Guide to best practices for ocean CO_2 measurements. PICES Special Publication.

DIEL, S. & BRETELER, W. C. M. K. 1986. Growth and development of *Calanus* spp. (Copepoda) during spring phytoplankton succession in the North Sea. *Marine Biology,* **91** (1), 85 - 92.

DINESHRAM, R., THIYAGARAJAN, V., LANE, A., ZINIU, Y., XIAO, S. & LEUNG, P. T. Y. 2013. Elevated CO_2 alters larval proteome and its phosphorylation status in the commercial oyster, *Crassostrea hongkongensis*. *Marine biology,* **160**, 2189 - 2205.

DINESHRAM, R., WONG, K. K. W., XIAO, S., YU, Z., QIAN, P. Y. & THIYAGARAJAN, V. 2012. Analysis of Pacific oyster larval proteome and its response to high-CO_2. *Marine Pollution Bulletin,* **64**, 2160 - 2167.

DURBIN, E. G. & DURBIN, A. G. 1992. Effects of temperature and food abundance on grazing and short-term weight change in the marine copepod *Acartia hudsonica*. *Limnology and Oceanography,* **37** (2), 361 - 378.

EDVARDSEN, A., PEDERSEN, J. M., SLAGSTAD, D., SEMENOVA, T. & TIMONIN, A. 2006. Distribution of overwintering *Calanus* in the North Norwegian Sea. *Ocean Science,* **3**, 25 - 53.

EDWARDS, M., BEAUGRAND, G., JOHN, A. W. G., JOHNS, D. G., LICANDRO, P., MCQUATTERS-GOLLOP, A. & REID, P. C. 2009. Ecological Status Report: results from the CPR survey 2007/2008. SAHFOS technical report. Plymouth, UK.

ERIKSSON, J. & FENYÖ, D. 2011. Modeling Mass Spectrometry-Based Protein Analysis. *In:* WU, C. & CHEN, C. (eds.) *Bioinformatics for Comparative Proteomics.* New York, USA: Humana Press.

FABRY, V. J., SEIBEL, B. A., FEELY, R. A. & ORR, J. C. 2008. Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES Journal of Marine Science,* **65** (3), 414 - 432.

FEDER, M. E. & WALSER, J. C. 2005. The biological limitations of transcriptomics in elucidating stress and stress responses. *Journal of Evolutionary Biology,* **18** (4), 901 - 910.

FIELDS, P. A., ZUZOW, M. J. & TOMANEK, L. 2012. Proteomic responses of blue mussel (*Mytilus*) congeners to temperature acclimation. *The Journal of Experimental Biology,* **215** (7), 1106 - 1116.

FITZER, S. C., CALDWELL, G. S., CLOSE, A. J., CLARE, A. S., UPSTILL-GODDARD, R. C. & BENTLEY, M. G. 2012. Ocean acidification induces multi-generational decline in copepod naupliar production with possible conflict for reproductive resource allocation. *Journal of Experimental Marine Biology and Ecology,* **418 - 419**, 30 - 36.

FORSTER, J. & HIRST, A. G. 2012. The temperature-size rule emerges from ontogenetic differences between growth and development rates. *Functional Ecology,* **26** (2), 483 - 492.

FORSTER, J., HIRST, A. G. & WOODWARD, G. 2011. Growth and development rates have different thermal responses. *The American Naturalist,* **178** (5), 668 - 678.

FRANGOULIS, C., CHRISTOU, E. & HECQ, J. 2005. Comparison of marine copepod outfluxes: Nature, rate, fate and role in the carbon and nitrogen cycles. *Advances in Marine Biology*, **47**, 253 - 309.

GARCIA-REYERO, N. & PERKINS, E. J. 2011. Systems biology: leading the revolution in ecotoxicology. *Environmental Toxicology and Chemistry*, **30** (2), 265 - 273.

GE HEALTCARE LIFE SCIENCE, Ettan DALTsix Large Vertical system. [Online]. Available: http://www.gelifesciences.com/gehcls_images/GELS/Images/TechSupportImages/Files/80649249_1.jpeg [Accessed 26. April 2014].

GOODING, R. A., HARLEY, C. D. & TANG, E. 2009. Elevated water temperature and carbon dioxide concentration increase the growth of a keystone echinoderm. *Proceedings of the National Academy of Sciences,* **106** (23), 9316 - 9321.

GUNNARSSON, F. 2010. *Sublethal effects of low pH in two fish species (Gasterosteus aculeatus and Gadus morhua).* MSc thesis, Department of Plant and Environmental Sciences, Gothenburg university.

HANSEN, B. H., ALTIN, D., NORDTUG, T. & OLSEN, A. J. 2007. Suppression subtractive hybridization library prepared from the copepod *Calanus finmarchicus* exposed to a sublethal mixture of environmental stressors. *Comparative Biochemistry and Physiology Part D,* 250 - 256.

HANSSEN, A. E. 2014. *Interaction Effects of Ocean Acidification and Warming on the Fecundity of the Marine Copepod Calanus finmarchicus.* MSc thesis, Department of Biology, Norwegian University of Science and Technology.

HAUTON, C., TYRRELL, T. & WILLIAMS, J. 2009. The subtle effects of sea water acidification on the amphipod *Gammarus locusta*. *Biogeosciences,* **6** (8), 1479 - 1489.

HEATH, M. R., RASMUSSEN, J., AHMED, Y., ALLEN, J., ANDERSON, C. I. H., BRIERLEY, A. S., BROWN, L., BUNKER, A., COOK, K., DAVIDSON, R., FIELDING, S., GURNEY, W. S. C., HARRIS, R., HAY, S., HENSON, S., HIRST, A. G., HOLLIDAY, N. P., INGVARSDOTTIR, A., IRIGOIEN, X., LINDEQUE, P., MAYOR, D. J., MONTAGNES, D., MOFFAT, C., POLLARD, R., RICHARDS, S., SAUNDERS, R. A., SIDEY, J., SMERDON, G., SPEIRS, D., WALSHAM, P., WANIEK, J., WEBSTER, L. & WILSON, D. 2008. Spatial demography of *Calanus finmarchicus* in the Irminger Sea. *Progress in Oceanography,* **76** (1), 39 - 88.

HELAOUËT, P. & BEAUGRAND, G. 2007. Macroecology of *Calanus finmarchicus* and *C. helgolandicus* in the North Atlantic Ocean and adjacent seas. *Marine Ecology Progress Series,* **345**, 147 - 165.

HILDEBRANDT, N., NIEHOFF, B. & SARTORIS, F. J. 2014. Long-term effects of elevated CO_2 and temperature on the Arctic calanoid copepods *Calanus glacialis* and *C. hyperboreus*. *Marine Pollution Bulletin,* **80** (1 – 2), 59 - 70.

HOEGH-GULDBERG, O., MUMBY, P. J., HOOTEN, A. J., STENECK, R. S., GREENFIELD, P., GOMEZ, E., HARVELL, C. D., SALE, P. F., EDWARDS, A. J., CALDEIRA, K., KNOWLTON, N., EAKIN, C. M., IGLESIAS-PRIETO, R., MUTHIGA, N., BRADBURY, R. H., DUBI, A. & HATZIOLOS, M. E. 2007. Coral reefs under rapid climate change and ocean acidification. *Science,* **318** (5857), 1737 - 1742.

HOFMANN, G. E. & TODGHAM, A. E. 2010. Living in the now: physiological mechanisms to tolerate a rapidly changing environment. *Annual Review of Physiology,* **72**, 127 - 145.

HOUGHTON, J. 2009. *Global warming – The complete briefing*, Cambridge, UK, Cambridge University Press.

HOUGHTON, J. T., DING, Y., GRIGGS, D. J., NOGUER, M., VAN DER LINDEN, P. J., DAI, X., MASKELL, K. & JOHNSON, C. A. 2001. IPCC Climate change 2001: The scientific basis. Cambridge University Press.

HUNTLEY, M. E. & LOPEZ, M. D. G. 1992. Temperature-dependent production of marine copepods: a global synthesis. *The American Naturalist,* **140** (2), 201 - 242.

HÅKEDAL, O. J. 2013. *Effects on Scope for Growth due to elevated carbon dioxide in the copepod Calanus finmarchicus.* MSc thesis, Department of Biology, Norwegian University of Science and Technology.

IRIGOIEN, X. 2004. Some ideas about the role of lipids in the life cycle of *Calanus finmarchicus*. *Journal of Plankton Research,* **26** (3), 259 - 263.

JOHANNESSEN, I. S. 2013. *Combined effect of elevated carbon dioxide and temperature on Calanus finmarchicus.* MSc thesis, Department of Biology, Norwegian University of Science and Technology.

JÓNASDÓTTIR, S. H. 1999. Lipid content of *Calanus finmarchicus* during overwintering in the Faroe–Shetland Channel. *Fisheries Oceanography,* **8** (s1), 61 - 72.

KAARTVEDT, S. 1996. Habitat preference during overwintering and timing of seasonal vertical migration of *Calanus finmarchicus*. *Ophelia,* **44** (1 - 3), 145 - 156.

KAARTVEDT, S. 2000. Life history of Calanus finmarchicus in the Norwegian Sea in relation to planktivorous fish. *ICES Journal of Marine Science,* **57** (6), 1819 - 1824.

KANIEWSKA, P., CAMPBELL, P. R., KLINE, D. I., RODRIGUEZ-LANETTY, M., MILLER, D. J., DOVE, S. & HOEGH-GULDBERG, O. 2012. Major cellular and physiological impacts of ocean acidification on a reef building coral. *PLoS ONE,* **7** (4), 1 - 13.

KIM, H., HWANG, N. R. & LEE, K. 2007. Heat shock responses for understanding diseases of protein denaturation. *Molecules and cells,* **23** (2), 123 - 131.

KIM, I. C., CHO, H. H., LEE, J. S. & LEE, H. K. 2008. Molecular cloning and expression of the mitochondrial 60-kDa heat shock protein from an Arctic copepod *Calanus glacialis*. *Gene and Genomics* **30** (2), 101 - 111.

KITA, J., KIKKAWA, T., ASAI, T. & ISHIMATSU, A. 2013. Effects of elevated CO_2 on reproductive properties of the benthic copepod *Tigriopus japonicus* and gastropod *Babylonia japonica*. *Marine Pollution Bulletin,* **73**, 402 - 408.

KOZLOVA, T., PEREZGASGA, L., REYNAUD, E. & ZURITA, M. 1997. The *Drosophila melanogaster* homologue of the hsp60 gene is encoded by the essential locus l (1)10Ac and is differentially expressed during fly development. *Development Genes and Evolution*, **207** (4), 253 - 263.

KREBS, R. A. & FEDER, M. E. 1997. Deleterious consequences of Hsp70 overexpression in *Drosphilla melanogaster* larvae. *Cell Stress & Chaperones*, **2** (1), 60 - 71.

KROEKER, K. J., KORDAS, R. L., CRIM, R. N. & SINGH, G. G. 2010. Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. *Ecology Letters,* **13** (11), 1419 - 1434.

KURIHARA, H. 2008. Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates. *Marine Ecology Progress Series,* **373**, 275 - 284.

KURIHARA, H. & ISHIMATSU, A. 2008. Effects of high CO_2 seawater on the copepod (*Acartia tsuensis*) through all life stages and subsequent generations. *Marine Pollution Bulletin,* **56** (6), 1086 - 1090.

KURIHARA, H., SHIMODE, S. & SHIRAYAMA, Y. 2004a. Effects of raised CO_2 concentration on the egg production rate and early development of two marine copepods (*Acartia steueri* and *Acartia erythraea*). *Marine Pollution Bulletin,* **49** (9), 721 - 727.

KURIHARA, H., SHIMODE, S. & SHIRAYAMA, Y. 2004b. Sub-lethal effects of elevated concentration of CO_2 on planktonic copepods and sea urchins. *Journal of Oceanography*, **60**, 743 - 750.

LAMANDA, A. 2010. *RuBPS staining protocol I (quality)* [Online]. Available: http://www.ruthenium.wg.am/rubps_staining_procedures/ [Accessed 21. February 2014].

LANE, L. 2013. *Protein purification by phenol extraction* [Online]. Available: http://lclane.net/text/proteinphenol.html [Accessed 28. January 2013].

LANGENBUCH, M., BOCK, C., LEIBFRITZ, D. & PÖRTNER, H.-O. 2006. Effects of environmental hypercapnia on animal physiology: A ^{13}C NMR study of protein synthesis rates in the marine invertebrate *Sipunculus nudus*. *Comparative Biochemistry and Physiology Part A,* **144** (4), 479 - 484.

LEWIS, C. N., BROWN, K. A., EDWARDS, L. A., COOPER, G. & FINDLAY, H. S. 2013. Sensitivity to ocean acidification parallels natural pCO_2 gradients experienced by Arctic copepods under winter sea ice. *Proceedings of the National Academy of Sciences,* **110** (51), E4960 - E4967.

LI, W. & GAO, K. 2012. A marine secondary producer respires and feeds more in a high CO_2 ocean. *Marine pollution bulletin,* **64** (4), 699 - 703.

LINDQUIST, S. & CRAIG, E. A. 1988. The heat-shock proteins. *Annual Review of Genetics,* **22** (1), 631 - 677.

LISCHKA, S. & RIEBESELL, U. 2012. Synergistic effects of ocean acidification and warming on overwintering pteropods in the Arctic. *Global Change Biology,* **18** (12), 3517 - 3528.

LUETHI, D., LE FLOCH, M., BEREITER, B., BLUNIER, T., BARNOLA, J.-M., SIEGENTHALER, U., RAYNAUD, D., JOUZEL, J., FISCHER, H., KAWAMURA, K. & STOCKER, T. F. 2008. High-resolution carbon dioxide concentration record 650,000-800,000 years before present. *Nature,* **453** (7193), 379 - 382.

MANEJA, R. H., DINESHRAM, R., THIYAGARAJAN, V., SKIFTESVIK, A. B., FROMMEL, A. Y., CLEMMESEN, C., GEFFEN, A. J. & BROWMAN, H. I. 2014. Proteome of Atlantic herring (*Clupea harengus* L.) larvae is resistant to elevated CO_2. *Marine Pollution Bulletin,* **86** (1 - 2), 154 - 160.

MAUCHLINE, J. 1998. *Advances in marine biology - The biology of calanoid copepods*, California, USA, Academic Press.

MAYOR, D. J., EVERETT, N. R. & COOK, K. B. 2012. End of century ocean warming and acidification effects on reproductive success in a temperate marine copepod. *Journal of Plankton Research,* **34** (3), 258 - 262.

MAYOR, D. J., MATTHEWS, C., COOK, K., ZUUR, A. F. & HAY, S. 2007. CO_2-induced acidification affects hatching success in *Calanus finmarchicus*. *Marine Ecology Progress Series,* **350**, 91 - 97.

MCCONVILLE, K., HALSBAND, C., FILEMAN, E. S., SOMERFIELD, P. J., FINDLAY, H. S. & SPICER, J. I. 2013. Effects of elevated CO_2 on the reproduction of two calanoid copepods. *Marine Pollution Bulletin,* **73** (2), 428 - 434.

MCCULLOCH, M., FALTER, J., TROTTER, J. & MONTAGNA, P. 2012. Coral resilience to ocean acidification and global warming through pH up-regulation. *Nature Climate Change,* **2**, 623 - 627.

MELZNER, F., GUTOWSKA, M. A., LANGENBUCH, M., DUPONT, S., LUCASSEN, M., THORNDYKE, M. C., BLEICH, M. & PÖRTNER, H.-O. 2009. Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences,* **6** (3), 2313 - 2331.

MELZNER, F., STANGE, P., TRÜBENBACH, K., THOMSEN, J., CASTIES, I., PANKNIN, U., GORB, S. N. & GUTOWSKA, M. A. 2011. Food supply and seawater pCO_2 impact calcification and internal shell dissolution in the blue mussel *Mytilus edulis*. *PLoS ONE,* **6** (9), e24223.

MICHAELIDIS, B., OUZOUNIS, C., PALERAS, A. & PÖRTNER, H.-O. 2005. Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovincialis*. *Marine Ecology Progress Series,* **293** (2), 109 - 118.

MILLER, C. B., COWLES, T. J., WIEBE, P. H., COPLEY, N. J. & GRIGG, H. 1991. Phenology in *Calanus funmarchicus*; hypotheses about control mechanisms. *Marine Ecology Progress Series*, **72**, 79 - 91.

MILLER, C. B., JOHNSON, J. K. & HEINLE, D. R. 1977. Growth rules in the marine copepod genus *Acartia*. *Limnology and Oceanography,* **22** (2), 326 - 335.

MILLER, C. B., MORGAN, C. A., PRAHL, F. G. & SPARROW, M. A. 1998. Storage lipids of the copepod *Calanus finmarchicus* from Georges Bank and the Gulf of Maine. *Limnology and Oceanography*, **43** (3), 488 - 497.

MUKHERJEE, J., WONG, K. K. W., CHANDRAMOULI, K. H., QIAN, P.-Y., LEUNG, P. T. Y., WU, R. S. S. & THIYAGARAJAN, V. 2013. Proteomic response of marine invertebrate larvae to ocean acidification and hypoxia during metamorphosis and calcification. *The Journal of Experimental Biology,* **216**, 4580 - 4589.

NAKAMURA, M., MORITA, M., KURIHARA, H. & MITARAI, S. 2011. Expression of hsp70, hsp90 and hsf1 in the reef coral *Acropora digitifera* under prospective acidified conditions over the next several decades. *Biology Open,* **1** (2), 75 - 81.

NATIONAL RESEARCH COUNCIL. 2010. *Ocean acidification: a national strategy to meet the challenges of a changing ocean,* Washington DC, USA, National Academies Press.

NEJSTGAARD, J. C., GISMERVIKZ, I. & SOLBERG, P. T. 1997. Feeding and reproduction by *Calanus finmarchicus*, and microzooplankton grazing during mesocosm blooms of diatoms and the coccolithophore Emiliania huxleyi. *Marine Ecology Progress Series,* **147**, 197 - 217.

NIEHOFF, B., KLENKEL, U., HIRCHEL, H.-J., IRIGOIEN, X., HEAD, R. & HARRIS, R. 1999. A high frequency time series at Weathership M, Norwegian Sea, during the 1997 spring bloom: the reproductive biology of *Calanus finmarchicus*. *Marine Ecology Progress Series,* **176**, 81 - 92.

NIEHOFF, B., SCHMITHÜSEN, T., KNÜPPEL, N., DAASE, M., CZERNY, J. & BOXHAMMER, T. 2013. Mesozooplankton community development at elevated CO_2 concentrations: results from a mesocosm experiment in an Arctic fjord. *Biogeosciences* **10** (3), 1391 - 1406.

NOYES, P. D., MCELWEE, M. K., MILLER, H. D., CLARK, B. W., VAN TIEM, L. A., WALCOTT, K. C., ERWIN, K. N. & LEVIN, E. D. 2009. The toxicology of climate change: Environmental contaminants in a warming world. *Environment International,* **35** (6), 971 - 986.

O'CONNOR, M. I., BRUNO, J. F., GAINES, S. D., HALPERN, B. S., LESTER, S. E., KINLAN, B. P. & WEISS, J. M. 2007. Temperature control of larval dispersal and the implications for marine ecology, evolution, and conservation. *Proceedings of the National Academy of Sciences,* **104** (4), 1266 - 1271.

O'DONNELL, M. J., HAMMOND, L. M. & HOFMANN, G. E. 2009. Predicted impact of ocean acidification on a marine invertebrate: elevated CO_2 alters response to thermal stress in sea urchin larvae. *Marine Biology*, **156** (3), 439 - 446.

OHMAN, M. & HIRCHE, H.-J. 2001. Density-dependent mortality in an oceanic copepod population. *Nature,* **412** (6847), 638 - 641.

PEDERSEN, S. A., HANSEN, B. H., ALTIN, D. & OLSEN, A. J. 2013. Chronic exposure of the North Atlantic copepod *Calanus finmarchicus* (Gunnerus, 1770) to CO_2-acidified seawater; effects on survival, growth and development. *Biogeosciences Discussions,* **10**, 5273 - 5300.

PEDERSEN, S. A., HÅKEDAL, O. J., SALABERRIA, I., TAGLIATI, A., GUSTAVSON, L. M., JENSSEN, B. M., OLSEN, A. J. & ALTIN, D. 2014a. Multigenerational exposure to ocean acidification during food limitation reveals consequences for copepod scope for growth and vital rates. *Environmental Science and Technology,* **48**, 12275 - 12284.

PEDERSEN, S. A., VÅGE, V. T., OLSEN, A. J., HAMMER, K. M. & ALTIN, D. 2014b. Effects of elevated carbon cioxide (CO_2) concentrations on early developmental stages of the marine copepod *Calanus finmarchicus* Gunnerus (Copepoda: Calanoidae). *Journal of Toxicology and Environmental Health, Part A,* **77** (9 - 11), 535 - 549.

PEDERSEN, S. N. & LUNDEBYE, A.-K. 1996. Metallothionein and stress protein levels in shore crabs (*Carcinus maenas*) along a trace metal gradient in the Fal estuary (UK). *Marine Environmental Research,* **42** (1 - 4), 241 - 246.

PELLETIER, G., LEWIS, E. & WALLACE, D. 2007. CO2SYS.XLS: a calculator for the CO_2 system in seawater for microsoft excel/VBA. *Washington State Department of Ecology*.

PEPIN, P. & HEAD, E. J. H. 2009. Seasonal and depth-dependent variations in the size and lipid contents of stage 5 copepodites of *Calanus finmarchicus* in the waters of the Newfoundland Shelf and the Labrador Sea. *Deep Sea Research Part I,* **56** (6), 989 - 1002.

PERRY, A. L., LOW, P. J., ELLIS, J. R. & REYNOLDS, J. D. 2005. Climate change and distribution shifts in marine fishes. *Science,* **308** (5730), 1912 - 1915.

PIERSON, J. J., BATCHELDER, H., SAUMWEBER, W., LEISING, A. & RUNGE, J. 2013. The impact of increasing temperatures on dormancy duration in *Calanus finmarchicus*. *Journal of Plankton Research,* **35** (3), 504 - 512.

PODOLSKY, R. D. & EMLET, R. B. 1993. Separating the effects of temperature and viscosity on swimming and water movement by sand dollar larvae (*Dendraster excentricus*). *The Journal of Experimental Biology,* **176** (1), 207 - 221.

PÖRTNER, H.-O. 2008. Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. *Marine Ecology Progress Series,* **373**, 203 - 217.

PÖRTNER, H.-O. & FARRELL, A. P. 2008. Physiology and climate change. *Science,* **322** (5902), 690 - 692.

PÖRTNER, H.-O. & KNUST, R. 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science,* **315** (5808), 95 - 97.

PÖRTNER, H.-O., LANGENBUCH, M. & REIPSCHLÄGER, A. 2004. Biological impact of elevated ocean CO_2 concentrations: lessons from animal physiology and earth history. *Journal of Oceanography*, **60** (4), 705 - 718.

PÖRTNER, H.-O., REIPSCHLÄGER, A. & HEISLER, N. 1998. Acid-base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level. *The Journal of Experimental Biology*, **201** (1), 43 - 55.

REECE, R. J. 2004. *Analysis of Genes and Genomes*, West Sussex, UK, John Wiley & Sons.

RICE, E., DAM, H. G. & STEWART, G. 2015. Impact of climate change on estuarine zooplankton: surface water warming in Long Island Sound is associated with changes in copepod size and community structure. *Estuaries and Coasts*, **38** (1), 13 - 23.

RIES, J. B., COHEN, A. L. & MCCORKLE, D. C. 2009. Marine calcifiers exhibit mixed responses to CO_2-induced ocean acidification. *Geology*, **37** (12), 1131 - 1134.

ROFF, D. A. & FAIRBAIRN, D. J. 2007. The evolution of trade-offs: where are we? *Journal of Evolutionary Biology*, **20** (2), 433 - 447.

ROSA, R. & SEIBEL, B. A. 2008. Synergistic effects of climate-related variables suggest future physiological impairment in a top oceanic predator. *Proceedings of the National Academy of Sciences*, **105** (52), 20776 - 20780.

RUNGE, J. A. 1988. Should we expect a relationship between primary production and fisheries? The role of copepod dynamics as a filter of trophic variability. *Hydrobiologia*, **167/168**, 61 - 71.

SERAFINI, L., HANN, J. B., KÜLTZ, D. & TOMANEK, L. 2011. The proteomic response of sea squirts (genus *Ciona*) to acute heat stress: A global perspective on the thermal stability of proteins. *Comparative Biochemistry and Physiology Part D*, **6** (3), 322 - 334.

SOLOMON, S. 2007. *Climate change 2007: The physical science basis: Working group I contribution to the fourth assessment report of the IPCC*, Cambridge, UK, Cambridge University Press.

SOMMER, U., ADRIAN, R., BAUER, B. & WINDER, M. 2012. The response of temperate aquatic ecosystems to global warming: novel insights from a multidisciplinary project. *Marine Biology*, **159** (11), 2367 - 2377.

SPEES, J. L., CHANG, S. A., SNYDER, M. J. & CHANG, E. S. 2002. Thermal acclimation and stress in the American lobster, *Homarus americanus*: equivalent temperature shifts elicit unique gene expression patterns for molecular chaperones and polyubiquitin. *Cell Stress and Chaperones*, **7** (1), 97 - 106.

SPICER, J. I., TAYLOR, A. C. & HILL, A. D. 1988. Acid-base status in the sea urchins *Psammechinus miliaris* and *Echinus esculentus* (Echinodermata: Echinoidea) during emersion. *Marine Biology*, **99** (4), 527 - 534.

STEINACHER, M., JOOS, F., FROELICHER, T. L., PLATTNER, G.-K. & DONEY, S. C. 2009. Imminent ocean acidification in the Arctic projected with the NCAR global coupled carbon cycle-climate model. *Biogeosciences*, **6** (4), 515 - 533.

THOMSEN, J., CASTIES, I., PANSCH, C., KÖRTZINGER, A. & MELZNER, F. 2013. Food availability outweighs ocean acidification effects in juvenile *Mytilus edulis*: laboratory and field experiments. *Global Change Biology,* **19** (4), 1017 - 1027.

TIMAKOV, B. & ZHANG, P. 2001. The hsp60B gene of *Drosophila melanogaster* is essential for the spermatid individualization process. *Cell stress and Chaperones,* **6** (1), 71 - 77.

TODGHAM, A. E. & HOFMANN, G. E. 2009. Transcriptomic response of sea urchin larvae *Strongylocentrotus purpuratus* to CO_2-driven seawater acidification. *The Journal of Experimental Biology,* **212** (16), 2579 - 2594.

TOMANEK, L. 2011. Environmental proteomics: changes in the proteome of marine organisms in response to environmental stress, pollutants, infection, symbiosis, and development. *Annual Review of Marine Science,* **3**, 373 - 399.

TOMANEK, L. 2014. Proteomics to study adaptations in marine organisms to environmental stress. *Journal of Proteomics,* **105**, 92 - 106.

TOMANEK, L. & ZUZOW, M. J. 2010. The proteomic response of the mussel congeners *Mytilus galloprovincialis* and *M. trossulus* to acute heat stress: implications for thermal tolerance limits and metabolic costs of thermal stress. *The Journal of Experimental Biology,* **213** (20), 3559 - 3574.

TOMANEK, L., ZUZOW, M. J., IVANINA, A. V., BENIASH, E. & SOKOLOVA, I. M. 2011. Proteomic response to elevated PCO_2 level in eastern oysters, *Crassostrea virginica*: evidence for oxidative stress. *The Journal of Experimental Biology,* **214** (11), 1836 - 1844.

TURLEY, C., BLACKFORD, J., WIDDICOMBE, S., LOWE, D., NIGHTINGALE, P. & REES, A. 2006. Reviewing the impact of increased atmospheric CO_2 on oceanic pH and the marine ecosystem. *In:* SCHELLNHUBER, H. J., CRAMER, W., NAKICENOVIC, N., WIGLEY, T. & YOHE, G. (eds.) *Avoiding dangerous climate change.* Cambridge, UK: Cambridge University Press.

UYE, S.-I. 1988. Temperature-dependent development and growth of *Calanus sinicus* (Copepoda: Calanoida) in the laboratory. *Hydrobiologia,* **167/168**, 285 - 193.

VAN DER HAVE, T. M. & DE JONG, G. 1996. Adult size in ectotherms: temperature effects on growth and differentiation. *Journal of Theoretical Biology,* **183** (3), 329 - 340.

VEHMAA, A., BRUTEMARK, A. & ENGSTRÖM-ÖST, J. 2012. Maternal effects may act as an adaptation mechanism for copepods facing pH and temperature changes. *PLoS ONE,* **7** (10), e48538.

VEHMAA, A., HOGFORS, H., GOROKHOVA, E., BRUTEMARK, A., HOLMBORN, T. & ENGSTRÖM-ÖST, J. 2013. Projected marine climate change: effects on copepod oxidative status and reproduction. *Ecology and Evolution,* **3** (13), 4548 - 4557.

VIANT, M. R., WERNER, I., ROSENBLUM, E. S., GANTNER, A. S., TJEERDEMA, R. S. & JOHNSON, M. L. 2003. Correlation between heat-shock protein induction and reduced metabolic condition in juvenile steelhead trout

(*Oncorhynchus mykiss*) chronically exposed to elevated temperature. *Fish Physiology and Biochemistry,* **29** (2), 159 - 171.

VISSER, A. W. & JÓNASDÓTTIR, S. H. 1999. Lipids, buoyancy and the seasonal vertical migration of *Calanus finmarchicus*. *Fisheries Oceanography,* **8** (s1), 100 - 106.

VÅGE, V. T. 2011. *Effects of high CO_2 concentrations on the early developmental stages of the marine copepod Calanus finmarchicus.* MSc thesis, Department of Biology, Norwegian University of Science and Technology.

WALTHER, T. C. & MANN, M. 2010. Mass spectrometry–based proteomics in cell biology. *The Journal of cell biology,* **190** (4), 491 - 500.

WERNER, I. & NAGEL, R. 1997. Stress proteins HSP60 and HSP70 in three species of amphipods exposed to cadmium, diazinon, dieldrin and fluoranthene. *Environmental Toxicology and Chemistry,* **16** (11), 2393 - 2403.

WEYDMANN, A., SOREIDE, J. E., KWASNIEWSKI, S. & WIDDICOMBE, S. 2012. Influence of CO_2-induced acidification on the reproduction of a key Arctic copepod *Calanus glacialis*. *Journal of Experimental Marine Biology and Ecology,* **428**, 39 - 42.

WHITELEY, N. M. 2011. Physiological and ecological responses of crustaceans to ocean acidification. *Marine Ecology Progress Series,* **430**, 257 - 271.

WHITELEY, N. M. & TAYLOR, E. W. 1992. Oxygen and acid-base disturbances in the hemolymph of the lobster *Homarus gammarus* during commercial transport and storage. *Journal of Crustacean Biology,* **12** (1), 19 - 30.

WIACEK, M., UDDIN, N., KIM, H.-J. & ZUBRZYCKI, I. Z. 2013. Proteome changes in response to ecologically viable environmental variation in *Calanus sinicus*. *Protein and Peptide Letters,* **20** (1), 78 - 87.

WONG, K. K. W., LANE, A. C., LEUNG, P. T. Y. & THIYAGARAJAN, V. 2011. Response of larval barnacle proteome to CO_2-driven seawater acidification. *Comparative Biochemistry and Physiology Part D,* **6** (3), 310 - 321.

WÆHRE, A. 2014. *Interaction Effects of Ocean Acidification and Warming on the Marine Copepod Calanus finmarchicus.* MSc thesis, Department of Biology, Norwegian University of Science and Technology.

XIAOWAN, T., YUNYAN, N., XIAOQIANG, P., CHUN, Y. & ANPING, H. 2009. Determination of total alkalinity and calcium concentration of seawater rapidly and automaticly with small-amount samples. *The 3rd international conference on bioinformatics and biomedical engineering,* Beijing, China, IEEE, 1 - 4.

XU, Q. & QIN, Y. 2012. Molecular cloning of heat shock protein 60 (PtHSP60) from *Portunus trituberculatus* and its expression response to salinity stress. *Cell Stress and Chaperones,* **17** (5), 589 - 601.

ZEEBE, R. E. & WOLF-GLADROW, D. 2001. *CO_2 in seawater: equilibrium, kinetics, isotopes,* Elsevier.

ZERVOUDAKI, S., FRANGOULIS, C., GIANNOUDI, L. & KRASAKOPOULOU, E. 2013. Effects of low pH and raised temperature on egg production, hatching and

metabolic rates of a Mediterranean copepod species (*Acartia clausi*) under oligotrophic conditions. *Mediterranean Marine Science*, **15** (1), 74 - 83.

ZHANG, D., LI, S., WANG, G. & GUO, D. 2011. Impacts of CO_2-driven seawater acidification on survival, egg production rate and hatching success of four marine copepods. *Acta Oceanologica Sinica*, **30** (6), 86 - 94.

ZHANG, D., LI, S., WANG, G., GUO, D., XING, Z. & ZHANG, S. 2012. Biochemical responses of the copepod *Centropages tenuiremis* to CO_2-driven acidified seawater. *Water Science & Technology*, **65** (1), 30 - 37.

Appendixes

Appendix A

A.1. Biometry data

Length and area were measured for each copepod. Raw data and calculations are presented in Table A.1. Measurements of length and area were used to calculate oil sac volume (Equation A.1), body volume (Equation A.2) and oil sac percentage (%, Equation A.3).

$$\text{Oil sac volume} = \frac{\pi * \text{oil sac area}}{4 * \text{oil sac length}} \quad \text{(Equation A.1)}$$

$$\text{Body volume} = \frac{\pi * \text{body area}}{4 * \text{body length}} \quad \text{(Equation A.2)}$$

$$\text{Oil sac \%} = \frac{\text{oil sac volume}}{\text{body volume}} * 100\ \% \quad \text{(Equation A.3)}$$

Animals marked in red indicates missing results, caused by either the copepod being dead or that the stomach covered parts of the oil sac, making it difficult to obtain accurate measurements.

Table A.1. Biometric measurements of body and oil sac of *Calanus finmarchicus* (CV-stage). Included are the measured length and area data, and the calculated volume and oil sac percentage of body. Ambient = A1, B1 and C1, acidified = A4, B4, and C4, warming = A2, B2 and C2, and acidified + warming = A3, B3 and C3.

Animal	Body length (mm)	Oil sac length (mm)	Body area (mm^2)	Oil sac area (mm^2)	Body volume (mm^3)	Oil sac volume (mm^3)	Volume (%)
A1-1	1.977	1.525	0.920	0.212	0.336	0.0231	6.88
A1-2	2.138	1.938	1.066	0.284	0.417	0.0327	7.83
A1-3	2.017	1.548	0.958	0.207	0.357	0.0217	6.08
A1-4	1.936	1.382	0.896	0.185	0.326	0.0194	5.97
A1-5	2.084	1.571	1.062	0.218	0.425	0.0237	5.59

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Table A.1. Continued

Animal	Body length (mm)	Oil sac length (mm)	Body area (mm^2)	Oil sac area (mm^2)	Body volume (mm^3)	Oil sac volume (mm^3)	Volume (%)
A1-6	2.133	1.628	1.086	0.25	0.434	0.0301	6.94
A1-7	2.167	1.878	1.152	0.322	0.481	0.0433	9.02
A1-8	2.24	1.87	1.190	0.308	0.496	0.0398	8.02
A1-9	2.112	1.538	1.050	0.222	0.410	0.0252	6.14
A1-10	2.165	1.66	1.188	0.279	0.512	0.0368	7.19
A1-11	1.938	1.812	0.867	0.248	0.304	0.0266	8.75
A1-12	2.053	1.808	1.013	0.269	0.392	0.0314	8.01
A1-13	1.983	1.224	0.931	0.146	0.343	0.0137	3.98
A1-14	2.11	0.647	1.049	0.090	0.409	0.0098	2.40
A1-15	2.154	2.027	1.106	0.319	0.446	0.0394	8.84
A2-1	1.97	1.126	0.891	0.128	0.316	0.0114	3.61
A2-2	1.855	1.138	0.792	0.097	0.265	0.0065	2.45
A2-3	1.865	0.772	0.761	0.062	0.244	0.0039	1.60
A2-4	1.826		0.765		0.252		
A2-5	1.933	1.043	0.867	0.095	0.305	0.0068	2.23
A2-6	1.960	1.163	0.872	0.091	0.305	0.0056	1.84
A2-7	1.927	1.352	0.863	0.132	0.303	0.0101	3.33
A2-8	1.877		0.850		0.302		
A2-9	2.011	1.891	0.896	0.250	0.313	0.0259	8.28
A2-10	2.060	1.199	0.947	0.089	0.342	0.0052	1.52
A2-11	1.868	1.314	0.823	0.141	0.285	0.0119	4.17
A2-12							
A2-13	1.935		0.846		0.290		
A2-14	1.919	1.155	0.847	0.135	0.293	0.0124	4.22
A2-15							
A3-1	1.892	0.803	0.711	0.058	0.210	0.0033	1.57
A3-2	2.061	1.354	0.908	0.168	0.314	0.0164	5.21
A3-3	1.884	0.74	0.848	0.086	0.300	0.0078	2.62
A3-4	1.980	1.263	0.923	0.155	0.338	0.0149	4.42
A3-5	2.020	1.516	0.949	0.243	0.350	0.0306	8.74
A3-6	1.914	0.918	0.797	0.066	0.261	0.0037	1.43
A3-7	1.985	1.884	0.971	0.391	0.373	0.0637	17.08
A3-8	1.924	1.385	0.879	0.202	0.315	0.0231	7.34
A3-9	2.003	1.283	0.918	0.140	0.330	0.0120	3.63
A3-10	1.846	0.808	0.811	0.109	0.280	0.0115	4.13
A3-11	1.852	1.107	0.814	0.121	0.281	0.0104	3.70
A3-12	1.892	1.642	0.846	0.214	0.297	0.0219	7.37
A3-13	2.027	1.975	0.990	0.439	0.380	0.0766	20.18
A3-14	1.906		0.796		0.261		
A3-15	1.919	0.953	0.805	0.074	0.265	0.0045	1.70

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Table A.1. Continued

Animal	Body length (mm)	Oil sac length (mm)	Body area (mm^2)	Oil sac area (mm^2)	Body volume (mm^3)	Oil sac volume (mm^3)	Volume (%)
A4-1	2.065	1.758	1.062	0.263	0.429	0.0309	7.20
A4-2	2.050	1.841	1.060	0.312	0.430	0.0415	9.65
A4-3	1.922	1.461	0.892	0.248	0.325	0.0330	10.17
A4-4	2.032	1.702	0.966	0.237	0.360	0.0259	7.19
A4-5	1.916	1.197	0.807	0.129	0.267	0.0109	4.09
A4-6	2.093	1.91	1.045	0.283	0.410	0.0329	8.04
A4-7	2.004	1.680	0.937	0.282	0.344	0.0372	10.80
A4-8	1.940	1.011	0.873	0.114	0.308	0.0101	3.27
A4-9	2.115	2.022	0.953	0.333	0.337	0.0431	12.77
A4-10	1.983	1.849	1.087	0.270	0.468	0.0309	6.62
A4-11	2.069	1.911	0.976	0.321	0.361	0.0423	11.71
A4-12	1.926	1.308	0.854	0.127	0.297	0.0097	3.26
A4-13	2.128	1.721	1.091	0.279	0.439	0.0355	8.09
A4-14	2.045	1.855	0.973	0.285	0.363	0.0344	9.46
A4-15	1.851	0.905	0.775	0.076	0.255	0.0050	1.97
B1-1	2.038	1.484	0.997	0.208	0.383	0.0229	5.98
B1-2	1.884	1.221	0.815	0.078	0.277	0.0039	1.41
B1-3	2.228	1.658	1.238	0.316	0.540	0.0473	8.76
B1-4	2.092	1.972	1.082	0.458	0.439	0.0835	19.01
B1-5	2.043	1.539	1.031	0.207	0.408	0.0219	5.35
B1-6	2.246	1.744	1.205	0.274	0.507	0.0338	6.66
B1-7	2.078	1.296	1.000	0.148	0.378	0.0133	3.51
B1-8	2.032	1.879	0.971	0.313	0.364	0.0409	11.24
B1-9	1.937	1.378	0.871	0.116	0.307	0.0077	2.49
B1-10	2.039	1.43	0.984	0.221	0.373	0.0268	7.19
B1-11	2.022	1.156	0.947	0.124	0.348	0.0104	3.00
B1-12	2.032	1.718	0.917	0.232	0.325	0.0246	7.57
B1-13	1.958	1.369	0.898	0.171	0.323	0.0168	5.19
B1-14	2.052	1.471	1.006	0.233	0.387	0.0290	7.48
B1-15	2.084	1.507	0.984	0.210	0.365	0.0230	6.30
B2-1	1.802	1.652	0.754	0.143	0.248	0.0097	3.92
B2-2	2.039	1.221	0.991	0.114	0.378	0.0084	2.21
B2-3	1.911		0.868		0.309		
B2-4	2.006	1.721	1.015	0.286	0.403	0.0373	9.25
B2-5	1.914	1.01	0.874	0.077	0.313	0.0046	1.47
B2-6	1.950	1.54	0.840	0.148	0.284	0.0112	3.93
B2-7	1.912	1.029	0.828	0.083	0.281	0.0053	1.87
B2-8	1.909	1.255	0.849	0.136	0.296	0.0116	3.90
B2-9	1.995	1.401	0.956	0.171	0.360	0.0164	4.56
B2-10	2.026	1.549	0.956	0.197	0.354	0.0197	5.55
B2-11	1.946	1.002	0.850	0.064	0.291	0.0032	1.10

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Table A.1. Continued

Animal	Body length (mm)	Oil sac length (mm)	Body area (mm^2)	Oil sac area (mm^2)	Body volume (mm^3)	Oil sac volume (mm^3)	Volume (%)
B2-12	1.898	1.104	0.818	0.091	0.277	0.0059	2.13
B2-13	1.828		0.750		0.242		
B2-14	1.936	0.831	0.881	0.073	0.315	0.0050	1.60
B2-15	1.955	1.381	0.845	0.188	0.287	0.0201	7.01
B3-1	2.128	1.906	1.101	0.293	0.447	0.0354	7.91
B3-2	1.898	1.483	0.855	0.158	0.302	0.0132	4.37
B3-3	1.964	1.21	0.885	0.131	0.313	0.0111	3.56
B3-4	2.001	1.455	0.982	0.175	0.378	0.0165	4.37
B3-5	1.936	1.555	0.929	0.198	0.350	0.0198	5.66
B3-6	1.840	0.944	0.808	0.072	0.279	0.0043	1.55
B3-7	2.044	1.698	0.959	0.219	0.353	0.0222	6.28
B3-8	1.933	1.315	0.857	0.138	0.298	0.0114	3.81
B3-9	2.035	1.529	0.987	0.158	0.376	0.0128	3.41
B3-10	2.106	1.539	1.031	0.186	0.396	0.0176	4.45
B3-11	1.832		0.770		0.254		
B3-12	1.893	1.003	0.833	0.098	0.288	0.0075	2.61
B3-13	1.983	1.209	0.928	0.096	0.341	0.0060	1.76
B3-14	1.804	1.477	0.767	0.200	0.256	0.0213	8.30
B3-15	1.983	1.492	0.941	0.193	0.351	0.0196	5.59
B4-1	2.035	1.566	1.022	0.216	0.403	0.0234	5.80
B4-2	2.105		1.087		0.441		
B4-3	2.026	1.58	1.008	0.224	0.394	0.0249	6.33
B4-4	2.035	1.236	0.955	0.144	0.352	0.0132	3.74
B4-5	2.013	1.478	0.985	0.210	0.378	0.0234	6.19
B4-6	2.019	1.544	0.994	0.219	0.384	0.0244	6.35
B4-7	1.974	1.493	0.918	0.197	0.335	0.0204	6.09
B4-8	1.962	1.176	0.920	0.118	0.339	0.0093	2.74
B4-9	2.056	1.552	1.002	0.245	0.383	0.0304	7.92
B4-10	2.069	1.699	1.019	0.227	0.394	0.0238	6.04
B4-11	2.054	1.631	1.008	0.246	0.388	0.0291	7.50
B4-12	2.071	1.51	1.006	0.193	0.384	0.0194	5.05
B4-13	2.093	0.962	0.993	0.089	0.370	0.0065	1.75
B4-14	2.014	1.609	0.944	0.203	0.347	0.0201	5.79
B4-15	2.042	1.483	0.996	0.209	0.381	0.0231	6.06
C1-1	2.212	1.223	1.009	0.107	0.361	0.0073	2.03
C1-2	2.117	1.646	0.935	0.270	0.324	0.0348	10.72
C1-3	2.050	1.524	0.837	0.200	0.268	0.0206	7.68
C1-4	2.025	1.385	0.864	0.185	0.289	0.0194	6.70
C1-5	2.087	1.393	0.928	0.197	0.324	0.0219	6.75
C1-6	2.151	1.903	0.986	0.295	0.355	0.0359	10.12
C1-7	1.957	1.398	0.804	0.172	0.259	0.0166	6.41

Table continued on next page.

Table A.1. Continued

Animal	Body length (mm)	Oil sac length (mm)	Body area (mm^2)	Oil sac area (mm^2)	Body volume (mm^3)	Oil sac volume (mm^3)	Volume (%)
C1-8	2.170	1.471	0.955	0.181	0.330	0.0175	5.30
C1-9	2.026		0.796		0.246		
C1-10	1.986	1.382	0.826	0.160	0.270	0.0145	5.39
C1-11	1.962	1.316	0.794	0.144	0.252	0.0124	4.90
C1-12	1.984	1.192	0.790	0.153	0.247	0.0154	6.24
C1-13	2.274	2.074	1.054	0.338	0.383	0.0432	11.28
C1-14	2.003	1.255	0.851	0.156	0.284	0.0152	5.36
C1-15	1.199	1.385	0.784	0.197	0.402	0.0220	5.47
C2-1							
C2-2	1.945	1.354	0.871	0.158	0.306	0.0145	4.73
C2-3	2.026	1.069	0.908	0.101	0.319	0.0075	2.34
C2-4	1.997	1.195	0.847	0.144	0.282	0.0136	4.83
C2-5	1.902	1.343	0.871	0.134	0.313	0.0105	3.35
C2-6	1.919	0.885	0.744	0.073	0.226	0.0047	2.09
C2-7	1.095	1.095	0.874	0.109	0.548	0.0085	1.56
C2-8	1.960	1.238	0.847	0.115	0.287	0.0084	2.92
C2-9	1.901	1.535	0.845	0.196	0.295	0.0196	6.66
C2-10	1.804	1.237	0.799	0.108	0.278	0.0074	2.66
C2-11	1.953	1.298	0.855	0.130	0.294	0.0102	3.48
C2-12	1.872	1.348	0.783	0.149	0.257	0.0129	5.03
C2-13	2.021	1.264	0.971	0.116	0.366	0.0084	2.28
C2-14	1.979	1.33	0.924	0.169	0.339	0.0169	4.98
C2-15	1.919	1.463	0.889	0.226	0.323	0.0274	8.48
C3-1	1.844	0.949	0.828	0.088	0.292	0.0064	2.19
C3-2	1.996	1.375	1.025	0.170	0.413	0.0165	3.99
C3-3	1.855	0.753	0.864	0.051	0.316	0.0027	0.86
C3-4	2.108	1.588	1.081	0.207	0.435	0.0212	4.87
C3-5	1.901	1.454	0.839	0.171	0.291	0.0158	5.43
C3-6	1.950	0.968	0.857	0.088	0.296	0.0063	2.12
C3-7	2.053	1.051	1.000	0.086	0.382	0.0055	1.44
C3-8	1.984	1.528	0.971	0.182	0.373	0.0170	4.56
C3-9	2.049	1.79	0.997	0.300	0.381	0.0395	10.36
C3-10	1.999	1.766	0.956	0.242	0.359	0.0260	7.25
C3-11	1.777	0.753	0.73	0.051	0.235	0.0027	1.15
C3-12	1.934	1.577	0.962	0.245	0.376	0.0299	7.95
C3-13	1.869	1.202	0.802	0.082	0.270	0.0044	1.63
C3-14	1.944	1.231	0.884	0.119	0.316	0.0090	2.86
C3-15	1.907	1.341	0.862	0.155	0.306	0.0141	4.60
C4-1	2.055	1.552	1.002	0.257	0.384	0.0334	8.71
C4-2	1.981	1.725	0.957	0.245	0.363	0.0273	7.53
C4-3	2.060	1.566	1.048	0.224	0.419	0.0252	6.01

Table continued on next page.

Table A.1. Continued

Animal	Body length (mm)	Oil sac length (mm)	Body area (mm^2)	Oil sac area (mm^2)	Body volume (mm^3)	Oil sac volume (mm^3)	volume (%)
C4-4	2.064	1.688	0.987	0.210	0.371	0.0205	5.54
C4-5	1.932	1.464	0.87	0.219	0.308	0.0257	8.36
C4-6	2.006	1.23	0.905	0.144	0.321	0.0132	4.13
C4-7	2.127	1.785	1.11	0.245	0.455	0.0264	5.81
C4-8	1.994	1.155	0.935	0.134	0.344	0.0122	3.55
C4-9	1.795	0.967	0.76	0.097	0.253	0.0076	3.02
C4-10	2.102	1.785	1.026	0.254	0.393	0.0284	7.22
C4-11	2.096	1.938	1.026	0.295	0.394	0.0353	8.94
C4-12	2.035	1.666	1.003	0.246	0.388	0.0285	7.35
C4-13	1.970	1.604	0.935	0.214	0.348	0.0224	6.43
C4-14	2.069	1.873	1.045	0.305	0.414	0.0390	9.41
C4-15	2.088	1.553	1.045	0.218	0.411	0.0240	5.85

Appendix B

B.1. Protein quantification

Protein concentration was quantified to know the amount of protein to be used in the 2-DE gels. The standard curve was used to calculate the protein concentration (Figure B.1). Average amount of protein in each copepod can be seen in Table B.1. It can here be seen that in the ambient and acidified treated copepod the amount of proteins are higher than for the warming and acidified + warming treated copepods.

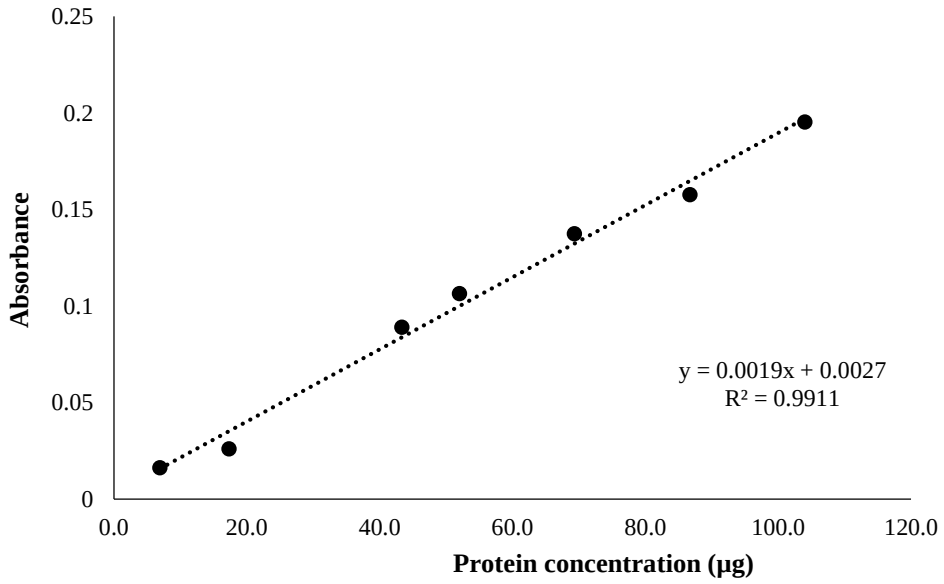

Figure B.1. Standard curve showing absorbance plotted against protein concentration (µg)

Table B.1. Protein concentration (µg) in *Calanus finmarchicus* (CV-stage) exposed for acidification and warming, separately and in combination.

Treatment	Protein conc. (µg) pr. copepod
Ambient	17.07 ± 0.86
Acidified	18.60 ± 1.61
Warming	15.13 ± 1.78
Acidified + warming	15.23 ± 0.38

Appendix C

C.1. Normalized spot volumes

Spot volumes were measured in the DIGE gel. The normalized volumes can be observed in Table C.1.

Table C.1. Normalized volume of protein spots extracted from *Calanus finmarchicus* (CV-stage) exposed for acidified and warming treatments, separately and combined.

Protein	Ambient	Acidified	Warming	Acidified + warming
MHSP60 (spot 321)				
1	1022017.3	2750125.8	845295.6	734172.3
2	1495124.0	2701998.1	544987.0	765779.7
3	1116998.3	1665293.6	1812808.2	851960.5
MHSP60 (spot 407)				
1	1332968.2	1581042.1	1104872.4	1290845.2
2	1773379.8	3207642.2	940534.5	1069308.7
3	1623758.6	1723724.9	1276298.1	756434.0
Gylderaldehyde-3-phosphate dehydrogenase (spot 607)				
1	10582168.6	4823519.8	7944844.1	7991378.7
2	5363314.6	5055828.0	7445196.0	7993951.7
3	7465410.6	4220205.9	8440551.1	6046508.1
Histone H3 (spot 614)				
1	2814076.0	1745846.5	2550025.3	2689180.5
2	1973674.4	1979396.2	2598455.1	2588183.2
3	2524495.7	1517127.8	2972433.0	2024350.8
Actin (spot 1097)				
1	3310167.1	2184061.6	2264311.8	1666883.9
2	3231213.9	2567774.3	2163601.6	1721028.9
3	2783594.2	3384389.5	3112317.4	2292464.3
Glucose-6-phosphate isomerase (spot 2139)				
1	56151621.8	115233347.9	190186110.1	178385883.8
2	120336075.9	128353077.3	134729240.9	142344452.7
3	76852302.4	113071068.4	107970057.3	172155007.7
Protein 531				
1	68877431.9	46777116.3	69792937.9	80118686.7
2	51983514.6	56660387.3	75218589.4	73308684.4
3	46523813.2	45658958.9	55478105.3	72209191.8
Protein 589				
1	16752480.6	42492373.0	47367367.7	58519828.8
2	28967717.5	35151227.3	65546150.5	57671248.5
3	33741108.1	30509847.4	34994645.5	49319756.0
Protein 684				
1	22582834.3	33785120.8	38401315.4	30585290.7
2	21644916.6	35248349.7	40554838.2	41082567.7
3	19192435.5	21648088.2	28356829.0	40573998.8
Protein 1288				
1	746871.0	640699.3	1305665.7	1536234.8
2	828224.9	671642.5	1262322.5	1631400.2
3	723177.8	800357.2	647432.1	1107300.4

Table continued on next page

Table C.1 Continued

Protein		Ambient	Acidified	Warming	Acidified + warming
Protein 2140					
	1	54364428.9	35121609.0	52592499.4	49106149.1
	2	54915898.2	41137561.9	45329452.7	47391704.7
	3	54076869.9	46429963.0	63666339.3	47676600.9
Protein 2153					
	1	8936990.7	10682802.4	10396419.2	15302361.1
	2	9184804.8	11847027.8	12269813.9	21203473.6
	3	7353718.8	95169109.0	9514927.4	11995413.0
Protein 2155					
	1	10095768.4	40480164.3	50104527.1	47139608.9
	2	20606539.2	47053787.7	66116336.2	69923856.0
	3	21435375.8	18974134.2	26791063.4	48104407.7
Protein 2260					
	1	45065343.0	75159489.8	126081735.0	128640575.2
	2	62819473.3	73743990.2	98064115.1	101690571.7
	3	54942710.5	58503128.2	61257152.5	110666348.7

Appendix D

D.1. Gel analysis

Figure D.1. shows a picture taken of the preparative gel.

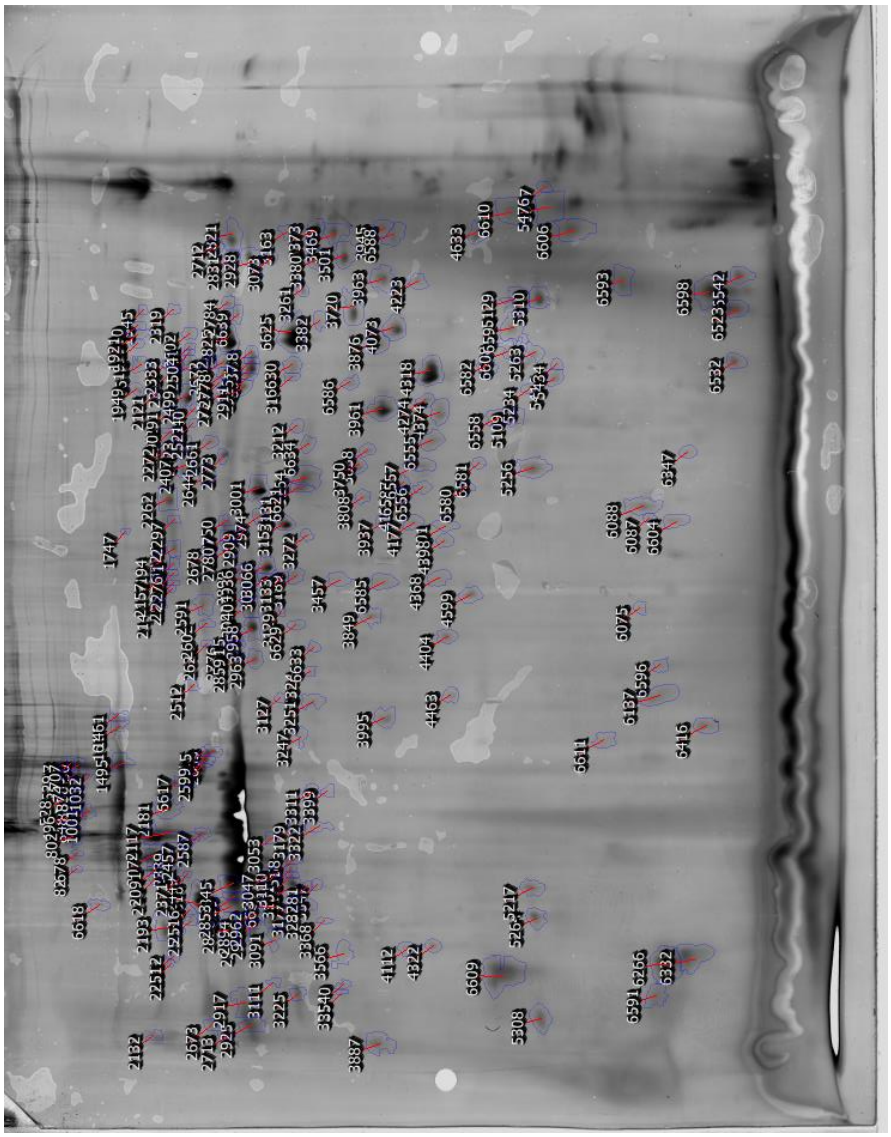

Figure D.1. Preparative gel image from protein extracts from *Calanus finmarchicus* (CV-stage) in pH range 3 - 11. Each protein spot are marked with a number.

To determine the location of the proteins a standards curve was used, which were obtained from GE Healthcare (Figure D.2). Set-up of DIGE gels can be seen in Table D.2.

Table D.2. Set-up of DIGE gels**.** It was used 1µl CyDy to 50 µg protein (total of 150 µg protein in each gel).

	Cy2	**Cy3**	**Cy3**
Gel 1	Pooled standard	C1	C2
Gel 2	Pooled standard	A1	A3
Gel 3	Pooled standard	C4	B1
Gel 4	Pooled standard	A4	B4
Gel 5	Pooled standard	C3	A2
Gel 6	Pooled standard	B2	B3

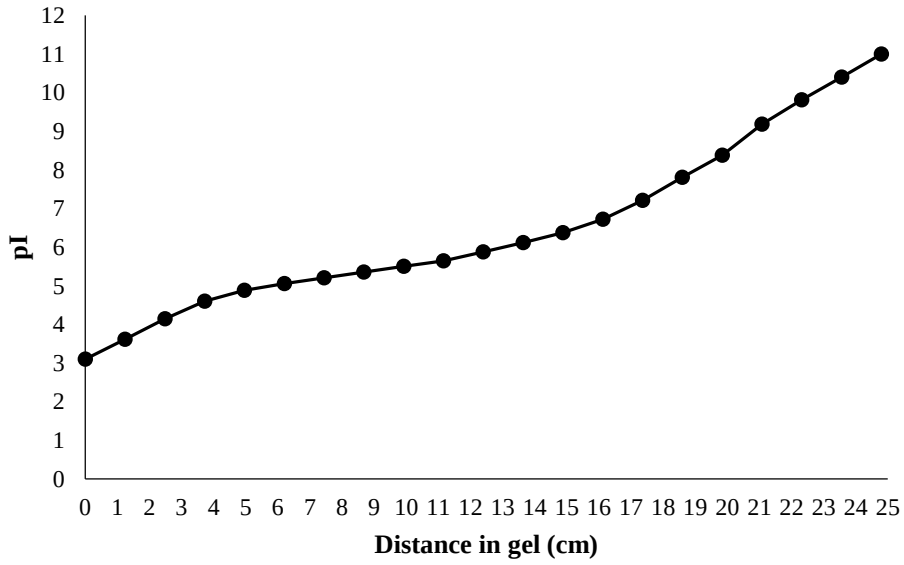

Figure D.2. Curve showing pI against distance (cm) in 2-DE gel.

Appendix E

E.1. Fragment spectrums

One peptide from each of the proteins that were found in both the DIGE and preparative gel can be observed on the following pages. The first six are from proteins that were tentatively identified and are examples of what is meant with good coverage on the fragment spectra, while the last two (spot 531 and 2140) are from unidentified proteins, and have low coverage on the fragment spectra.

E.1.1. Mitochondrial heat shock protein 60 (Spot 321)

In Table E.1 a peptide from mHSP60 with sequence IGLQIVAVK can be seen. The ions in the peptide can be seen in the fragment spectrum in Figure E.1.

Table E.1. Fragmented ion series. Red and blue color shows the matched ions.

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	114.09135	57.54931	I			9
2	171.11282	86.06005	G	827.53496	414.27112	8
3	284.19689	142.60208	L	770.51349	385.76038	7
4	412.25547	206.63137	Q	657.42942	329.21835	6
5	525.33954	263.17341	I	529.37084	265.18906	5
6	624.40796	312.70762	V	416.28677	208.64702	4
7	695.44508	348.22618	A	317.21835	159.11281	3
8	794.51350	397.76039	V	246.18123	123.59425	2
9			K	147.11281	74.06004	1

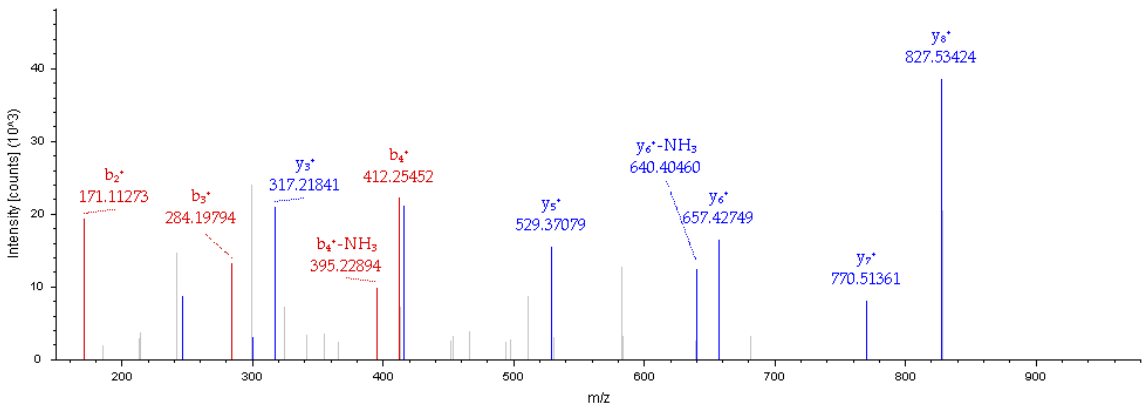

Figure E.1. Fragment spectrum of peptide from mHSP60.

E.1.2. Mitochondrial heat shock protein 60 (Spot 407)

In Table E.2 a peptide from mHSP60 with sequence IGGSSEVEVNEK can be seen. The ions in the peptide can be seen in the fragment spectrum in Figure E.2.

Table E.2. Fragmented ion series. Red and blue color shows the matched ions.

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	114.09135	57.54931	I			12
2	171.11282	86.06005	G	1134.52738	567.76733	11
3	228.13429	114.57078	G	1077.50591	539.25659	10
4	315.16632	158.08680	S	1020.48444	510.74586	9
5	402.19835	201.60281	S	933.45241	467.22984	8
6	531.24095	266.12411	E	846.42038	423.71383	7
7	630.30937	315.65832	V	717.37778	359.19253	6
8	759.35197	380.17962	E	618.30936	309.65832	5
9	858.42039	429.71383	V	489.26676	245.13702	4
10	972.46332	486.73530	N	390.19834	195.60281	3
11	1101.50592	551.25660	E	276.15541	138.58134	2
12			K	147.11281	74.06004	1

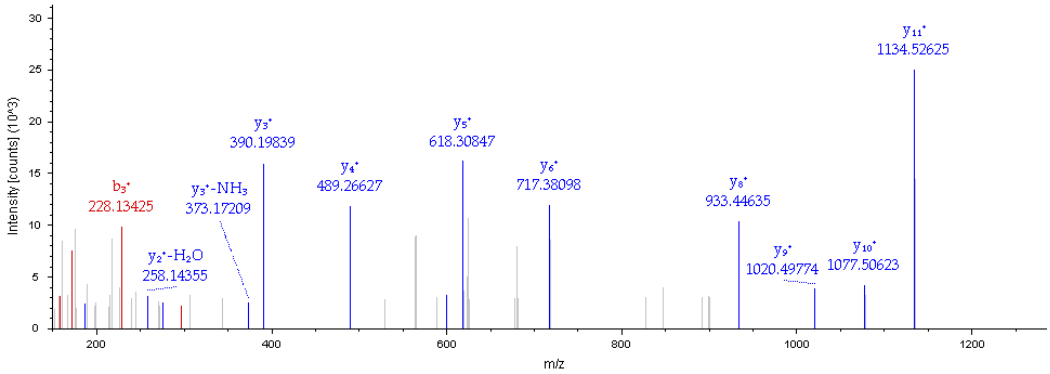

Figure E.2. Fragment spectrum of peptide from mHSP60.

E.1.3. Glyceraldehyde-3-phosphate dehydrogenase (Spot 607)

In Table E.3 a peptide from GAPDH with sequence VPTPDVSVVDLTVR can be seen. The ions in the peptide can be seen in the fragment spectrum in Figure E.3.

Table E.3. Fragmented ion series. Red and blue color shows the matched ions.

#1	a^+	a^{2+}	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	72.08078	36.54403	100.07570	50.54149	V			14
2	169.13355	85.07041	197.12847	99.06787	P	1397.76354	699.38541	13
3	270.18123	135.59425	298.17615	149.59171	T	1300.71077	650.85902	12
4	367.23400	184.12064	395.22892	198.11810	P	1199.66309	600.33518	11
5	482.26095	241.63411	510.25587	255.63157	D	1102.61032	551.80880	10
6	581.32937	291.16832	609.32429	305.16578	V	987.58337	494.29532	9
7	668.36140	334.68434	696.35632	348.68180	S	888.51495	444.76111	8
8	767.42982	384.21855	795.42474	398.21601	V	801.48292	401.24510	7
9	866.49824	433.75276	894.49316	447.75022	V	702.41450	351.71089	6
10	981.52519	491.26623	1009.52011	505.26369	D	603.34608	302.17668	5
11	1094.60926	547.80827	1122.60418	561.80573	L	488.31913	244.66320	4
12	1195.65694	598.33211	1223.65186	612.32957	T	375.23506	188.12117	3
13	1294.72536	647.86632	1322.72028	661.86378	V	274.18738	137.59733	2
14					R	175.11896	88.06312	1

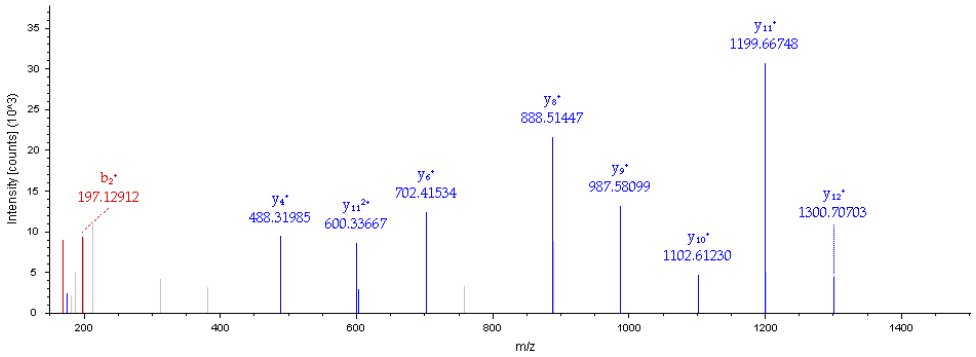

Figure E.3. Fragment spectrum of peptide from GAPDH.

E.1.4. Histone H3 (Spot 1097)

In Table E.4 a peptide from histone H3 with sequence STELLIR can be seen. The ions in the peptide can be seen in the fragment spectrum in Figure E.4.

Table E.4. Fragmented ion series. Red and blue color shows the matched ions.

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	88.03931	44.52329	S			7
2	189.08699	95.04713	T	744.46145	372.73436	6
3	318.12959	159.56843	E	643.41377	322.21052	5
4	431.21366	216.11047	L	514.37117	257.68922	4
5	544.29773	272.65250	L	401.28710	201.14719	3
6	657.38180	329.19454	I	288.20303	144.60515	2
7			R	175.11896	88.06312	1

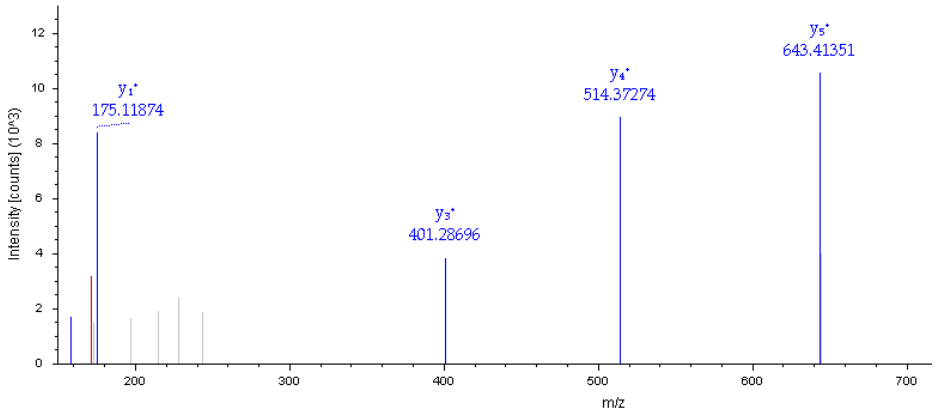

Figure E.4. Fragment spectrum of peptide from histone H3.

E.1.5. Actin (Spot 1097)

In Table E.5 a peptide from actin with sequence GYSFTTTAEREIVR can be seen. The ions in the peptide can be seen in the fragment spectrum in Figure E.5.

Table E.5. Fragmented ion series. Red and blue color shows the matched ions.

#1	b^+	b^{2+}	b^{3+}	Seq.	y^+	y^{2+}	y^{3+}	#2
1	58.02875	29.51801	20.01443	G				14
2	221.09207	111.04967	74.36887	Y	1572.80170	786.90449	524.93875	13
3	308.12410	154.56569	103.37955	S	1409.73838	705.37283	470.58431	12
4	455.19252	228.09990	152.40236	F	1322.70635	661.85681	441.57363	11
5	556.24020	278.62374	186.08492	T	1175.63793	588.32260	392.55083	10
6	657.28788	329.14758	219.76748	T	1074.59025	537.79876	358.86827	9
7	758.33556	379.67142	253.45004	T	973.54257	487.27492	325.18571	8
8	829.37268	415.18998	277.12908	A	872.49489	436.75108	291.50315	7
9	958.41528	479.71128	320.14328	E	801.45777	401.23252	267.82411	6
10	1114.51640	557.76184	372.17698	R	672.41517	336.71122	224.80991	5
11	1243.55900	622.28314	415.19118	E	516.31405	258.66066	172.77620	4
12	1356.64307	678.82517	452.88587	I	387.27145	194.13936	129.76200	3
13	1455.71149	728.35938	485.90868	V	274.18738	137.59733	92.06731	2
14				R	175.11896	88.06312	59.04450	1

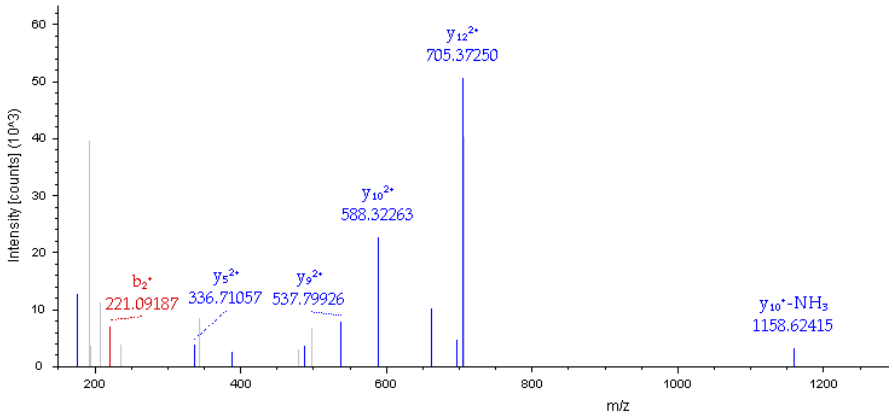

Figure E.5. Fragment spectrum of peptide from actin.

E.1.6. Glucose-6-phospate isomerase (Spot 2139)

In Table E.6 a peptide from G6P isomerase with sequence LVQEFGIDK can be seen. The ions in the peptide can be seen in the fragment spectrum in Figure E.6.

Table E.6. Fragmented ion series. Red and blue color shows the matched ions.

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	114.09135	57.54931	L			9
2	213.15977	107.08352	V	935.48332	468.24530	8
3	341.21835	171.11281	Q	836.41490	418.71109	7
4	470.26095	235.63411	E	708.35632	354.68180	6
5	617.32937	309.16832	F	579.31372	290.16050	5
6	674.35084	337.67906	G	432.24530	216.62629	4
7	787.43491	394.22109	I	375.22383	188.11555	3
8	902.46186	451.73457	D	262.13976	131.57352	2
9			K	147.11281	74.06004	1

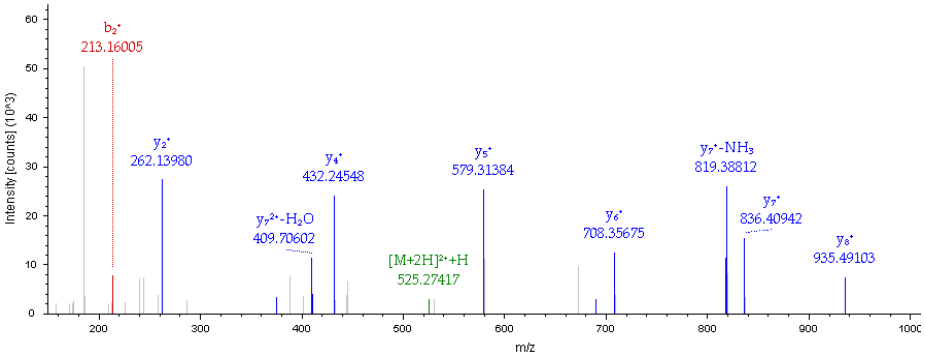

Figure E.6. Fragment spectrum of peptide from G6P isomerase.

E.1.7. Spot 531

In Table E.7 a peptide from spot 531 with sequence AGIQLSPTFVK can be seen. The ions in the peptide can be seen in the fragment spectrum in Figure E.7.

Table E.7. Fragmented ion series. Red and blue color shows the matched ions.

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	72.04440	36.52584	A			11
2	129.06587	65.03657	G	1089.63032	545.31880	10
3	242.14994	121.57861	I	1032.60885	516.80806	9
4	370.20852	185.60790	Q	919.52478	460.26603	8
5	483.29259	242.14993	L	791.46620	396.23674	7
6	570.32462	285.66595	S	678.38213	339.69470	6
7	667.37739	334.19233	P	591.35010	296.17869	5
8	768.42507	384.71617	T	494.29733	247.65230	4
9	915.49349	458.25038	F	393.24965	197.12846	3
10	1014.56191	507.78459	V	246.18123	123.59425	2
11			K	147.11281	74.06004	1

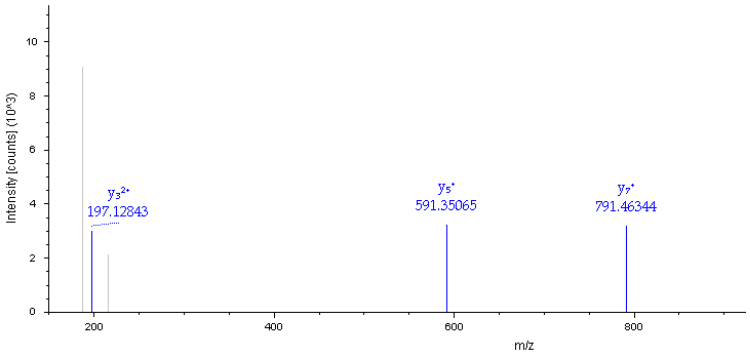

Figure E.7. Fragment spectrum of peptide from spot 531.

E.1.8. Spot 2140

In Table E.8 a peptide from spot 2140 with sequence DIPRASYNFR can be seen. The ions in the peptide can be seen in the fragment spectrum in Figure E.8.

Table E.8. Fragmented ion series. Red and blue color shows the matched ions.

#1	b^+	b^{2+}	Seq.	y^+	y^{2+}	#2
1	148.04269	74.52498	M-Oxidation			11
2	263.06964	132.03846	D	1238.62769	619.81748	10
3	376.15371	188.58049	I	1123.60074	562.30401	9
4	473.20648	237.10688	P	1010.51667	505.76197	8
5	629.30760	315.15744	R	913.46390	457.23559	7
6	700.34472	350.67600	A	757.36278	379.18503	6
7	787.37675	394.19201	S	686.32566	343.66647	5
8	950.44007	475.72367	Y	599.29363	300.15045	4
9	1064.48300	532.74514	N	436.23031	218.61879	3
10	1211.55142	606.27935	F	322.18738	161.59733	2
11			R	175.11896	88.06312	1

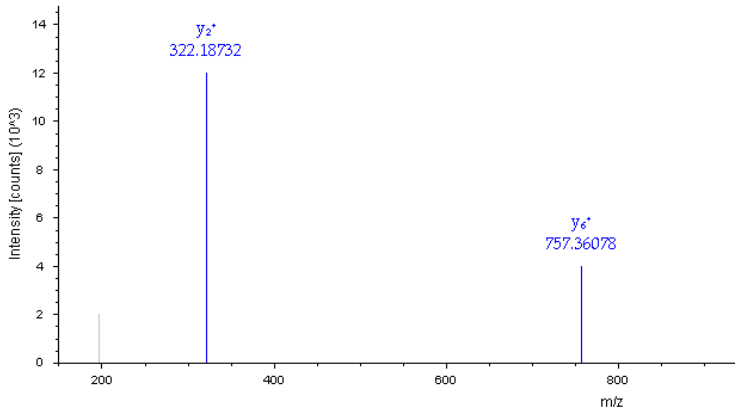

Figure E.8. Fragment spectrum of peptide from spot 2140.

Appendix F

F.1. Statistical analysis

F.1.1. Test of homogeneity of Variance

Prior to the *post-hoc* analysis, homogeneity of variance was tested (Table F.1, and F.2). All of the biometric variables passed. For the proteins all passed, except from mHSP60 (321) and protein 1288. MHSP60 was therefore log-transformed prior to the *post-hoc* analysis. Protein 1288 did not pass the test even when log-transformed nor with any other transformations. The *post-hoc* test Games-Howell, which do not assume equal variance, was therefore used.

Table F.1. Test of homogeneity of Variance, biometry data

Spot ID	Levene Statistic	Significance
Body length	1.685	0.247
Lipid sac length	0.938	0.466
Volume (% of body)	3.981	0.052

Table F.2. Test of homogeneity of Variance, protein data. Significant results are marked in bold.

Spot ID	Levene Statistic	Significance
mHSP60 (321)	3.134	0.087
mHSP60 (407)	20.156	0.171
GAPDH (607)	3.103	0.089
Histone H3 (614)	0.836	0.511
Actin (1097)	1.089	0.408
G6P isomerase (2139)	2.159	0.171
Protein 531	1.648	0.254
Protein 589	1.414	0.308
Protein 684	2.695	0.117
Protein 1288	**5.887**	**0.020**
Protein 2140	3.298	0.079
Protein 2153	3.263	0.080
Protein 2155	1.090	0.407
Protein 2260	1.909	0.207

F.1.2. Fisher's least significant difference test (LSD)

The LSD *post hoc* analysis can be seen in Table F.3 and F.4 for the biometry data and the protein data, respectively.

Table F.3. Fisher's least significant difference test (LSD), biometric variables. Significant results are marked in bold. A = ambient (11 °C, 390 ppm), OA = acidified (11 °C, 2080 ppm), T = warming (14 °C, 390 ppm) and OA + T = acidified + warming treatment (14 °C, 2080 ppm).

Body length	OA + T	T	OA	A
A	**0.005**	**0.001**	0.763	-
OA	**0.003**	**0.001**	-	
T	0.256	-		
OA + T	-			
Lipid sac length	OA + T	T	OA	A
A	**0.001**	**<0.001**	0.251	-
OA	**0.005**	**<0.001**	-	
T	0.103	-		
OA + T	-			
Volume (% of body)	OA + T	T	OA	A
A	0.077	**0.003**	0.722	-
OA	0.072	**0.006**	-	
T	0.136	-		
OA + T	-			

Table F.4. Fisher's least significant difference test (LSD), identified proteins. Significant results are marked in bold. A = ambient (11 °C, 390 ppm), OA = acidified (11 °C, 2080 ppm), T = warming (14 °C, 390 ppm) and OA + T = acidified + warming treatment (14 °C, 2080 ppm).

mHSP60 (321)	OA + T	T	OA	A	*mHSP60 (407)*	OA + T	T	OA	A
A	0.179	0.431	0.051	-	A	0.074	0.132	0.230	-
OA	**0.005**	**0.014**	-		OA	**0.010**	**0.018**	-	
T	0.537	-			T	0.715	-		
OA + T	-				OA + T	-			
GAPDH	OA + T	T	OA	A	*Histone H3*	OA + T	T	OA	A
A	0.711	0.910	**0.032**	-	A	0.990	0.337	**0.031**	-
OA	0.058	**0.027**	-		OA	**0.032**	**0.007**	-	
T	0.630	-			T	0.331	-		
OA + T	-				OA + T	-			
Actin	OA + T	T	OA	A	*G6P isomerase*	OA + T	T	OA	A
A	**0.012**	0.152	0.323	-	A	**0.009**	**0.033**	0.178	-
OA	0.061	0.612	-		OA	0.088	0.308	-	
T	0.138	-			T	0.417	-		
OA + T	-				OA + T	-			
Protein 531	OA + T	T	OA	A	*Protein 589*	OA + T	T	OA	A
A	**0.024**	0.154	0.410	-	A	**0.007**	**0.020**	0.261	-
OA	**0.007**	**0.040**	-		OA	**0.042**	0.132	-	
T	0.266	-			T	0.479	-		
OA + T	-				OA + T	-			
Protein 684	OA + T	T	OA	A	*Protein 1288*	OA + T	T	OA	A
A	**0.009**	**0.015**	0.093	-	A	0.125	0.589	0.731	-
OA	0.170	0.278	-		OA	0.098	0.488	-	
T	0.739	-			T	0.599	-		
OA + T	-				OA + T	-			

Table continued on next page

Table F.4. Table continued

Protein 2140	OA + T	T	OA	A	*Protein 2153*	OA + T	T	OA	A
A	0.188	0.898	**0.016**	-	A	**0.006**	0.315	0.324	-
OA	0.145	**0.019**	-		OA	**0.030**	0.983	-	
T	0.227	-			T	**0.031**	-		
OA + T	-				OA + T	-			
Protein 2155	OA + T	T	OA	A	*Protein 2260*	OA + T	T	OA	A
A	**0.012**	**0.031**	0.158	-	A	**0.005**	**0.029**	0.361	-
OA	0.131	0.326	-		OA	**0.020**	0.128	-	
T	0.543	-			T	0.261	-		
OA + T	-				OA + T	-			