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# Global Climate Change and the Southern Ocean: How Antarctic Fishes Physiologically Respond to a Changing Environment from the Cellular to the Organismal Level

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GLOBAL CLIMATE CHANGE AND THE SOUTHERN OCEAN: HOW ANTARCTIC  
FISHES PHYSIOLOGICALLY RESPOND TO A CHANGING ENVIRONMENT  
FROM THE CELLULAR TO THE ORGANISMAL LEVEL

by

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Submitted in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy in

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## DEDICATION

I dedicate this to my Mama; the best role-model anyone could ask for

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## ABSTRACT

Studies have projected that future changes in sea surface temperature and  $p\text{CO}_2$  levels will impact higher latitudes to a greater extent than in temperate regions. For notothenioid fishes of the Southern Ocean, evolution in extremely stable, cold waters has resulted in several adaptations which have left these fishes poorly prepared for global climate change. I have analyzed the metabolic and cellular response of *Trematomus bernacchii*, *Pagothenia borchgrevinki* and *Trematomus newnesi* to a long-term, multi-stressor scenario relevant to the predicted changes in the Southern Ocean. By combining whole animal respirometry with cellular level analysis of energy allocation, osmoregulatory mechanisms and cellular damage, I aimed to determine if acclimation to increased sea surface temperature ( $4^\circ\text{C}$ ), increased seawater  $p\text{CO}_2$  levels ( $1000\ \mu\text{atm}$ ), or a combination of these two parameters result in energetic trade-offs and exacerbated cellular damage. The data suggest a synergistic relationship exists between elevated temperature and  $p\text{CO}_2$ , as the combination of these variables further elevates metabolic rates and delays the acclamatory response. Overall, long-term acclimation to experimental treatments resulted in a novel discovery: despite evolving in the same environment and on the same time-scale, these three species of notothenioid differ in their physiological response to global climate change, and defend different biochemical pathways when confronted with a changing environment. While *T. bernacchii*, *P. borchgrevinki*, and *T. newnesi* all showed small acclamatory capacities, there appear to be energetic trade-offs associated

with this acclimation, and overall, it may not be possible for energetic demands to be met over long time scales, which could result in long-term impacts to population numbers.



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## LIST OF ABBREVIATIONS

ANOVA .....	Analysis of Variance
CA .....	Carbonic Anhydrase
CAT .....	Catalase
NKA .....	Na <sup>+</sup> /K <sup>+</sup> ATPase
PBS .....	Phosphate-buffered Saline
PC .....	Protein Carbonyl
<i>p</i> CO <sub>2</sub> .....	Partial Pressure of CO <sub>2</sub>
RMR .....	Resting Metabolic Rate
ROS .....	Reactive Oxygen Species
SOD .....	Superoxide Dismutase
SST .....	Sea Surface Temperature
TBS .....	Tris-buffered Saline

## INTRODUCTION

Approximately 25 million years ago, shifting of the Earth's continental plates caused the opening of the Drake Passage, a channel of water found between the modern-day Antarctica Peninsula and southern-most tip of South America, as well as the formation of the Antarctic Circumpolar Current (ACC). These geological changes, along with a region of down-welling within the ACC known as the Polar Front, began the progressive cooling of waters of the Southern Ocean, and subsequent isolation of the Antarctic Continental Shelf (Eastman, 1993). This isolation has resulted in one of the coldest, most stable oceanic environments on the planet.

The dominant fish-fauna of the Southern Ocean, the notothenioids, have evolved in these frigid waters, and therefore possess a suite of unique adaptations which allow them to survive extreme cold. The presence of anti-freeze glycoproteins (DeVries, 1969) and higher serum osmolalities compared to temperate species (O'Grady and DeVries, 1982; Gonzalez-Cabrera et al., 1995; Guynn et al., 2002; Brauer et al., 2005) both contribute to freeze-avoidance. Notothenioid fish constantly express heat shock proteins, which are thought to contribute to protein folding at low temperatures (Hofmann et al., 2000; Place et al., 2004). Finally, these fish utilize a number of metabolic adaptations including increased mitochondrial density and the use of lipid peroxidation as an energy source (Lin et al., 1974; Clarke et al., 1984; Johnston et al., 1998; Pörtner et al., 2005). While evolution in the Southern Ocean has resulted in numerous traits ideal for extreme

stenothermy, it has also left the notothenioids potentially vulnerable to any environmental changes. Given the narrow window in which these fish exist (and hence, physiologically perform), the notothenioids may experience negative effects associated with global climate change sooner than other species. Therefore, it can be argued these endemic fish can be considered “potential forecasters” of how other organisms may respond to factors of global climate change.

The Earth’s overall temperature has risen  $\sim 0.6^{\circ}\text{C}$  in the last 50 years (Walther et al., 2002). This change represents an approximately 30% change from pre-industrial levels (Caldeira and Wickett, 2003) and can largely be attributed to an exponential global increase in atmospheric  $\text{CO}_2$ . This increase has been partially off-set by the absorption of  $\text{CO}_2$  by the world’s oceans. However, this absorption also causes a change in seawater chemistry, resulting in a drop in pH levels, termed “ocean acidification.” According to the International Panel on Climate Change (IPCC) A1F1 scenario, which predicts oceanic  $p\text{CO}_2$  levels in a largely fossil-fuel based economy, the world’s oceans will experience and increase in  $p\text{CO}_2$  levels exceeding 990 ppm by the year 2100 (IPCC, 2007).

It has been well documented that the poles are changing faster than temperature regions (Orr et al., 2005; McNeil and Matear, 2008; Fabry et al., 2008; Mathis et al., 2011). The biogeochemical processes occurring in the waters of the Southern Ocean coupled with the natural deep-water entrapment of  $\text{CO}_2$  which occurs during the winter months in the absence of primary productivity have amplified acidification in this region (McNeil and Matear, 2008), and it is estimated organisms of the Southern Ocean will experience detrimental impacts related to ocean acidification by the year 2050; 50 years sooner than other regions of the planet (McNeil and Matear, 2008; McNeil et al., 2010).

Additionally, the mean surface temperature in waters around the Antarctic Peninsula have rise about 2.5°C since the 1950's (Turner et al. 2005), over four times the global temperature increase.

These lines of evidence suggest that the organisms inhabiting the Southern Ocean may be experiencing environmental changes much sooner than other regions of the planet. Evolution in the stable environment of the Southern Ocean has resulted in species which are likely limited in their capacity to respond to any environmental variation (Peck et al., 2005; Clarke et al., 2007). It is therefore imperative that we gain an understanding of how ecologically dominant species, such as the notothenioids, respond to factors associated with global climate change. The purpose of this research was to investigate how an increase in temperature and/or  $p\text{CO}_2$  affected the physiology of three Antarctic fish species, *Trematomus bernacchii*, *Pagothenia borchgrevinki* and *Trematomus newnesi*. The broad objectives of this research were to determine the long-term effects of a multi-stressor scenario, and investigate the acclamatory capacity of notothenioid fishes.

In order to examine the acclamatory capacity of these notothenioid fishes, I took a “top-down” approach, first focusing on the whole organism, and then exploring the effects of experimental treatments on a cellular level. Traditionally, a whole-organism response to an environmental stress has been viewed as a means to directly link the effects of the stress to the organism's fitness and overall survival (Fry, 1947; Schmidt-Nielsen, 1984). While studying a whole-organism response provides useful information, it provides no insight on the organism's cellular-level response. The bioenergetics of an organism are critical to understanding their response and tolerance to environmental stress (Sokolova et al., 2012; Sokolova, 2013; Todgham and Stillman, 2013).

For fish, being placed into an increased temperature and/or increased  $p\text{CO}_2$  environment results in an increased energy demand, as the costs of basal maintenance are increased due to the fact biochemical reaction rates occur faster at higher temperatures (Fry, 1947; Hochachka and Somero, 2002). This increase in energy demand and increased basal maintenance cost likely leads to energetic trade-offs resulting in a decrease in aerobic scope (The Oxygen Limited Thermal Tolerance hypothesis, Pörtner, 2010). Examining the cellular-level pathways of the notothenioids can illustrate a different picture than simply investigating a whole-organism response. Establishing where additional energy is needed as well as being used provides insight into if these fish are re-allocating energy away from growth and reproduction in order to compensate for increased basal maintenance costs. Examining pathways related to acid-base balance, energetics, as well as oxidative damage and antioxidant capacity, and combining those with whole organism oxygen consumption measurements provides a novel view on the abilities of these fish to respond to Global Climate Change. I hypothesized that placing these fish in any changed environment would heighten the stress response, but given the previous work performed on the notothenioids, temperature would be the driving factor behind it. I also believed that using multiple stressors would manifest in a synergistic effect. Overall, placing these fish in a high temperature and/or high  $p\text{CO}_2$  environment would result in more energy needed to defend acid base balance, combat oxidative stress and damage, and compensate for the additional energy required to acclimate to a changed environment.



Specific objectives of this research were to:

1. Establish short-term oxygen consumption levels by measuring resting metabolic rates (Chapter 1).
2. Determine the levels of cellular stress and damage caused by experimental treatments as well as assess antioxidant capacity (Chapter 2).
3. Investigate physiological compensation relating to osmoregulation and ion balance (Chapter 3).
4. Discern if notothenioid fish have the capacity to acclimate to future predicted ocean conditions, and if that acclimation comes at the cost of depleted energy stores and overall body condition (Chapter 4).

## CHAPTER 1

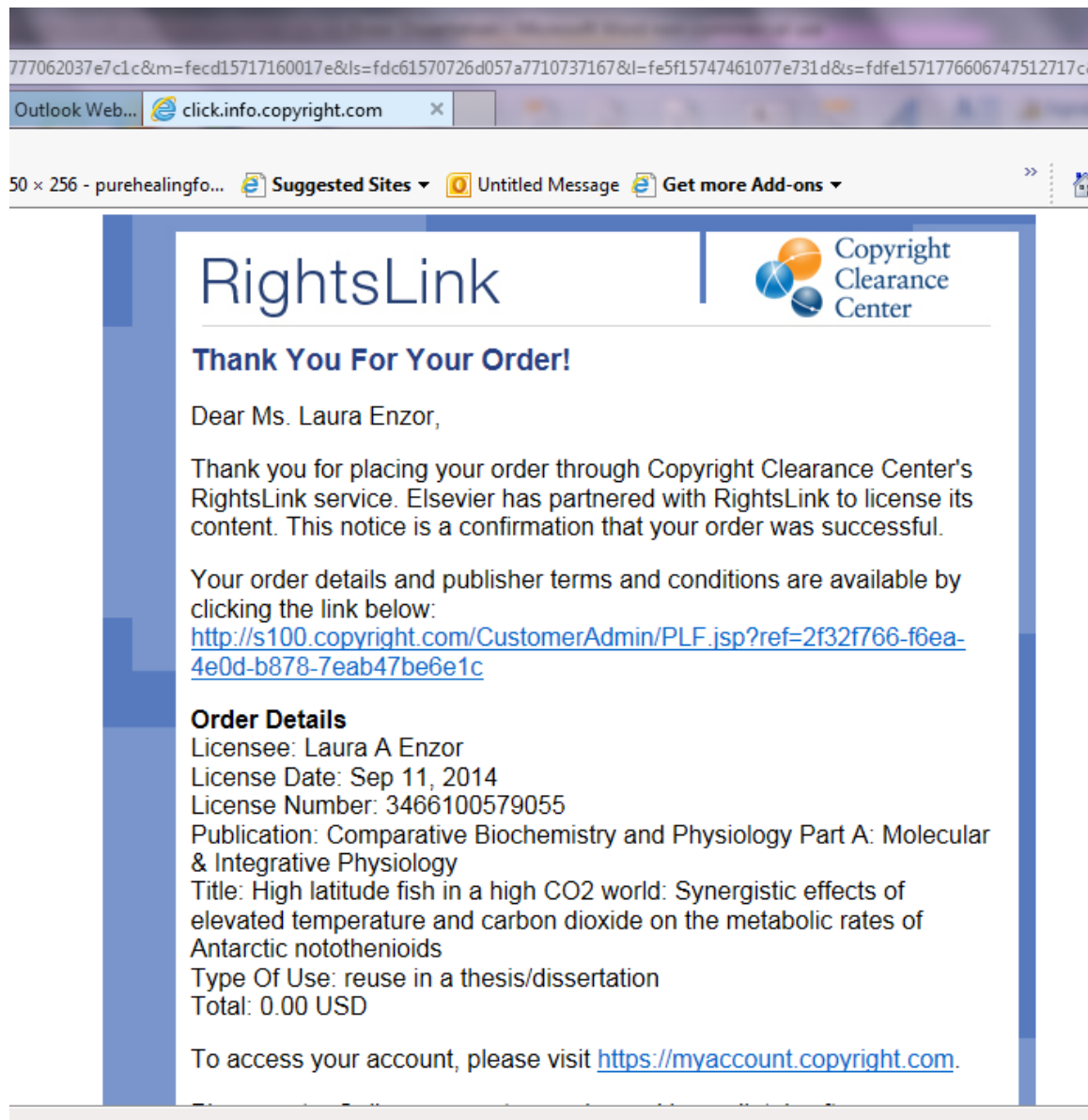
# HIGH LATITUDE FISH IN A HIGH CO<sub>2</sub> WORLD: SYNERGISTIC EFFECTS OF ELEVATED TEMPERATURE AND CARBON DIOXIDE ON THE METABOLIC RATES OF ANTARCTIC NOTOTHENIIDS<sup>1</sup>

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<sup>1</sup> Enzor, L.E., M. L. Zippay, and S.P. Place. *Comparative Biochemistry and Physiology*. 164A(1): 154-161.

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## 1.1 ABSTRACT

Although the physiological response of teleost fishes to increased temperature has been well documented, there is only a small body of literature that examines the effects of ocean acidification on fish under ecologically relevant scenarios. Furthermore, little data exists which examines the possible synergistic effects of increased sea surface temperatures and  $p\text{CO}_2$  levels, although it is well established that both will committedly change in the coming centuries. In this study we examined the effects of increased temperature, increased  $p\text{CO}_2$ , and a combination of these treatments on the resting metabolic rate (RMR) of four species of notothenioid fish, *Trematomus bernacchii*, *T. hansonii*, *T. newnesi*, and *Pagothenia borchgrevinki*, acclimated to treatment conditions for 7, 14 or 28 days. While most species appear capable of rapidly acclimating to increased  $p\text{CO}_2$ , temperature continues to impact RMR's for up to 28 days. One species in particular, *T. newnesi*, displayed no acclamatory response to any of the treatments regardless of acclimation time and may have a reduced capacity to respond to environmental change. Furthermore, we present evidence that temperature and  $p\text{CO}_2$  act synergistically to further elevate the RMR and slow acclimation when compared to temperature or  $p\text{CO}_2$  increases alone.

## 1.2 INTRODUCTION

When studying the effects of global climate change, the roles of temperature and CO<sub>2</sub> are invariably linked. In the last 50 years, the Earth's overall temperature has warmed by ~ 0.6°C (Walther et al., 2002), representing nearly a 30% change relative to pre-industrial values (Caldeira and Wickett, 2003, Vitousek et al., 1997) which can be attributed to the recent exponential increase of atmospheric CO<sub>2</sub>. This rapid increase has been partially offset by the absorption of CO<sub>2</sub> by the world's oceans; however, this is expected to lead to a co-committed drop in ocean pH and alteration of seawater chemistry termed "ocean acidification." Under the Intergovernmental Panel on Climate Change (IPCC) A1F1 prediction scenario, the world's oceans will experience an increase in *p*CO<sub>2</sub> levels upwards of 990 ppm by the year 2100 (IPCC, 2012). Furthermore, it is projected that changes in sea surface temperature and *p*CO<sub>2</sub> levels will impact higher latitudes to a greater extent than temperate regions (Walther et al., 2002; Orr et al., 2005; Fabry et al., 2008; Halpern et al., 2008) and is expected to occur on a faster time-scale (Turner et al., 2005; McNeil and Matear, 2008; McNeil et al., 2010). The Southern Ocean is particularly vulnerable due its unique biogeochemical processes and natural deep-water entrainment of CO<sub>2</sub> that occurs during the extended winter months. It is anticipated the Southern Ocean will experience detrimental impacts related to ocean acidification by the year 2050; 50 years sooner than the IPCC projected year of 2100 (McNeil and Matear, 2008; McNeil et al., 2010). Additionally, the mean surface temperature of Faraday Station, Antarctica has risen about 2.5°C since the 1950's (Turner et al., 2005), compared to the 0.6°C increase seen in the Earth's overall temperature (Walther et al., 2002). While these ecosystems as a whole may adapt to large-scale changes, it is unclear exactly how these

changes will occur, and more importantly, at what cost. To understand this, we must address how key organisms will respond to global changes as well as investigate the costs associated with adaptation.

A major portion of the Southern Ocean fauna is comprised of fishes of the perciform suborder Notothenioidei (Gon and Heemstra, 1990; Eastman, 1993). These fishes began to radiate into Antarctic waters in the early Tertiary, gradually adapting to the progressive cooling, which set in after the opening of the Drake passage and the formation of the circumpolar current some 14-25 million years ago (Eastman, 1993). Isolation of the Antarctic continental shelf by the Polar Front has produced arguably the coldest, most oceanographically stable environment on the planet. However, in opposition to this highly stenothermic environment, the profound environmental extremes produced by the transition from 24 hours of sunlight to complete darkness over the winter months results in significant variation in primary productivity. As a result, Antarctic marine organisms inhabiting these ice-laden waters face unique metabolic and physiological challenges for survival and persistence. The impacts of low temperatures and seasonally limited food availability have long been recognized as primary selective forces driving the evolution of the many endemic species found in Antarctica today (Clarke, 1992; Peck et al., 2004; Pörtner, 2006; Clarke et al., 2007; Peck et al., 2009). In addition to the high degree of endemism produced by food availability and temperature, a wide-array of specialized physiological adaptations, spanning multiple cellular pathways, have arisen in specific genera or families of Antarctic fish, including chaperonins (Pucciarelli et al., 2006), heat shock proteins (Hofmann et al., 2000; Place et al., 2004),

heme proteins (O'Brien and Sidell, 2000; Sidell and O'Brien, 2006), tubulin kinetics (Detrich et al., 2000), and anti-freeze proteins (Devries, 1969; Cheng et al., 2006).

The Southern Ocean's rigid stability may have resulted in an ecosystem filled with endemic fauna that are poorly poised to deal with rapid climate variation (Peck, 2005; Clarke et al., 2007). For instance, extreme stenothermy has made Antarctic fishes very susceptible to stress induced by warming, with upper thermal limits reported around 6 °C in *Trematomus bernacchii*, *T. hansonii* and *Pagothenia borchgrevinkii* (Somero and DeVries, 1967). Additionally, these fish have lost a ubiquitous cellular response to the cytotoxic effects of thermal denaturation of proteins that has been conserved across all taxa (Hofmann et al., 2000; Place et al., 2004; Place and Hofmann, 2005). While some cold-adapted species show an ability to alter their thermal sensitivities, significant inter-specific variation in these responses exist (Podrabsky and Somero, 2006). In addition to thermal tolerance, some notothenioids also display thermal flexibility in O<sub>2</sub> consumption (Robinson and Davison, 2008a,b). However, these changes in oxygen consumption do not appear to confer increased thermal tolerance (Podrabsky and Somero, 2006) and the thermal response of O<sub>2</sub> consumption also appears to be inter-specific (Somero et al., 1968). These previous findings have highlighted the need to take a multi-species comparative approach to understand the capacity of Antarctic notothenioids' ability to tolerate environmental variation. Lending urgency to the need to understand species-specific capacities is the likelihood that the Antarctic ecosystem will reach a critical tipping point far sooner than ecosystems north of the Antarctic Polar Front, perhaps as soon as 2050 (McNeil and Mearns, 2008; McNeil et al., 2010). Thus, the Antarctic ecosystem may be particularly vulnerable to climate variations and it is imperative that

we begin to understand the potential impacts on ecologically dominant species such as the notothenioids.

While numerous studies have been performed on the ability of notothenioid fishes to adapt to increases in temperature (Somero and DeVries, 1967; Forster et al., 1987; Davison et al., 1990; Pörtner et al., 2008; Robinson and Davison, 2008a,b); only a handful of studies to date have examined how fish respond to ecologically relevant increases in seawater  $p\text{CO}_2$ , and only one study has included species from the particularly vulnerable high latitude ecosystems. Studies have shown shunting energy to increase osmoregulation can affect basic processes such as growth and otolith formation (Ishimatsu et al., 2008; Munday et al., 2011), as well as various cellular processes (Langenbuch and Pörtner, 2003). Currently, few studies exist that examine the additive effects of temperature and ocean acidification on piscine species. The rate at which oceanic temperatures and  $p\text{CO}_2$  levels are shifting leave little time for evolutionary change; thus species must rely on current physiological plasticity in order to adapt to increases in temperature and  $p\text{CO}_2$  levels. Therefore, the question is raised if notothenioid fishes are reallocating energy stores in order to survive extreme cold temperatures, do they possess the metabolic scope necessary to respond to climate perturbations? In this study we examined the metabolic response of several notothenioid species to a multi-stressor scenario of increased temperature and  $p\text{CO}_2$ , and report how Antarctic fishes respond to the additive effect of global climate change factors under an ecologically relevant scenario.



### 1.3 METHODS

#### Collection of study specimens

Benthic Antarctic Notothenioids, *Trematomus bernacchii* (Boulenger, 1902), *T. hansonii* (Boulenger, 1902), *T. newnesi* (Boulenger, 1902) and the cryopelagic Antarctic Notothenioid, *Pagothenia borchgrevinki* (Boulenger, 1902), were collected in McMurdo Sound, Antarctica (77°53'S, 166°40'E), from October to November of 2011.

*Trematomus bernacchii*, *P. borchgrevinki*, and *T. newnesi* were caught by hook and line and *T. hansonii* fish were collected using baited fish traps set on the substrate at a depth of ~300 m. All fish were maintained in a 4,100 L flow-through aquarium near ambient seawater temperatures (-1.5°C) and acclimated for one week prior to being placed in experimental tanks. While in the acclimation tank, fish were fed frozen anchovy every other day.

#### Experimental Design

While little to no information is available regarding changes to  $p\text{CO}_2$  levels in McMurdo Sound during winter months, there appears to be little temporal change to  $p\text{CO}_2$  levels over the austral summer. In 2011, Hofmann and colleagues estimated  $p\text{CO}_2$  levels in McMurdo Sound based on observed pH, salinity and *in situ* temperature recorded at 1 hr intervals from October to December and these measurements served as a target value for the control  $p\text{CO}_2$  settings in our experimental tanks. At Hut Point, mean  $p\text{CO}_2$  was estimated at  $413 \pm 8$  ppm ( $\pm$  s.d.) with a range of 391 to 427 ppm and at Cape Evans, mean  $p\text{CO}_2$  was estimated at  $426 \pm 16$  ppm with a range of 358 to 450 ppm (Matson and Hofmann, unpub data). These data are representative of the values we recorded for incoming seawater in the aquarium facilities at McMurdo Station which held

steady around 417 ppm (Table 1). We used four identical, 1240-L experimental tanks maintained at different temperatures and/or  $p\text{CO}_2$  to examine the combined effects of temperature and  $\text{CO}_2$  on the resting metabolic rate of fishes acclimated from 7 to 28 days. The four experimental treatments consisted of (1) a control treatment held near ambient seawater temperature and  $p\text{CO}_2$  ( $-1\text{ }^\circ\text{C}$ , 415 ppm), (2) an ambient low temperature + high  $p\text{CO}_2$  treatment ( $-1\text{ }^\circ\text{C}$ , 1000 ppm), (3) a high temperature + low  $p\text{CO}_2$  treatment ( $+4\text{ }^\circ\text{C}$ , 415 ppm), and a high temperature + high  $p\text{CO}_2$  treatment ( $+4\text{ }^\circ\text{C}$ , 1000 ppm). Fish ( $n=4$  fish per time point, per treatment) were placed in experimental tanks for a period of 7, 14 or 28 days. In these cold adapted species, food can impact resting metabolic rates for 14 days after the last meal (Davison et al., 1990) and previous work performed by Robinson and Davison (2008a,b) has shown withholding food for 28 days in Antarctic fish does not affect metabolic rate measurements. As fish were acclimated in treatment tanks for varying time periods, food was withheld from experimental tanks for the duration of the experiment to ensure the resting metabolic rates were not impacted by the organism's specific dynamic action. Given the extended influence of specific dynamic action of feeding on metabolic rates in these species, there was potential for our first experimental time point to be affected by fish which were fed prior to introduction to the experimental tanks. However, no significant differences were found between control fish across acclimation times, suggesting only resting metabolic rates of fish were measured.

#### Manipulation of seawater conditions

Temperature and  $p\text{CO}_2$  levels were manipulated within the experimental tanks using a combination of thermostated titanium heaters (Process Technology, Brookfield, CT, USA) and a modified  $p\text{CO}_2$  generation system first described by Fangue et al. (2010)

to blend pure CO<sub>2</sub> with CO<sub>2</sub>-free atmospheric air at precise  $p\text{CO}_2$  concentrations. Briefly, atmospheric air was pumped through a chilled condensing coil and passed through drying columns filled with drierite to remove all moisture. Next, CO<sub>2</sub> was scrubbed from the air using a series of columns filled with Sodasorb<sup>®</sup>. CO<sub>2</sub>-free air and pure CO<sub>2</sub> were then mixed to desired levels using digital mass flow controllers (Sierra Instruments, Monterey, CA, USA), and infused into seawater using venturri injectors. Treated air was delivered directly into experimental tanks as well as 45-gallon header tanks used to equilibrate seawater and allow for continuous exchange of treated seawater within the experimental tanks.

Experimental tanks as well as incoming seawater were sampled daily to determine temperature, pH (total scale), salinity, total alkalinity (T<sub>A</sub>) and oxygen saturation.

Seawater parameters for the duration of the experiment are reported in Table 1.

Additionally, experimental tanks were tested daily for water quality (ammonia, nitrite and nitrate levels) of which, no significant increase in any nitrogenous waste were noticed in any of the experimental tanks for the duration of the experiment (data not shown).

#### Evaluation of Metabolic Rates

Resting metabolic rates (RMR's) were determined using an automated intermittent respirometry system (Loligo Systems, Denmark). Respirometry chambers were housed in covered 99-L tanks receiving continuous flow of treated seawater directly from the experimental tanks to maintain consistency among acclimation conditions and conditions under which metabolic rates were recorded. In addition, the 99-L tanks used for metabolic measurements of warm acclimated fish were fitted with glass aquarium heaters to maintain constant acclimation temperatures while cold 99-L tanks were

submerged within an 850-L sea table with continuous flow of ambient seawater to maintain lower temperatures. Fish were placed in respirometry chambers with flush pumps running for 10 - 12 h prior to determination of oxygen consumption rates. Oxygen consumption measurements were collected over a 20 min interval followed by a 5 min flush cycle to re-oxygenate the respirometry chamber. Following the adjustment period to the respirometry chamber, respiration rates ( $\dot{M}O_2$ ) were measured continuously over a three-hour period at the same time each day. Once no discernible chamber effect could be observed, as indicated by no significant changes in oxygen consumption over a 1h period and  $r^2$  values of  $>0.95$  for the slope describing the rate of oxygen consumption, mean  $\dot{M}O_2$  values were calculated by averaging five sequential measurements. Additionally, values whose slope deviated from an  $r^2$  of  $>0.95$  were excluded, as they are likely indication of fish activity in the chamber during that particular measurement period. Mass specific oxygen consumption rates were standardized to a 100-g fish (Steffensen, 2005; Robinson and Davison, 2008a,b) using a mass exponent of -0.25 (Schmidt-Nielsen, 1984). As we did not have enough individuals to experimentally determine the mass-exponent for our study species, we chose to be conservative with our measurements and utilize the more general model of -0.25 as a mass exponent. A study performed by Clarke and Johnston (1999) indicated that a wide range of teleost fish, whether polar or temperate, scale similarly.

### Statistics

We used two-way Analysis of Variance (ANOVA) to test for significant differences in the RMR of *T. bernacchii* acclimated to different treatments, length of acclimation (7, 14 or 28-days), and tested for an interaction between treatment and

acclimation time. For inter-species comparisons, we again utilized two-way ANOVA to test for differences within treatment, within species and for an interaction between treatment and species for 7-day (*T. bernacchii*, *T. hansonii* and *P. borchgrevinkii*) and 28-day (*T. bernacchii*, *T. hansonii*, *P. borchgrevinkii*, and *T. newnesii*) acclimation times. For comparisons that revealed a significant difference in the RMR ( $p < 0.05$ ), we utilized a Tukey's HSD multiple comparison test to identify the means that significantly differed from each other ( $p < 0.05$ ).

## 1.4 RESULTS

### Seawater Chemistry

During the course of the 3-month experimental run of the seawater system, we observed minor variation between the measured seawater parameters and the target treatment (Table 1.1). Although we observed temporal variation in the absolute temperature and  $p\text{CO}_2$  values, the treatments remained significantly different from one another over the course of the experiment. One-way ANOVA found the two high temperature treatments significantly differed from the two low temperature treatments as well as the ambient incoming seawater over the course of the experiment ( $p < 0.05$ ).

The two high  $p\text{CO}_2$  treatments also significantly differed from both low  $p\text{CO}_2$  treatments as well as incoming seawater ( $p < 0.05$ ). Low treatment  $p\text{CO}_2$  levels did not differ from those of incoming seawater ( $p > 0.05$ ). Significant differences were seen when comparing the low  $p\text{CO}_2$  + high temperature treatment to the low  $p\text{CO}_2$  + low temperature treatment and incoming seawater ( $p < 0.05$ ). This may have been a consequence of the reduced flow rates of ambient seawater necessary to maintain the

elevated temperature and the increased respiration rates of fish in response to increased temperature. No significant differences were found between the  $p\text{CO}_2$  levels for the high  $p\text{CO}_2$  treatments.

#### Intra-species comparison

For *T. bernacchii* specimens, acclimation to high  $p\text{CO}_2$  and high temperature resulted in elevated RMR's (Fig. 1.1). The response varied both between treatments and across acclimation periods, suggesting *T. bernacchii* may be differentially affected by pH and temperature changes in their environment (Fig. 1). Two-way ANOVA performed on the RMR's of *T. bernacchii* acclimated to different seawater treatments indicated there was an effect of treatment, acclimation time, and an interaction between treatment and acclimation time in this species (Table 1.2). Overall, RMR's in *T. bernacchii* acclimated to high temperature or high temperature + high  $p\text{CO}_2$ , were significantly higher than the RMR's in fish acclimated to either of the low temperature treatments (Table 1.3). Additionally, within a given acclimation period, fish acclimated to control conditions (low temperature + low  $p\text{CO}_2$ ) displayed lower RMR's when compared to all other treatments (low temperature + high  $p\text{CO}_2$ , high temperature + low  $p\text{CO}_2$ , and high temperature + high  $p\text{CO}_2$ , see Table 1.3).

*Trematomus bernacchii* displayed a relatively rapid compensation with respect to oxygen consumption when acclimated to high  $p\text{CO}_2$  and high temperatures (Fig. 1.1). Fish acclimated to these treatments show a consistent decline in RMR with specific oxygen consumption rates becoming nearly indistinguishable from control fish within a 28-day acclamatory period (Fig. 1.1). After a 7-day acclimation period, all experimental treatments resulted in significantly elevated RMR (low temperature + high  $p\text{CO}_2$  = 30.36

mg O<sub>2</sub>/kg/hr; high temperature + low pCO<sub>2</sub>= 39.56 mg O<sub>2</sub>/kg/hr; and high temperature + high pCO<sub>2</sub>= 46.28 mg O<sub>2</sub>/kg/hr) relative to control fish (12.80 mg O<sub>2</sub>/kg/hr; Fig. 1.1, Table 1.3). Within 14 days, there was no statistical difference between control (22.02 mg O<sub>2</sub>/kg/hr) and the low temperature + high pCO<sub>2</sub> treatment (22.21 mg O<sub>2</sub>/kg/hr), however after 28 days, the RMR of *T. bernacchii* acclimated to both high temperature treatments (high temperature + low pCO<sub>2</sub>, 23.67 mg O<sub>2</sub>/kg/hr; and high temperature + high pCO<sub>2</sub>, 27.26 mg O<sub>2</sub>/kg/hr) remained elevated above control values (9.72 mg O<sub>2</sub>/kg/hr; Table 1.3). These trends indicate that within 14 days, metabolic rates of fish acclimated to high pCO<sub>2</sub> seawater are indistinguishable from the metabolic rates of control fish, however elevated temperature continues to impact the RMR in *T. bernacchii* for upwards of a month after initial exposure.

*Pagothenia borchgrevinki* specimens also showed an effect of acclimation time and treatment when analyzed using two way ANOVA (Fig. 1.2, Table 1.2), but no interaction between acclimation time and treatment was found (Table 1.2). As with *T. bernacchii*, 7-day acclimated fish had elevated RMR's in the high temperature + high pCO<sub>2</sub> treatment (41.86 mg O<sub>2</sub>/kg/hr) when compared to the low temperature + high pCO<sub>2</sub> treatment (24.29 mg O<sub>2</sub>/kg/hr) and control treatment (25.64 mg O<sub>2</sub>/kg/hr; Fig. 1.2, Table 1.3). Fish acclimated in the high temperature + low pCO<sub>2</sub> treatment (38.84 mg O<sub>2</sub>/kg/hr) also showed elevated RMR's when compared to the low temperature + high pCO<sub>2</sub> treatment (24.29 mg O<sub>2</sub>/kg/hr; p=0.037), however no difference was found in the oxygen consumption of high temperature-acclimated fish and those in the control treatment (Table 1.3). This finding may be a result of the low n-value for these fish as the difference found was just below the significance level. Fish acclimated for 28-days to

experimental conditions showed elevated RMR's in the high temperature + high  $p\text{CO}_2$  treatment (33.29 mg  $\text{O}_2/\text{kg/hr}$ ) when compared to control RMR values (19.57 mg  $\text{O}_2/\text{kg/hr}$ ; Fig. 1.3, Table 1.3). No differences were found in the RMR's between fish acclimated in the high temperature + low  $p\text{CO}_2$  treatment (24.29 mg  $\text{O}_2/\text{kg/hr}$  and fish in the control tanks (Table 1.3).

Two-way ANOVA performed on the RMR's of *T. hansonii* showed only a treatment effect was present (Table 1.2). These fish followed the same trends as *T. bernacchii* and *P. borchgrevinki*; RMR's measured from the high temperature (38.35 mg  $\text{O}_2/\text{kg/hr}$ ) and high temperature + high  $p\text{CO}_2$  (33.51 mg  $\text{O}_2/\text{kg/hr}$ ) treatments were elevated when compared to control values (18.65 mg  $\text{O}_2/\text{kg/hr}$ ) after 7 days of acclimation (Fig. 1.2, Table 1.3). Oxygen consumption rates of fish acclimated for 28-days did not differ from control values for any experimental treatment (Fig. 1.3, Table 1.3).

#### Inter-species comparison

Despite the phylogenetic distance between the species used in this study and the variety of habitat niches occupied by these fish, we found no species effect after 7 days of acclimation (Fig. 1.2, Table 1.4). However, resting metabolic rates did differ as a function of treatment (Fig. 1.2, Table 1.4). No interaction between species and treatment was observed (Table 1.4). As previously seen with *T. bernacchii*, RMR's for *T. hansonii* and *P. borchgrevinki* were significantly lower in the two low temperature treatments compared to the two high temperature treatments, regardless of  $p\text{CO}_2$  levels (Fig. 1.2).

Unlike fish acclimated for shorter periods of time, a significant difference in the metabolic response was seen both between treatments and between species when



acclimated for 28 days (Fig. 1.3). A two-way ANOVA revealed a significant difference between the mean RMR as a function of treatment (Table 1.4), while no significant differences were seen between fish acclimated to high temperature alone and the combined stress of high temperature + high  $p\text{CO}_2$  (Fig. 1.3). However, RMR's in fish acclimated for 28 days were significantly different in control treatments when compared to the high temperature + high  $p\text{CO}_2$  treatment, as well as high temperature + low  $p\text{CO}_2$  treatments (Table 1.3). Additionally, there was a significant difference in RMR with respect to species (Table 1.4). Overall, RMR's of *T. bernacchii* (control= 9.72 mg  $\text{O}_2/\text{kg}/\text{hr}$ , low temperature + high  $p\text{CO}_2$ = 13.80 mg  $\text{O}_2/\text{kg}/\text{hr}$ , high temperature + low  $p\text{CO}_2$ = 23.67 mg  $\text{O}_2/\text{kg}/\text{hr}$ , and high temperature + high  $p\text{CO}_2$ = 27.26 mg  $\text{O}_2/\text{kg}/\text{hr}$ ) were lower when compared with the metabolic rates of *T. hansonii*, (control= 19.80 mg  $\text{O}_2/\text{kg}/\text{hr}$ , low temperature + high  $p\text{CO}_2$ = 33.35 mg  $\text{O}_2/\text{kg}/\text{hr}$ , high temperature + low  $p\text{CO}_2$ = 36.26 mg  $\text{O}_2/\text{kg}/\text{hr}$ , and high temperature + high  $p\text{CO}_2$ = 34.15 mg  $\text{O}_2/\text{kg}/\text{hr}$ ) and *T. newnesi* (control= 11.08 mg  $\text{O}_2/\text{kg}/\text{hr}$ , low temperature + high  $p\text{CO}_2$ = 31.76 mg  $\text{O}_2/\text{kg}/\text{hr}$ , high temperature + low  $p\text{CO}_2$ = 33.09 mg  $\text{O}_2/\text{kg}/\text{hr}$ , and high temperature + high  $p\text{CO}_2$ = 39.87 mg  $\text{O}_2/\text{kg}/\text{hr}$ ; Table 1.3), but when compared with *P. borchgrevinkii*, (control= 19.57 mg  $\text{O}_2/\text{kg}/\text{hr}$ , low temperature + high  $p\text{CO}_2$ = 22.48 mg  $\text{O}_2/\text{kg}/\text{hr}$ , high temperature + low  $p\text{CO}_2$ = 24.29 mg  $\text{O}_2/\text{kg}/\text{hr}$ , and high temperature + high  $p\text{CO}_2$ = 33.29 mg  $\text{O}_2/\text{kg}/\text{hr}$ ), *T. bernacchii* RMR's were not significantly different (Fig. 1.3). No interaction was found between treatment and species (Table 1.4).

Across all species, *T. newnesi* exhibited remarkably different patterns of oxygen consumption after 28 days of acclimation to various  $p\text{CO}_2$  and temperature treatments (Fig. 1.3). Unlike the other species tested, *T. newnesi* displayed elevated RMR's in

response to all treatments relative to control values even after 28 days of acclimation (Fig. 1.3). A two-way ANOVA revealed fish acclimated to the control treatment (11.08 mg O<sub>2</sub>/kg/hr) had significantly lower mean RMR's when compared to the low temperature + high pCO<sub>2</sub> (31.76 mg O<sub>2</sub>/kg/hr;), high temperature + low pCO<sub>2</sub> (33.09 mg O<sub>2</sub>/kg/hr), and high temperature + high pCO<sub>2</sub> treatments (39.87 mg O<sub>2</sub>/kg/hr; Table 1.3). These data suggest that unlike *T. bernacchii*, *T. hansonii* and *P. borchgrevinkii*, *T. newnesi* cannot adjust physiologically to increased levels of pCO<sub>2</sub> within the 28-day acclimation period used in this study.

## 1.5 DISCUSSION

Isolation of the Antarctic Polar Front has resulted in the evolution of marine organisms in an extremely stable environment that are often considered to be limited in their capacity to respond to environmental change (Peck, 2005; Clarke et al., 2007). Consequently, these organisms are often perceived as highly vulnerable to the predicted changes in environmental parameters as a result of anthropogenic disturbances. Waters of the Southern Ocean are expected to experience some of the earliest impacts of global climate change (Orr et al., 2005; McNeil and Matear, 2008). Therefore, inhabitants of this unique ecosystem are considered potential forecasters of biological impacts of climate change and thus we set out to examine the metabolic response of several species of the suborder Notothenioidei, the dominant fish fauna of the Antarctic ecosystem (Eastman, 1993), to ecologically relevant perturbations of seawater pCO<sub>2</sub> and temperature.

Control RMR's from fish in this study are representative of oxygen consumption rates previously measured. The measurements of RMR's in *T. bernacchii* (12.80 mg O<sub>2</sub>/kg/hr), *T. hansonii* (18.65 mg O<sub>2</sub>/kg/hr), and *P. borchgrevinkii* (25.64 mg O<sub>2</sub>/kg/hr),

were similar to those found by Steffensen; 17.3, 22.4 and 28.2 mg O<sub>2</sub>/kg/hr, respectively (2005). Davison and colleagues have reported slightly higher RMR's for *P. borchgrevinki* (32.8 mg O<sub>2</sub>/kg/hr, 1990), however these values were obtained at 0 °C and this may account for the small difference between our measurements. Our resting measurements of oxygen consumption in *T. newnesi* under ambient conditions (11.07 mg O<sub>2</sub>/kg/hr) were much lower than previously unpublished values of 41 mg O<sub>2</sub>/kg/hr (referenced in Steffensen, 2005), however we cannot speculate as to the differences between these two studies. The idea of metabolic cold adaptation has been long-debated in regard to Antarctic species. These data follow previously established findings that notothenioid resting metabolic rates routinely fall below values reported by Wohlschlag (1960; 1964) that helped establish the theory of metabolic cold adaptation in these fish. Our data find no evidence that would suggest metabolic cold adaptation of resting metabolic rates has occurred in these species and offers no further empirical support for Krogh's initial predictions (1914; 1916).

As environmental temperature increases, oxygen demand also increases and organisms must increase oxygen consumption in order to compensate for this increased demand. If demand is not met, tissues become hypoxic, causing protein synthesis to slow, ultimately halting growth and reproduction (Fry, 1947; Pörtner et al., 2005). The use of anaerobic metabolism has been documented as a common tool to combat the physiological stress that accompanies increased environmental temperature (Pörtner et al., 2005). Given the limited glycolytic capacity of the notothenioids, (Dunn and Johnston, 1986; Forster et al., 1987; Davison et al., 1988) there can be little doubt that the initial stress response involves restructuring of energy stores, and hence, an increase in

metabolic rate, until cellular homeostasis can again be achieved. Overall, each species examined in this study displayed an increase in resting metabolic rate above control conditions after a 7-day exposure to experimental treatments. These data are consistent with previous studies that have identified energetic shifts in cellular processes as a result of increased temperature in Antarctic notothenioids. Studies performed on the thermal tolerance of *P. borchgrevinki* acclimated to 4°C for short periods of time shows a marked increase in RMR (Wilson et al., 2002; Robinson and Davison, 2008a,b). In addition, work performed by Somero and DeVries (1967) illustrated that oxygen consumption of brain tissue from *T. bernacchii* increased by almost 2-fold at 4°C.

Similarly, it has been shown that elevated  $p\text{CO}_2$  environments require fish to spend more energy on physiological responses such as acid-base regulation and ventilation rates (Perry and Gilmour, 2002; Evans et al., 2005; Perry and Gilmour, 2006). The cost of baseline osmoregulation in fishes is estimated to be approximately 6-15% of resting metabolic rate (Kidder et al., 2006); therefore energy spent on increased acid-base regulation is likely to shunt energy away from growth and otolith formation (Ishimatsu et al., 2008; Munday et al., 2011), as well as other cellular processes such as protein synthesis (Langenbuch and Pörtner, 2003). In our study, both elevated temperature and  $p\text{CO}_2$  levels alone resulted in an upward shift in RMR within the first 7 days of exposure, suggesting a potential short-term energetic cost for physiological adaptations needed to restore proper cellular homeostasis.

Given the higher cost of ventilation amongst aquatic breathers (Dejours, 1981), is it thought fish will exhibit little to no respiratory acclimation when confronted by high  $p\text{CO}_2$  environments. Indeed, Atlantic salmon display significantly elevated ventilation

rates for upwards of 62 days when exposed to high  $p\text{CO}_2$  environments (Fivelstad et al., 1999; Hosfeld et al., 2008). This increase in RMR is likely an attempt to balance cellular energetics resulting from hypercapnic effects on respiratory gas exchange and acid-base balance in marine fish (for reviews see Perry and Gilmour, 2006; Brauner and Baker, 2009). Unlike Atlantic salmon, Antarctic notothenioids displayed a rapid acclamatory response when exposed to elevated  $p\text{CO}_2$  levels for an extended duration. Respiration rates quickly leveled off to those of control values within 14 days of exposure to hypercapnic conditions. Some of the discrepancy in the acclamatory response may in-part lie with the significant difference in the  $p\text{CO}_2$  levels fish were exposed to in the studies by Fivelstad et al. [12,000  $\mu\text{atm}$ ] (1999) and Hosfeld et al. [7,800  $\mu\text{atm}$ ] (2008). Recently, Esbaugh and colleagues (2012) reported a similar rapid physiological compensation for blood plasma acidosis resulting from low level hypercapnia in another marine fish, *Opsanus beta*, and suggested the potential impacts of ocean acidification on marine teleosts may not necessarily stem directly from acidosis, but the energetic costs associated with chronic exposure. Alternatively, short term studies performed on juvenile tropical (Munday et al., 2009) and coral reef fish (Nowicki et al., 2012), as well as juvenile Atlantic cod, *Gadus morhua*, (Melzner et al., 2009), have shown little to no detrimental effects when fish are reared in a hypercapnic environment at levels predicted for 2100. Taken together, these studies highlight the variability of sensitivities among species and across developmental stages. The longer acclimation times and ecologically relevant  $p\text{CO}_2$  levels used in this study may suggest many of these unique species of fish have the necessary metabolic scope to compensate for the expected shift in seawater pH and  $p\text{CO}_2$  levels. However, it remains to be determined if the rapid acclamatory response

to elevated  $p\text{CO}_2$  is indicative of a return to an optimal physiological status, or a result of energetic tradeoffs associated with a continued defense of acid-base regulation that could have potential long-term impacts on growth and fecundity.

As seawater temperature and  $p\text{CO}_2$  are expected to concomitantly change, we also assessed the potential synergistic impact these combined changes will have on oxygen consumption rates. The combination of these two experimental treatments resulted in further elevation of the RMR in most individuals; yet, we found no statistically significant interaction between elevated  $p\text{CO}_2$  and temperature. This implies that these two environmental stresses may potentially be additive in their effect, however, our data suggest temperature may be the major driver of metabolic rates in these fish. For instance, *T. bernacchii* appears to quickly acclimate to increased levels of  $p\text{CO}_2$ , with fish exposed to high  $p\text{CO}_2$  seawater at ambient temperatures showing no significant difference in RMR compared to control fish within a 14-day acclimation period. Temperature on the other hand, continues to impact RMR's in these fish even after a 28-day acclimation period. The trends identified for *T. bernacchii* held true for both *T. hansonii* and *P. borchgrevinki*, suggesting acclimation to elevated SST may come at a higher energetic cost in these notothenioids. Work performed by Robinson and Davison (2008a,b) illustrated *P. borchgrevinki* is capable of acclimating to 4°C within one month; we identified a similar trend in our study with respect to temperature effects on the RMR of this species. Oxygen consumption was initially elevated in fish acclimated to 4°C followed by a steady decrease over the 28-day acclimation period. When increases in SST and  $p\text{CO}_2$  are co-varied in an ecologically relevant manner, the RMR of *P. borchgrevinki* is further elevated, and in contrast to temperature alone, *P. borchgrevinki*

continued to display significantly elevated RMR's after a 28-day acclimation period. The additive effects of elevated  $p\text{CO}_2$  and temperature appear to slow the acclamatory response in *P. borchgrevinki*, and much like *T. bernacchii*, the RMR of fish in this treatment continue to expend relatively more energy than fish acclimated under control conditions. It was hypothesized by Pörtner (2008) that elevated  $\text{CO}_2$  levels may heighten an organisms' response to thermal stress; thus, the physiological stress of adapting to increased temperature and  $p\text{CO}_2$  concurrently could explain the deviation from the previously observed metabolic compensation in *P. borchgrevinki* (Franklin et al., 2007; Robinson and Davison, 2008a,b). These data provide some of the first evidence that changes in these two seawater parameters may act synergistically to impact the performance of marine teleosts for extended durations.

Unlike the other species used in this study, *T. newnesi* continues to display elevated RMR's after acclimation to increased  $p\text{CO}_2$ , temperature, and the combined treatment of temperature and  $p\text{CO}_2$ , even after 28 days. These data suggest this species may be particularly vulnerable to changes in seawater conditions and further investigation is needed to examine if *T. newnesi* requires a longer acclimation time or if they are not capable of acclimating to these experimental conditions at all. Several key differences between *T. newnesi* and other members of the family Nototheniidae have been noted in previous comparative studies, such as differences in morphology (Balushkin, 1984) and hemoglobin components (di Prisco et al., 1991). These differences caused Balushkin and others to reconsider the evolutionary relationship of *T. newnesi* to other members of the Nototheniidae family. Recent studies, however, have placed *T. newnesi* squarely within the Trematomid genus (Sanchez et al., 2007) and this

physiological deviation in the acclimation response of *T. newnesi* from the other notothenioids tested may be more representative of the rapid radiation and high plasticity of the Trematominae.

Although both temperature and hypercapnia have both received a significant amount of attention with respect to physiological impacts on marine teleosts, the current results are among a small handful of studies that demonstrate the potential synergistic impacts these linked environmental parameters will have on marine fish. Furthermore, this study demonstrates ecologically relevant changes in seawater temperature and  $p\text{CO}_2$  can significantly impact the physiological response of the highly endemic fishes of the Southern Ocean. Our results suggest that while these animals appear to have the necessary scope to adjust to near future changes in seawater temperature and  $p\text{CO}_2$ , there is an additive energetic cost to maintaining homeostasis in a high  $\text{CO}_2$  world evident by the extended acclimation time required by *P. borchgrevinki* under a realistic multi-stressor scenario. What is unclear from these initial studies is whether or not these physiological adjustments come in the form of energetic trade-offs with respect to other cellular functions. Notably, a particularly important determinant of what form these energetic costs may take is whether or not the availability of food is also impacted. As sufficient food availability may provide a means to potentially offset the energetic costs, it will be critical to have a better understanding of the impact climate variation will have on key species within these food webs as well. These studies have highlighted the need to further understand the energetic costs of long-term acclimation to ecologically relevant environmental changes in order to predict the potential downstream impacts of chronic elevation of atmospheric  $\text{CO}_2$ .



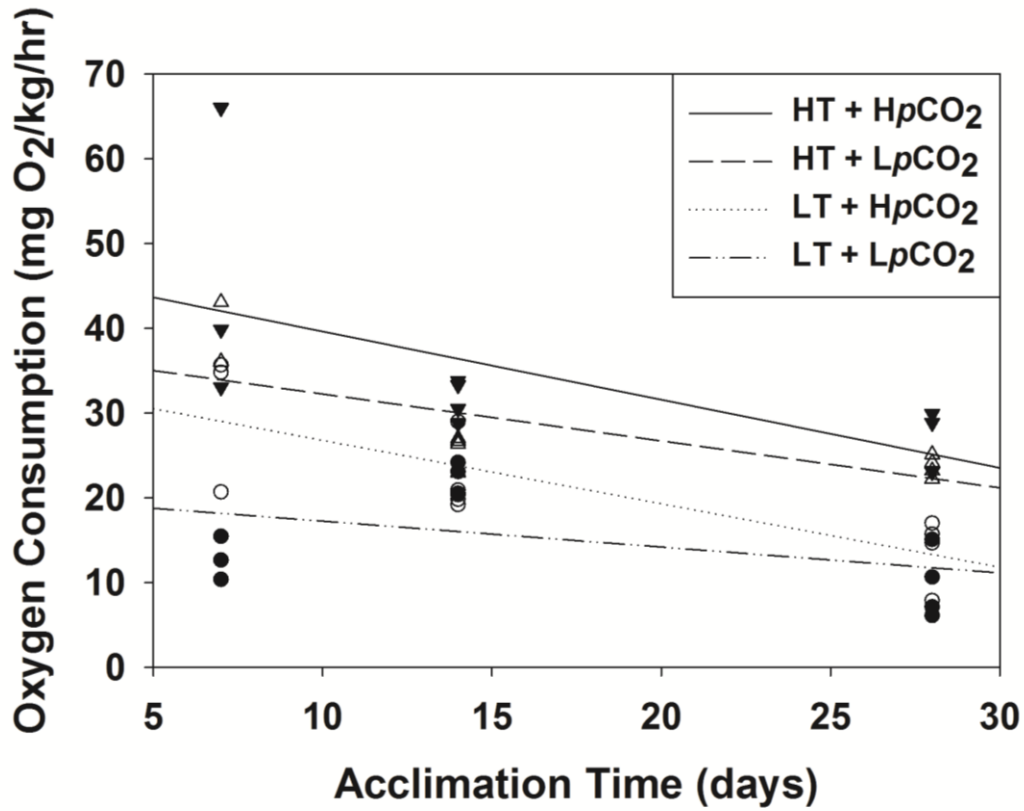


Figure 1.1. Resting Metabolic Rates (scaled to a 100-g fish, mass exponent of -0.25) for *T. bernacchii* acclimated at 7, 14 and 28 days to treatments of ambient conditions, (Low Temperature + Low  $p\text{CO}_2$ , LT + L $p\text{CO}_2$ ; indicated by closed circles), Low Temperature + High  $p\text{CO}_2$  (LT + H $p\text{CO}_2$ ; open circles), High Temperature + Low  $p\text{CO}_2$  (HT + H $p\text{CO}_2$ ; open triangles) and High Temperature + High  $p\text{CO}_2$  (HT + H $p\text{CO}_2$ ; closed triangles).

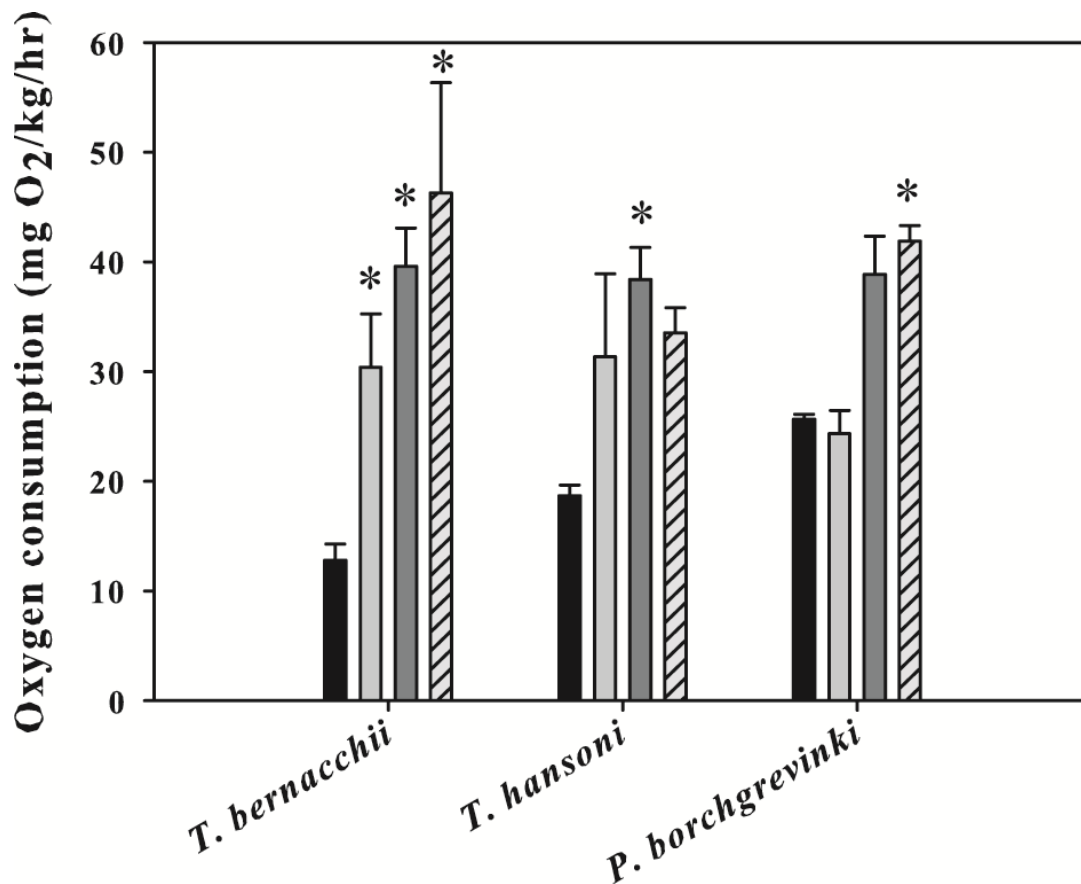


Figure 1.2. Resting Metabolic Rates ( $\pm$  SE; scaled to a 100-g fish, mass exponent of -0.25) for *T. bernacchii*, *T. hansonii*, and *P. borchgrevinki* acclimated at 7 days to treatments of ambient conditions, (Low Temperature + Low  $p\text{CO}_2$ ; black bars), Low Temperature + High  $p\text{CO}_2$  (light grey bars), High Temperature + Low  $p\text{CO}_2$  (dark grey bars) and High Temperature + High  $p\text{CO}_2$  (light grey bars with crosshatch's). Treatments found to be significantly different from control values are marked with asterisks.

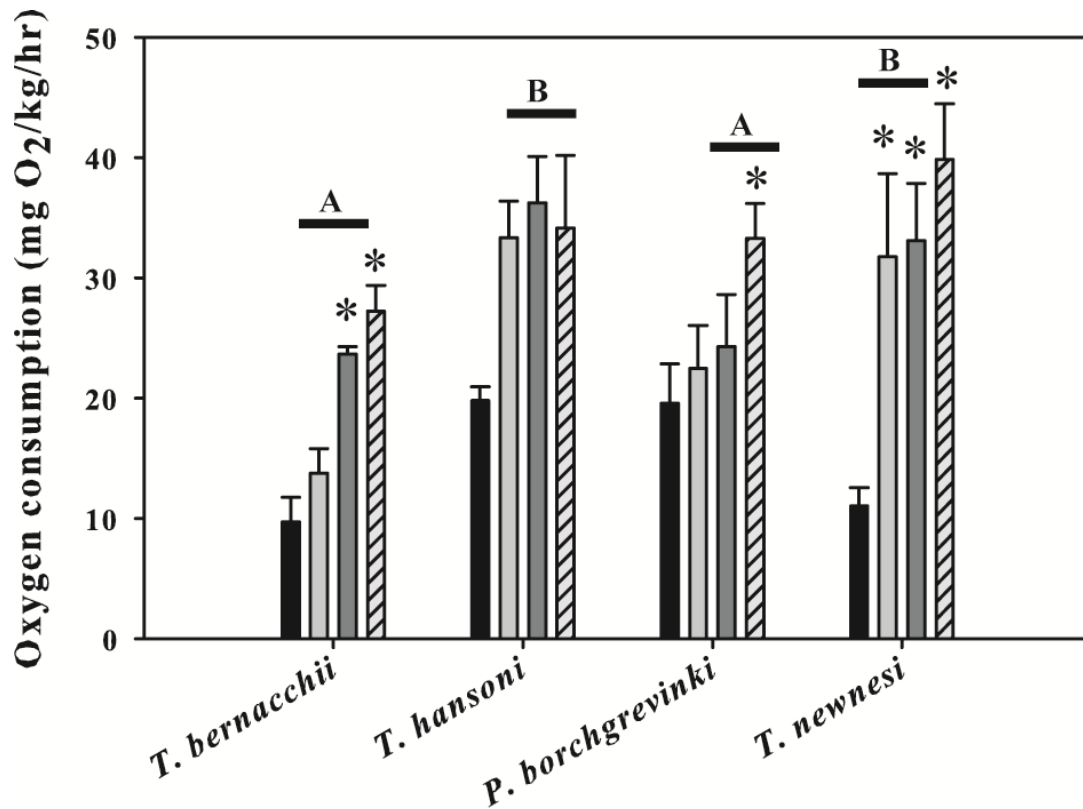


Figure 1.3. Resting Metabolic Rates ( $\pm$  SE; scaled to a 100-g fish, mass exponent of -0.25) for *T. bernacchii*, *T. hansonii*, *P. borchgrevinki*, and *T. newnesi* acclimated at 28 days to treatments of ambient conditions, (Low Temperature + Low  $p\text{CO}_2$ ; indicated by black bars), Low Temperature + High  $p\text{CO}_2$  (light grey bars), High Temperature + Low  $p\text{CO}_2$  (dark grey bars) and High Temperature + High  $p\text{CO}_2$  (light grey bars with crosshatch's). Letters indicates a significant species effect. Asterisks indicate a significant difference between the treatment and control values within a given species.

Table 1.1. Mean measurements of Total Alkalinity, pH,  $p\text{CO}_2$ , Dissolved oxygen and Temperature  $\pm$  SD over the course of the 2011 experiment

Sample	Total Alkalinity ( $\mu\text{mol/kg sol'n}$ )	pH	$p\text{CO}_2$ (ppm)	Oxygen (% air sat)	Temperature ( $^{\circ}\text{C}$ )
Incoming Seawater	2329.53 $\pm$ 9.34	8.015 $\pm$ 0.012	417.15 $\pm$ 12.26	90.926 $\pm$ 4.697	-1.24 $\pm$ 0.08
Low temperature + low $p\text{CO}_2$	2330.17 $\pm$ 8.96	7.944 $\pm$ 0.014	438.82 $\pm$ 16.08	93.80 $\pm$ 2.465	-0.61 $\pm$ 0.17
Low temperature + high $p\text{CO}_2$	2328.31 $\pm$ 11.04	7.685 $\pm$ 0.068	953.89 $\pm$ 50.38	93.635 $\pm$ 2.80	-0.45 $\pm$ 0.16
High temperature + low $p\text{CO}_2$	2342.81 $\pm$ 9.76	7.944 $\pm$ 0.015	525.11 $\pm$ 21.07	94.14 $\pm$ 2.20	4.02 $\pm$ 0.44
High temperature + high $p\text{CO}_2$	2341.87 $\pm$ 8.02	7.675 $\pm$ 0.036	1026.66 $\pm$ 9.03	94.983 $\pm$ 1.610	4.22 $\pm$ 0.56

Table 1.2. Results of 2-way ANOVA for *T. bernacchii* (acclimated at 7, 14 and 28 days), *P. borchgrevinki* (acclimated at 7 and 28-days), and *T. hansonii* (acclimated at 7 and 28 days). Values marked with an asterisk indicate a significant finding

Species	Acclimation Time	Treatment	Interaction
<i>T. bernacchii</i>	F = 17.186 df = 2 p <0.001*	F = 23.520 df = 3 p <0.001*	F = 3.188 df = 6 p = 0.015*
<i>P. borchgrevinki</i>	F = 11.153 df = 1 p = 0.004*	F = 10.088 df = 3 p <0.001*	F = 1.313 df = 3 p = 0.303
<i>T. hansonii</i>	F = 0.0177 df = 1 p = 0.8954	F = 5.188 df = 3 p = 0.007*	F = 0.0844 df = 3 p = 0.968

Table 1.3. Results of Tukey's HSD test for *T. bernacchii*, *T. hansonii*, *P. borchgrevinkii*, and *T. newnesi* acclimated at 7, 14 and 28 days. Treatment conditions of Low Temperature + High  $p\text{CO}_2$  (LT +  $\text{HpCO}_2$ ), High Temperature + Low  $p\text{CO}_2$  (HT +  $\text{LpCO}_2$ ) and High Temperature + High  $p\text{CO}_2$  (HT +  $\text{HpCO}_2$ ) are compared with control conditions of Low Temperature + Low  $p\text{CO}_2$ . Values marked with an asterisk indicate a significant finding

<i>T. bernacchii</i>	LT + $\text{HpCO}_2$	HT + $\text{LpCO}_2$	HT + $\text{HpCO}_2$
7-day acclimation	q = 5.229 p < 0.005*	q = 7.128 p < 0.001*	q = 9.969 p < 0.001*
14-day acclimation	q = 0.0627 p = 1.00	q = 1.281 p = 0.802	q = 3.287 p = 0.115
28-day acclimation	q = 1.402 p = 0.756	q = 4.794 p = 0.010*	q = 5.582 p = 0.002*

<i>T. hansonii</i>	LT + $\text{HpCO}_2$	HT + $\text{LpCO}_2$	HT + $\text{HpCO}_2$
7-day acclimation	q = 2.997 p = 0.178	q = 4.642 p = 0.017*	q = 3.501 p = 0.092
28-day acclimation	q = 2.607 p = 0.281	q = 3.167 p = 0.144	q = 2.761 p = 0.236

<i>P. borchgrevinkii</i>	LT + $\text{HpCO}_2$	HT + $\text{LpCO}_2$	HT + $\text{HpCO}_2$
7-day acclimation	q = 0.342 p = 0.995	q = 3.850 p = 0.063	q = 4.732 p = 0.018*
28-day acclimation	q = 0.900 p = 0.919	q = 1.462 p = 0.733	q = 4.541 p = 0.024*

<i>T. newnesi</i>	LT + $\text{HpCO}_2$	HT + $\text{LpCO}_2$	HT + $\text{HpCO}_2$
28-day acclimation	q = 5.079 p = 0.005*	q = 5.406 p = 0.003*	q = 7.072 p < 0.001*

Table 1.4. Results of 2-way ANOVA for fish species (*T. bernacchii*, *P. borchgrevinki* and *T. hansonii*) acclimated for 7-days and species (*T. bernacchii*, *P. borchgrevinki*, *T. hansonii* and *T. newnesi*) acclimated for 28-days. Values marked with an asterisk indicate a significant finding

Acclimation Time	Species	Treatment	Interaction
7-day acclimation	F = 0.274 df = 2 p = 0.762	F = 13.352 df = 3 p <0.001*	F = 1.322 df = 6 p = 0.282
28-day acclimation	F = 7.336 df = 3 p <0.001*	F = 13.920 df = 3 p <0.001*	F = 1.203 df = 9 p = 0.319

## CHAPTER 2

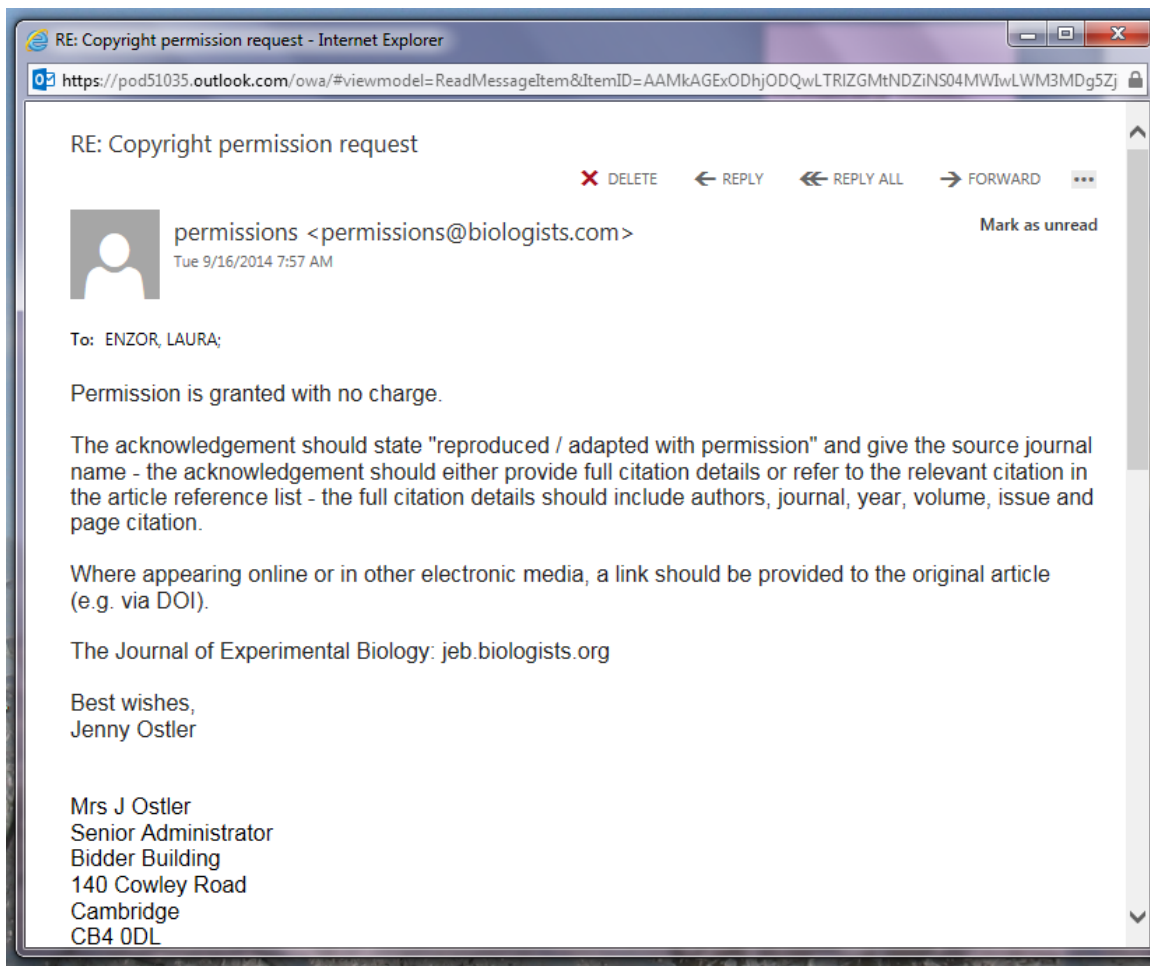
### IS WARMER BETTER? DECREASED OXIDATIVE DAMAGE IN NOTOTHENIOID FISH AFTER LONG-TERM ACCLIMATION TO MULTIPLE STRESSORS<sup>2</sup>

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<sup>2</sup> Enzor, L.A. and S.P. Place. *Journal of Experimental Biology*. 217: 3301-3310.

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## 2.1 ABSTRACT

Antarctic fish of the suborder Notothenioidei have evolved several unique adaptations to deal with subzero temperatures. However, these adaptations may come with physiological trade-offs, such as an increased susceptibility to oxidative damage. As such, the expected environmental perturbations brought on by global climate change have the potential to significantly increase the level of oxidative stress and cellular damage in these endemic fish. Previous single stressor studies of the notothenioids have shown they possess the capacity to acclimate to increased temperatures, but the cellular level effects remain largely unknown. Additionally, there is little information on the ability of Antarctic fish to respond to ecologically relevant environmental changes where multiple variables change con-comittently. We have examined the potential synergistic effects increased temperature and  $p\text{CO}_2$  have on the level of protein damage in *Trematomus bernacchii*, *Pagothenia borchgrevinki*, and *Trematomus newnesi*, and combined these measurements with changes in total enzymatic activity of catalase (CAT) and superoxide dismutase (SOD) in order to gauge tissue-specific changes in antioxidant capacity. Our findings indicate that total SOD and CAT activity levels displayed only small changes across treatments and tissues. Short-term acclimation to decreased seawater pH and increased temperature resulted in significant increases in oxidative damage. Surprisingly, despite no significant change in antioxidant capacity, cellular damage returned to near basal levels, and in *T. bernacchii*, significantly decreased, after long-term acclimation. Overall, these data suggest notothenioid fish currently maintain the antioxidant capacity necessary to offset predicted future ocean conditions, but it remains unclear if this capacity comes with physiological trade-offs.

## 2.2 INTRODUCTION

The highly endemic fishes of the suborder Notothenioidei represent the dominant perciform fish fauna and a significant portion of the biomass found in the Southern Ocean (Eastman, 1993). Contributing to their endemism, notothenioids have evolved under extreme cold conditions for several million years, and as such, several unique cellular and physiological alterations have arisen, such as the evolution of anti-freeze proteins (DeVries, 1969), the functional loss of heme proteins (O'Brien and Sidell, 2000; Sidell and O'Brien, 2006) and the constant expression of inducible heat shock proteins (Hofmann et al., 2000; Place et al., 2004). Additionally, evolution in this extreme cold environment has led to several unique metabolic adaptations including increased mitochondrial density and the utilization of lipids as a primary energy source (Lin et al., 1974; Clarke et al., 1984; Johnston et al., 1998; Pörtner et al., 2005). These adaptations have been suggested to potentially result in an increased susceptibility to reactive oxygen species (ROS; Abele and Puntarulo, 2004), which can be further exacerbated by environmental perturbations associated with global climate change, such as changes in temperature and pH (Cadenas, 1986; Lesser, 2006). If left unchecked, or allowed to form faster than they can be detoxified, ROS can cause significant damage to proteins, DNA and lipids (Abele and Puntarulo, 2004; Lesser, 2006) and may represent a significant energetic cost to basal cellular maintenance in Antarctic fish.

The effects of increased temperature on Antarctic fish have been well documented (Somero and DeVries, 1967; Forster et al., 1987; Davison et al., 1990; Pörtner, 2008; Robinson and Davison, 2008a; Robinson and Davison 2008b). At the level of the whole organism, an early study from Somero and DeVries (1967) illustrated a narrow thermal

window exists in which notothenioids can survive, however, more recent studies have shown that notothenioid fishes have retained the capacity to acclimate to increased temperatures (Seebacher et al., 2005; Franklin et al., 2007; Robinson and Davison, 2008a; Robinson and Davison, 2008b; Bilyk and DeVries, 2011; Bilyk et al., 2012). While these studies provide evidence that Antarctic fish are capable of acclimating to an increase in temperature, there is little information about the cost of acclimation on a cellular level. Of the studies that have examined the effects of temperature on oxidative damage in Antarctic fish, most have focused primarily on the hemoglobinless fish from the family Channichthyidae, with only three red-blooded notothenioids represented, *Gobionotothen gibberifrons*, *Notothenia rossii* and *Notothenia coriiceps* (Beers and Sidell, 2011; Mueller et al., 2011; Mueller et al., 2012; Machado et al., 2014). Moreover, with the exception of the study by Machado et al. which examined liver tissues of fishes acclimated up to 6-days, these studies utilized isolated mitochondria from fish exposed to their CT<sub>max</sub> for 2 hours. To-date, little information exists with respect to tissue specific levels of cellular damage in red-blooded notothenioids, especially when considering potential differences in chronic versus acute exposures.

In addition to elevated sea surface temperature (SST), a growing body of literature has focused on the impacts ocean acidification will have on marine teleosts, however, the majority of these studies have focused primarily on tropical or temperate species (Melzner et al., 2009; Munday et al., 2009a; Munday et al., 2009b; Munday et al., 2010; Munday et al., 2011; Esbaugh et al., 2012; Heuer et al., 2012; Nowicki et al., 2012; Bignami et al. 2013; Chivers et al., 2014; Munday et al., 2014; Murray et al., 2014; Pope et al., 2014). Despite projections that indicate changes in SST and *p*CO<sub>2</sub> levels will

impact higher latitudes faster and to a greater extent than temperate regions (Walther et al., 2002; Orr et al., 2005; Turner et al., 2005; McNeil and Matear, 2008; Fabry et al., 2008; Fabry et al., 2009; Halpern et al., 2008; McNeil et al., 2010; Mathis et al., 2011a; Mathis et al., 2011b), only a handful of studies have examined the effects of ecologically relevant increases in seawater  $p\text{CO}_2$  on high latitude marine teleosts (Hurst et al., 2012; Strobel et al., 2012; Enzor et al., 2013; Strobel et al., 2013a; Strobel et al., 2013b). Similar to impacts seen with elevated temperature, ocean acidification may also perturb oxidative stress in marine teleosts (Murphy, 2009; Tomanek et al., 2011) and may even exacerbate the detrimental effects of reactive oxygen species at the cellular level (Ezraty et al., 2011).

Currently, the potential synergistic effects of increased temperature and increased  $p\text{CO}_2$  in fish have only been examined in a small number of studies (Pankhurst and Munday, 2011; Nowicki et al., 2012; Strobel et al., 2012; Enzor et al., 2013; Strobel et al., 2013a; Strobel et al., 2013b; Gräns et al., 2014). We have previously shown increased temperature and  $p\text{CO}_2$  levels result in a rapid increase in resting metabolic rates in several species of Antarctic fish (Enzor et al., 2013). This rapid increase in respiration has the potential to negatively impact the organism through elevated production of ROS (Lesser, 2006). Therefore, given the known impacts of temperature and elevated  $p\text{CO}_2$  on metabolic rates in these fish, combined with the potential increased susceptibility of Antarctic fish to ROS production, we set out to test the hypothesis that Antarctic fish will experience a significant increase in oxidative stress and cellular damage caused by acclimation to a multi-stressor scenario of increased temperature and  $p\text{CO}_2$ . We first investigated the amount of oxidative damage by quantifying the level of protein

carbonyls from gill and liver tissue isolated from three notothenioid fish, *Trematomus bernacchii* (Boulenger, 1902), *T. newnesi* (Boulenger, 1902), and *Pagothenia borchgrevinki* (Boulenger, 1902) after long-term acclimation to both single and multi-stressor treatments. While little information is available on the cellular-level effects of temperature on *T. newnesi*, both *T. bernacchii* and *P. borchgrevinki* have previously shown a decreased capacity to respond to thermal stress at the cellular level (Hofmann et al., 2000, Place et al., 2004). However, it is unknown if the reduced capacity to respond to thermal insults extends to secondary stressors associated with increased temperature such as ROS production. Therefore we also assessed if acclimation resulted in an increase in key antioxidant defense mechanisms, which are ubiquitous among vertebrates (Lesser, 2006). To this end we further quantified changes in total catalase (CAT) and superoxide dismutase (SOD) enzymatic activity to discern if these species are capable of compensating for the additional oxidative stress that likely occurs when fish are acclimated to a multi-stressor scenario.

## 2.3 MATERIALS AND METHODS

### Collection of fish

*Trematomus bernacchii*, *Pagothenia borchgrevinki* and *Trematomus newnesi* were collected in McMurdo Sound, Antarctica between October and December, 2011 as well as September through December, 2012. Fish were caught using hook and line through 10-inch holes drilled through the sea ice and transported back to McMurdo Station in aerated coolers where they were housed in a flow-through aquaria maintained at ambient seawater temperature (-1.5°C). Fish were tank-acclimated under ambient

conditions for one week prior to being placed in experimental tanks. All fish were handled according to guidelines set forth by the University of South Carolina Institutional Animal Care and Use Committee (IACUC).

### Experimental Design

We used four, 1240 L experimental tanks to assess the combined effects of elevated temperature and  $p\text{CO}_2$  on *T. bernacchii*, *P. borchgrevinki* and *T. newnesi*. Our four experimental treatments consisted of a control tank which was held near ambient conditions ( $-1^\circ\text{C}$  and  $430\ \mu\text{atm}$ ), a low temperature + high  $p\text{CO}_2$  treatment ( $-1^\circ\text{C}/1000\ \mu\text{atm}$ ), a high temperature + low  $p\text{CO}_2$  treatment ( $+4^\circ\text{C}/430\ \mu\text{atm}$ ), and a high temperature + high  $p\text{CO}_2$  treatment ( $+4^\circ\text{C}/1000\ \mu\text{atm}$ ). In 2011 fish were placed in experimental tanks and acclimated for 28 days. Four fish per treatment were removed at 7d and 28d, after which fish were sacrificed and tissues were collected and immediately flash-frozen in liquid nitrogen. In 2012, the acclimation experiment was repeated and fish were acclimated for 42-56 days. Five fish per treatment were sampled as described above at 7d, 28d, and either 42d (*T. newnesi*) or 56d (*T. bernacchii* and *P. borchgrevinki*). One-way Analysis of Variance (ANOVA) showed no significant differences inter-annually between fish sampled from the same treatment, so data obtained from the 2011 and 2012 seasons were combined for all analyses. In total,  $n=9$  fish per treatment were analyzed from each species at the day 7 and day 28 time points. For *T. newnesi*,  $n=5$  fish per treatment were analyzed at the 42 day time point and for *T. bernacchii* and *P. borchgrevinki*,  $n=5$  fish per treatment were analyzed at the 56 day time point. Although the constraints of working in Antarctica prevented us from utilizing a fully replicated

tank design to control for tank effect, the treatment conditions established for each tank was alternated between the 2011 and 2012 field season.

#### Manipulation of seawater conditions

Temperature and  $p\text{CO}_2$  levels were manipulated within the experimental treatment tanks using a  $p\text{CO}_2$  generation system first described by Fanguie et al. (2010) and adapted for use with large-scale applications and combined with thermostated titanium heaters (Process Technology, Brookfield CT, USA; Enzor et al., 2013). Atmospheric air was pumped through drying columns (filled with drierite) to remove moisture, and air was scrubbed of  $\text{CO}_2$  using columns filled with Sodasorb. Pure  $\text{CO}_2$  and  $\text{CO}_2$ -free air were then blended using digital mass flow controllers and bubbled into header tanks that were continuously replenished with ambient seawater using venturri injectors, which in turn fed into experimental treatment tanks.

Temperature, pH (total scale), salinity, total alkalinity ( $T_A$ ) and oxygen saturation were measured daily from both incoming seawater as well as experimental treatment tanks. For  $p\text{CO}_2$  analysis, we followed the SOP as described in the Best Practices Guide (Riebesell et al., 2010) for the spectrophotometric determination of pH using m-cresol purple and measurement of total alkalinity via acid titration using a computer controlled T50 Titrator (Mettler Toledo, Columbus, OH, USA). Temperature was measured with a calibrated digital thermocouple (Omega Engineering Inc., Stamford, CT, USA) and salinity was measured using a YSI 3100 Conductivity meter (Yellow Springs, OH, USA).  $\text{CO}_2$  calc (Robbins et al. 2010), using the constants of Mehrbach et al. (1973) as refit by Dickson and Millero (1987), was used to calculate all other carbonate parameters. Oxygen saturation was recorded using a galvanic oxygen probe (Loligo Systems,



Denmark). Mean values ( $\pm$  SD) of temperature ( $^{\circ}\text{C}$ ) and  $p\text{CO}_2$  ( $\mu\text{atm}$ ) over the course of the experiment are reported in Table 2.1. Although inter-annual variation was apparent within the same treatments, one-way ANOVA showed no significant differences inter-annually between the same treatments. Additionally, treatment tanks were sampled daily for the presence of ammonia, nitrite and nitrates, with no significant increase in waste products noted over the course of the experiment (data not shown).

#### Measurement of Protein Carbonyls

Protein carbonyl formation was quantified using reagents and antibodies from a Cell BioLabs kit (STA-308) and a protocol described by Robinson et al. (1999). Proteins were extracted by homogenizing approximately 50 mg of gill or liver tissue in 500  $\mu\text{L}$  of SDS homogenization buffer (50 mM Tris-HCl, pH 6.8, 4% SDS, 1 mM EDTA). Briefly, protein extracts (5  $\mu\text{g}$ ) were diluted in 100  $\mu\text{L}$  of 1x Tris-Buffered Saline (TBS; 20 mM Tris base, 500 mM NaCl, pH 7.4), and immobilized directly onto a PVDF membrane using a BioRad DotBlot apparatus (Hercules, CA, USA). A single protein extract taken from a non-experimental animal was used as a protein standard in order to normalize spot density across all blots. All samples, including the protein standard, were randomly loaded in duplicate. Immediately before immunoblotting the membrane, a derivatization step was carried out whereby each membrane was incubated with a 1x dinitrophenylhydrazine (DNPH) solution for 5 min. Membranes were incubated in primary antibody (1:25,000 in antibody diluent solution [5% Non-fat Dry Milk, NFDM, in 1.0% TBST]) overnight at  $4^{\circ}\text{C}$ , and secondary antibody solution (1:5,000 in antibody diluent solution) for 1 h at room temperature. Membranes were incubated for 5 min in West Pico Super Signal and exposed on x-ray film (Thermo Scientific). DotBlot images

were scanned using a Fotodyne imaging system (Hartland WI, USA) and densitometry was performed using Image J software. The mean density of the duplicate measures for each sample was normalized across blots by dividing the mean density of each sample by that of the protein standard.

#### Enzyme activity measurements

*Super Oxide Dismutase (SOD)*- We utilized a SOD assay kit (19160-1KT-F, Sigma Aldrich) to quantify the level of total SOD activity (SOD1, SOD2 and SOD3) in gill and liver tissues of fish acclimated to the four treatments for 7, 28 or 42/56 days. A cleared protein extract was prepared by homogenizing approximately 100 mg of tissue in 100 mM phosphate buffer followed by centrifugation at 4,000 RPM for 10 min and transferring the supernatant to a fresh microcentrifuge tube. The total SOD activity in 20  $\mu$ L of the cleared protein extract was determined by measuring the conversion of Dojindo's water-soluble tetrazolium salt (WST-1) to a water-soluble formazan dye and measuring the subsequent change in absorbance at 450 nm using 96-well plate reader (Bio-Tek, Synergy HT, Winooski, VT, USA). Activities were then normalized to units of SOD per gram wet tissue weight and reported as I.U./gfw  $\pm$  SEM. One unit of SOD was defined as the amount of enzyme needed for 50% inhibition of formazan dye.

*Catalase (CAT)*- We used a spectrophotometric method to quantify catalase activity in gill and liver tissue by measuring the change in absorbance at 240 nm resulting from the disappearance of H<sub>2</sub>O<sub>2</sub> (Beers and Sizer, 1952). Total catalase activity in 5  $\mu$ L of cleared protein extract (prepared as described above), was combined with 300  $\mu$ L of H<sub>2</sub>O<sub>2</sub>-phosphate buffer solution (made by diluting 0.16 mL H<sub>2</sub>O<sub>2</sub> [30% w/v] to 100mL with a 67mM phosphate buffer solution, pH=7.0). Samples were read in a UV-transparent

96-well plate (UV-Star, Greiner Bio-One, Monroe, NC, USA) using a 96-well UV-Vis plate reader (Bio-Tek, Synergy HT, Winooski, VT, USA). Plates were scanned every 47 sec for 5 min, using the pathway correction of the plate reader. Total catalase activities were calculated using the following equation:

$$Activity = S * \left( \frac{V}{\epsilon} \right)$$

where S = the slope describing the rate of disappearance of H<sub>2</sub>O<sub>2</sub>, V = the volume of H<sub>2</sub>O<sub>2</sub> phosphate buffer added to each well, and  $\epsilon$  = the micromolar extinction coefficient of H<sub>2</sub>O<sub>2</sub> ( 0.0436 ml  $\mu$ mol<sup>-1</sup> cm<sup>-1</sup> at 240nm). Lastly, activities were normalized by gram wet tissue weight and are reported as I.U. /gfw  $\pm$  SEM.

### Statistics

A Shapiro–Wilk test indicated all data from this study exhibited a normal distribution and homogenous variance with the exception of the protein carbonyl data gathered from *T. bernacchii* (both tissues) and the gill tissues from *P. borchgrevinki*. In those instances in which the assumptions of normal distribution and variance were not met, we initially used the non-parametric Kruskal-Wallace statistical test, which indicated significance differences existed between our control fish and fish exposed to the stress treatments ( $p < 0.05$ ). As the non-parametric test allows us to draw no further insight into the interactions among treatments, and given that Analysis of Variance is considered a robust statistical test even within data sets that exhibit small deviations from normality (Lunney, 1970), we proceeded with the analyses using a three-way ANOVA. To test for significant differences between fish acclimated to specific treatments along with interactions among treatment variables, we used three-way ANOVA with time (7-days, 28-day and 42/56-days), temperature (-1 or 4°C) and  $p$ CO<sub>2</sub> treatment (400 or 1,000  $\mu$ atm)

as independent variables. For those comparisons that showed a significant interaction ( $p < 0.05$ ), we ran Holm-Sidak Multiple Comparison tests and generated interaction profiles by plotting the least square means for temperature x  $p\text{CO}_2$  at each experimental end point in order to visualize the nature of the interaction.

## 2.4 RESULTS

### Protein Carbonyl Protein Concentration

*T. bernacchii*: Overall, both elevated temperature and  $p\text{CO}_2$  had a significant effect on the level of oxidative damage to cells. Both gill and liver tissues showed main level effects of temperature,  $p\text{CO}_2$  and time ( $p < 0.001$ ; Fig. 2.1A and B). Additionally, both tissues displayed a significant 3-way interaction between time, temperature, and  $p\text{CO}_2$  ( $p < 0.001$ ). Short-term acclimation resulted in elevated protein carbonyl levels suggesting an initial increase in cell damage. Yet, over a long-term exposure, we observed protein carbonyl levels either returned to control values or significantly decreased, which may be an indicator of physiological compensation (Fig. 2.1A and B). Across acclimation groups, we observed a tissue specific shift in the effects of elevated temperature and  $p\text{CO}_2$  after 28 days of acclimation. In the gill tissue of fish sampled from the single stressor acclimation groups the level of protein carbonyl formation returned to control levels (Fig. 2.1A). In the combined treatment of elevated temperature and  $p\text{CO}_2$ , we observed a more rapid decline in the level of oxidatively damaged proteins in the gill tissue (Fig. 2.1A). By 56 days, the level of oxidative damage in gill tissue was significantly lower in all treatments compared to control levels (Fig. 2.1A). Conversely, protein carbonyl levels continued to increase in liver tissues isolated from fish in all three

stress treatments from the 28-day acclimation group (Fig. 2.1B). For fish acclimated for 56 days to the single stressor treatments, protein carbonyl levels were no longer distinguishable from control fish, however, the level of oxidative damage in liver tissues sampled from fish in the multi-stressor treatment remained slightly elevated (Fig. 2.1B).

The interaction plots generated for *T. bernacchii* gill tissue showed a significant interaction between temperature and  $p\text{CO}_2$  for each acclimation group and suggests this interaction is slightly antagonistic (Fig. 2.2A, C, E). Alternatively, only the 28 and 56-day acclimation groups displayed a significant interaction between temperature and  $p\text{CO}_2$  in the liver, and this interaction appears to shift from antagonistic at day 28 to synergistic by day 56 (Fig. 2.2 B, D, F).

*P. borchgrevinki*: Overall, protein carbonyl levels measured from gill and liver tissues from *P. borchgrevinki* showed a similar trend as tissues analyzed from *T. bernacchii*. Both gill and liver tissues from *P. borchgrevinki* showed a main-level effect of time, temperature and  $p\text{CO}_2$  (Fig. 2.1C and D;  $p < 0.001$ ) as well as a 3-way interaction between the three treatments (gill,  $p < 0.001$  and liver,  $p = 0.018$ ). Within the 7-day acclimation group we observed a marked increase in protein carbonyl formation followed by a general decline over time. Unlike the gill tissues from *T. bernacchii*, protein carbonyl levels in *P. borchgrevinki* gill tissue remained elevated above control levels after acclimation to the multi-stressor treatment for 28 days (Fig. 2.1C). By 56-days levels of oxidatively damaged proteins had returned to near control-values in all treatments (Fig. 2.1D).

While the general trends of protein carbonyl formation are similar between tissues from *T. bernacchii* and *P. borchgrevinki*, the interaction plots are markedly different.

Significant interactions between temperature and  $p\text{CO}_2$  were only observed on two occasions, the gill tissue from the 7-day acclimation group and the liver tissue for the 56-day acclimation group (Fig. 2.3 A-F). While the gill tissue interaction appears additive and the liver tissue interaction slightly antagonistic, all other interaction plots appeared additive or synergistic in nature, although these interactions are not statistically significant (Fig. 2.3 A-F).

*T. newnesi*: Both liver and gill tissues showed main level effects of time, temperature and  $p\text{CO}_2$  in *T. newnesi* ( $p < 0.001$ , Fig. 2.1). Additionally, liver tissues showed a three-way interaction between time, temperature and  $p\text{CO}_2$  ( $p = 0.002$ ). Like the other fish species examined, protein carbonyl levels in gill and liver tissues from *T. newnesi* increased within 7-days of acclimation to all stress treatments (Fig. 2.1E and F). In gill tissues there was a decline in oxidatively damaged proteins in fish acclimated to high  $p\text{CO}_2$  alone after 28-days, however in the high temperature treatments the level of oxidative damage remained elevated above control levels (Fig. 2.1E). By 42 days of acclimation to the treatments, levels of protein carbonyl formation remained slightly elevated above control values, especially in the multi-stressor treatment (Fig. 2.1E). Similar to the trends seen in the gill tissue of *T. newnesi*, liver tissues from 28-day acclimated fish showed a reduction in protein carbonyl levels in fish acclimated to the low temperature + high  $p\text{CO}_2$  treatment while levels of oxidative damage remained significantly elevated in fish from the two high temperature treatments (Fig. 2.1F). By 42-days protein carbonyl formation declined in the high temperature treatments compared to the 7-day acclimation group but overall oxidative damage remained significantly elevated above control values (Fig. 2.1F),

Unfortunately, due to logistical constraints, we were unable to obtain tissue samples from a 56-day acclimation group for *T. newnesi*. The general trends observed in fish acclimated for 42 days suggests protein carbonyl levels may continue to drop and eventually level off near control values, however physiological compensation may be slower in this species. Counter to what was observed for the other species, the interaction plots generated for *T. newnesi* at each acclimation time point suggests the relationship between temperature and  $p\text{CO}_2$  appears to be largely synergistic in nature, which may be contributing to the slower recovery observed in *T. newnesi* (Fig. 2.4 A-F).

#### Superoxide Dismutase Activity

We monitored total superoxide dismutase (SOD) enzyme activity in order to measure the effects elevated temperature and  $p\text{CO}_2$  had on the total antioxidant capacity of *T. bernacchii*, *P. borchgrevinki* and *T. newnesi*. Overall we observed only small fluctuations in the total activity of SOD across all three species with *T. bernacchii* gill and *T. newnesi* liver displaying the greatest variation across treatments (Fig. 2.5). Apart from *T. bernacchii* liver tissue, the range of enzyme activities was found to be highly similar among the three notothenioid species studied here (Fig. 2.5). Under no circumstances did we observe a significant interaction among the treatment factors (time, temperature and  $p\text{CO}_2$ ) for any of the fish species. Furthermore, for *T. bernacchii* and *P. borchgrevinki*, three-way ANOVA revealed no significant main level effects. In *T. newnesi*, there was no effect of either temperature or  $p\text{CO}_2$  in both gill and liver tissues, although SOD activity in *T. newnesi* did display a significant main effect of time in both tissues in which the 28-day acclimation groups displayed a small increase in SOD activity across all treatments ( $p=0.007$ ; Fig. 2.5).

### Catalase Activity

As a second measure of changes in antioxidant capacity in these species, we also measured catalase (CAT) activity in the gill and liver tissues of fish from each acclimation group.

The activity of catalase enzymes in *T. bernacchii* showed tissue specific differences in activity, with liver tissues displaying activity levels roughly twice that of levels found in the gill tissue, although no main effects or interactions were detected (Fig. 2.6A and B). For *P. borchgrevinki*, we found no significant effect temperature or  $p\text{CO}_2$  in either gill or liver tissue across all acclimation groups (Fig. 2.6C and D). We did observe a main effect of time in gill tissue ( $p=0.016$ ), and further analysis showed 7-day acclimated fish were different from 56-day fish in the high temperature treatments ( $p=0.015$ ; Fig. 2.6C).

Catalase activity within the gill tissues collected from *T. newnesi* showed a main effect of temperature ( $p=0.033$ ). This effect is noted when comparing the 28-day and 42-day acclimated fish, in which the catalase activity in fish acclimated to the high temperature treatments are lower when compared to the low temperature treatment levels ( $p=0.042$ ; Fig. 2.6E). Liver tissues from *T. newnesi* showed the most variability over the course of the experiment and a main effect of time was seen ( $p<0.001$ ; Fig. 2.6F). We also noted a highly tissue-specific response in *T. newnesi*, as catalase activity levels were significantly lower compared to control values after 7 days of acclimation to both single stressors as well as the multi-stressor treatment (Fig. 2.6F). Activity levels did increase from 7 to 28-days of acclimation across all three stress treatments, however catalase activity never exceed control values in fish exposed to any of the stressors (Fig. 2.6F).



## 2.5 DISCUSSION

The physiological adaptations that allowed notothenioids to flourish under extreme cold conditions may have also come with an inherent physiological cost in the form of susceptibility to oxidative stress (Abele and Puntarulo, 2004). Their increased mitochondrial densities as well as reliance upon lipid peroxidation as a sustained energy source may have pre-disposed these organisms to experience relatively high levels of oxidative damage compared to temperate fish species as mitochondria are the primary site of reactive oxygen species formation and  $\beta$ -oxidation of lipids also propagates ROS formation. If Antarctic fish have a pre-existing susceptibility to oxidative stress, then the predicted increases in oceanic temperatures coupled with ocean acidification in the polar regions could overwhelm their antioxidant capacity and significantly impact the health of notothenioid fish populations. Therefore, we attempted to assess the impacts these two potential stressors had on the antioxidant capacity of three Antarctic notothenioids, *T. bernacchii*, *P. borchgrevinki*, and *T. newnesi*. Overall, our data revealed all three species displayed non-significant changes in antioxidant capacity and long-term acclimation to the experimental conditions actually led to a decrease in cellular damage for some fish. Below we provide a brief discussion of the observed changes and how they relate to previous studies. In doing so, we propose two competing interpretations of the potential physiological relevance of these observed changes.

### *Tissue specific changes in antioxidant capacity*

The acclimation conditions used in our study did in-fact result in an initial elevation of oxidative damage across all three species, an indication that predicted changes for the Southern Ocean are capable of disrupting cellular homeostasis in these

fish over short time scales. Although temperature increases have long been known to impact oxidative stress in organisms (Lesser, 2006), ocean acidification has recently emerged as a potentially more pressing issue in the world's oceans and to date, we still have a poor understanding of the cellular level impact ecologically relevant increases in seawater  $p\text{CO}_2$  will have on cellular homeostasis. Increases in  $p\text{CO}_2$  are specifically hypothesized to exacerbate oxidative stress by directly affecting mitochondrial function (Murphy, 2009; Tomanek et al., 2011), and recent studies have indeed shown that hypercapnia can negatively affect mitochondrial capacities in Antarctic fish (Strobel et al., 2012; Strobel et al., 2013b). We found that oxidative damage was most apparent within the first 7 days of acclimation to the treatment conditions which coincides with a spike in the resting metabolic rate (RMR) of all three species under these same acclimation conditions (Enzor et al., 2013). However, after long-term acclimation, oxygen consumption rates began to decline across treatments in *T. bernacchii* and *P. borchgrevinki*, (Enzor et al., 2013; Enzor and Place, in prep), which also coincide with the observed changes in antioxidant activity and decrease in levels of protein damage reported in this study. We previously observed resting metabolic rates in *T. newnesi* acclimated to elevated temperature and  $p\text{CO}_2$  remained significantly elevated even in fish acclimated up to 42-days. These data also correlate with our current findings that levels of protein damage in the multi-stressor treatment remain significantly higher compared to control fish over the same acclimation period (Enzor et al., 2013, Enzor and Place, in prep). These data are strong indicators that the increased level of damaged protein is closely tied to metabolic production of ROS and that metabolic capacity will likely play a significant role in long-term adaptation to these predicted changes.

The observed lack of significant changes in activity of the two antioxidant enzymes examined in these fish illustrate similar trends when compared to previous studies and may indicate notothenioid fish maintain a sufficient level of antioxidant defense to compensate for the predicted climate change scenarios for the Southern Ocean. Similar to the findings in our study, Mueller and colleagues reported SOD and CAT activity in the red-blooded notothenioid, *Notothenia coriiceps*, appeared relatively temperature insensitive (Mueller et al., 2012). Machado and colleagues also determined that exposure to elevated temperature in *N. coriiceps* and *N. rossii* did not elicit any changes in SOD or CAT activities (Machado et al., 2014). Furthermore, similar thermal responses have also been observed in non-notothenioid species with respect to SOD and CAT activity, with heart and liver tissues from cold-acclimated *Fundulus heteroclitus* and *Lepomis macrochirus* showing no increase in antioxidant activity levels (Grim et al., 2010).

#### Temporal changes in the oxidative damage response

By assessing the cellular response over a relatively long-term acclimation period, our data illustrate temporally different physiological responses within notothenioid fish. When the short-term acclimation responses are considered (~7 d), the lack of changes in SOD and CAT activity would suggest the cellular stress response was insufficient to offset ROS production in these fish as we found a significant increase in protein damage after just 7 days of acclimation to all treatments. However, our data show that after an initial increase in protein carbonyl formation, levels of protein damage in all treatment groups returned close to basal levels or significantly decreased after 56 days of acclimation in *T. bernacchii* and *P. borchgrevinkii*. These long-term acclimations also

highlight physiological differences among these closely related species as this study and previous measurements of RMR suggest *T. newnesi* in particular, may require a significantly longer acclimation period to physiologically compensate for environmental perturbations (Enzor et al. 2013, Enzor and Place, in prep).

In addition to providing insight into the basic cellular response of these endemic fish to global climate change, perhaps more importantly, these data highlight the importance of considering multiple stressors over longer time scales. Recent studies by DeVries and colleagues suggest high-latitude fish have retained a level of thermal plasticity that is on-par with temperate species despite evolution under a constant cold environment, however the physiological response of the cold-adapted species were relatively slow to develop and would likely have gone undetected with short term physiological measures (Bilyk et al., 2012; Bilyk and DeVries, 2011). Similarly, the cellular response we observed in these notothenioid species appears to be the result of long-term physiological adjustments as significant decreases in protein damage could not be detected before two months of acclimation in most cases. Furthermore, when the long-term acclimation responses are considered, the interpretation of the SOD and CAT activity shifts from potentially insufficient, to a view that a robust response may not be necessary to alleviate the potential impacts global climate change may have on the physiological production of ROS in these fish. Taken together, these data indicate functional changes at the cellular level may be absent, or at least too small to detect during acute exposures and are suggestive that studies considering short-term cellular responses may be underestimating the adaptive capability of these stenothermal species.

Lastly, these long-term acclimations give us important insight into the tissue level differences in susceptibility to ROS production. We found protein carbonyl formation in liver tissues remained elevated above control fish for a much longer period of time. Similar tissue specific responses have been reported for other tissues that differ significantly in their functional capacity (Mueller et al., 2012) and it is likely the temporal differences reported here are grounded in the functional importance of each tissue, as liver tissue is characterized by high metabolic activity and is a primary site for lipid peroxidation (Pörtner et al., 2005). While these studies give important insight into the likely drivers of tissue level responses, they come up short when attempting to draw conclusions with respect to organism level impacts. For instance, when considering only the short-term response of these notothenioids, we see tissue specific responses that are anti-correlated and would make extrapolation to a broader understanding of the whole organism response impossible. Alternatively, given the long-term considerations of these tissue specific responses, we see that although levels of protein damage in the liver remained elevated even after 28 days across species, these levels also begin to drop and most returned to the basal levels seen in control animals. Thus, the extended measurements allow us to extend these cellular level data to the whole organism and generate the prediction that given enough time, the organism as a whole is fully capable of offsetting the effects of the stressors.

#### *Predicting winners and losers in global climate change scenarios*

In general, we found acclimation to multiple stressors had no significant long-term negative effects on the accumulation of cellular damage in these fish. Furthermore,

*T. bernacchii* in particular showed a substantial decrease in oxidative damage as measured by protein carbonyl formation. We argue these findings can be interpreted in one of two ways. First, as our measurements of both SOD and CAT activity demonstrate a little to no increase in antioxidant capacity over time, it seems these fish already maintain sufficient anti-oxidant capacity to offset the oxidative stress that cellular perturbations associated with global climate change are expected to produce. Furthermore, given the significant decrease in protein damage seen in *T. bernacchii*, it seems some Antarctic species may benefit at the cellular level from small increases in temperature in the sense that protein turnover may decrease over the long run and thus energy expenditure may be reduced after the initial cellular restructuring period.

The initial spike in oxidative damage observed in this study along with the increased metabolic rates previously observed within the first week of acclimation (Robinson and Davison, 2008a; Robinson and Davison, 2008b; Strobel et al., 2012; Enzor et al., 2013) may signal a surge in protein synthesis and turnover as the cellular environment is restructured. The slow decline of metabolic rates seen in previous studies, connected with the precipitous drop-off in damaged proteins seen in this study, may in-turn signal the initial energy expenditure has led to a more stable cellular environment. We now have data to support the idea that protein homeostasis in these fish may in-fact become more stable at elevated temperatures. Our recent work has shown that the decrease in metabolic activity and protein carbonyl formation seen in *T. bernacchii* and *P. borchgrevinki* correlates with a 3-fold reduction in the expression Hsc71, a molecular chaperone that plays an important role in maintaining protein homeostasis (Lindquist, 1986; Feder and Hofmann, 1999). This would suggest there is a significant reduction in

the need for protein folding capacity in the cells of these fish at elevated temperature and hence, a decrease in demand for ATP production (Huth and Place, 2013). In addition to changes seen at the cellular-level, there is some evidence at the level of the whole organism that performance in these cold-adapted notothenioids may be optimal at elevated temperatures. Davison and colleagues have described similar effects of temperature on the RMR of *Pagothenia borchgrevinki* and also found elevated temperature increases metabolic scope in these cold-adapted fish, with a maximal factorial scope occurring at +3°C (Seebacher et al. 2005; Lowe and Davison, 2006; Franklin et al. 2007; Robinson and Davison, 2008a; Robinson and Davison, 2008b). Thus, in the context of balancing energy expenditures and protecting metabolic scope, at least some Antarctic fish may perform better under future Southern Ocean conditions than previously predicted.

On the other hand, an alternative interpretation of these data could reflect an entirely different scenario and the potential implications for climate change impacts on notothenioid fishes. As oxidative damage is directly related to changes in metabolic rate, the drop-off in damaged protein could be related to an overall decrease in ROS production as a secondary consequence resulting from energetic trade-offs necessary for acclimation to the experimental conditions. A growing body of literature has previously documented that increases in temperature and  $p\text{CO}_2$  levels can negatively impact energetically costly processes such as protein synthesis in fish (Langenbuch and Pörtner, 2003; Abele and Puntarulo, 2004; Lesser, 2006; Vinagre et al., 2012). Growth rates in some fish appear to decrease under ocean acidification conditions (Foss et al., 2003; Ishimatsu et al., 2008), and these changes in growth rates may result as a consequence of

energetic trade-offs associated with a notable increase in acid-base regulation in high  $p\text{CO}_2$  environments (Esbaugh et al., 2012). Furthermore, even when fish display a capacity to compensate for ocean acidification at the cellular level, the combination of multiple stressors may eventually overwhelm the organism. For instance, Reid et al. (1997) discovered that juvenile rainbow trout, *Oncorhynchus mykiss*, displayed compensation at the cellular level for small amounts of environmental acidification. However, when faced with increases in temperature and  $p\text{CO}_2$  simultaneously, this cellular level compensation came at the expense of growth potential in these fish as rates of protein synthesis and turnover were dramatically reduced (Reid et al., 1997). In a study assessing the impact of ecologically relevant increases in temperature and  $p\text{CO}_2$  on reef fish, Munday and colleagues report these two environmental parameters act synergistically to decrease the aerobic scope of tropical fish (Munday et al., 2009a). Furthermore, recent studies on the Antarctic fish *N. rossii* have shown that although these fish have a limited capacity to respond to ocean warming and ocean acidification, their response is highlighted by uncompensated mitochondrial enzyme activities that result in increased energy demands met largely by mobilizing energy stores in the liver; lending additional information to the supposition that ocean acidification may lead to long-term energetic impacts in notothenioid fishes (Strobel et al., 2013a; Strobel et al., 2013b). Together, these studies further suggest that physiological compensations identified at the cellular level could have significant impacts on performance at the level of the whole animal. Thus it is possible the decrease in RMR previously reported in these fish are achieved through energetic trade-offs aimed at reducing the overall cellular



oxygen demand while meeting the elevated costs of basal maintenance under these conditions.

When interpreted under this latter scenario, our data would suggest that although physiologically capable of compensating for the environmental changes at the cellular level, these fish might still be vulnerable at the level of the whole organism as physiological trade-offs may have significant impacts on long-term growth and reproduction. As the exact interpretation of our findings cannot currently be discerned, further investigation is necessary to determine if these fish experience energetic trade-offs as a result of the increased challenges to cellular homeostasis generally associated with global climate change.

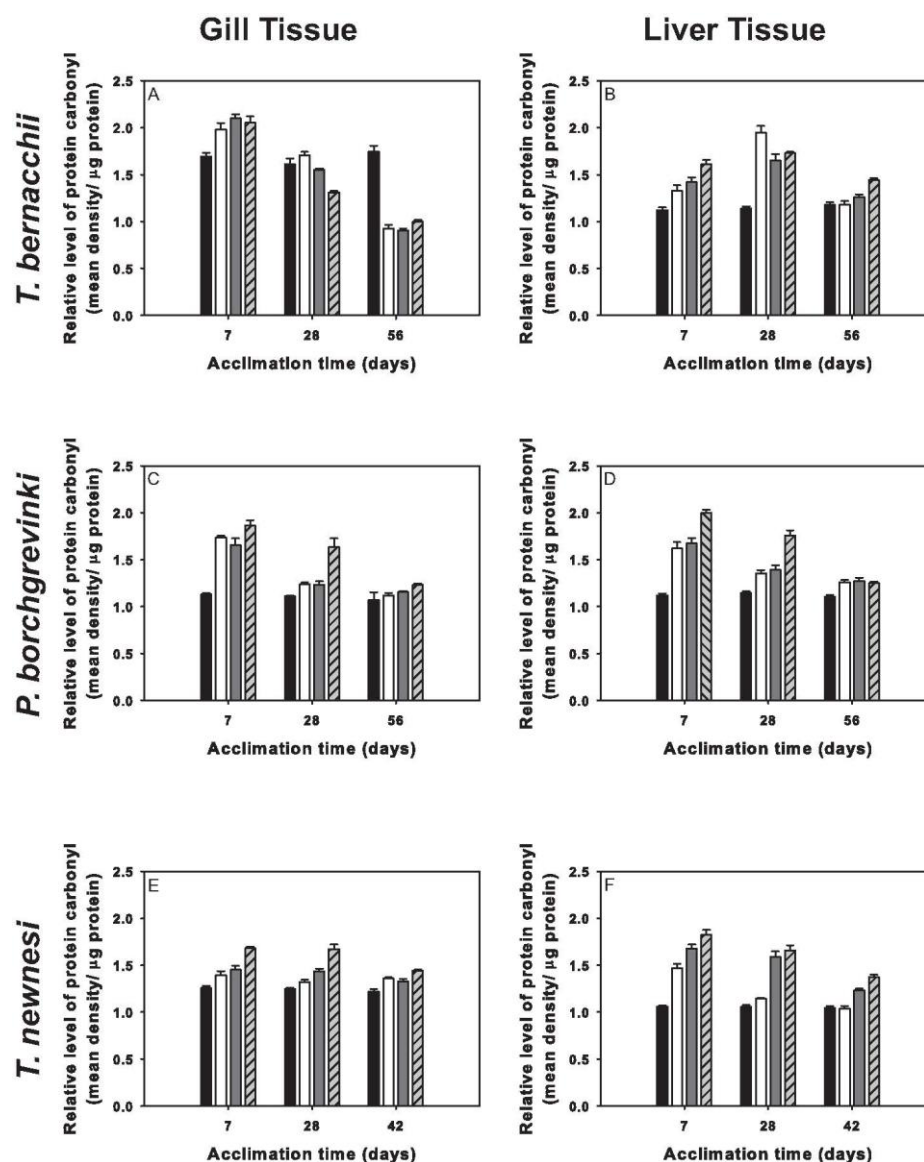


Figure 2.1. Protein Carbonyl Concentration ( $\pm\text{SE}$ ) of *T. bernacchii* gill and liver tissues (A and B), *P. borchgrevinki* gill and liver tissues (C and D) and *T. newnesi* gill and liver tissues (E and F) acclimated at 7, 28 and 42/56 days to treatments of ambient conditions, (Low Temperature + Low  $p\text{CO}_2$ ; black bars), Low Temperature + High  $p\text{CO}_2$  (white bars), High Temperature + Low  $p\text{CO}_2$  (dark grey bars) and High Temperature + High  $p\text{CO}_2$  (light grey bars with crosshatch's)

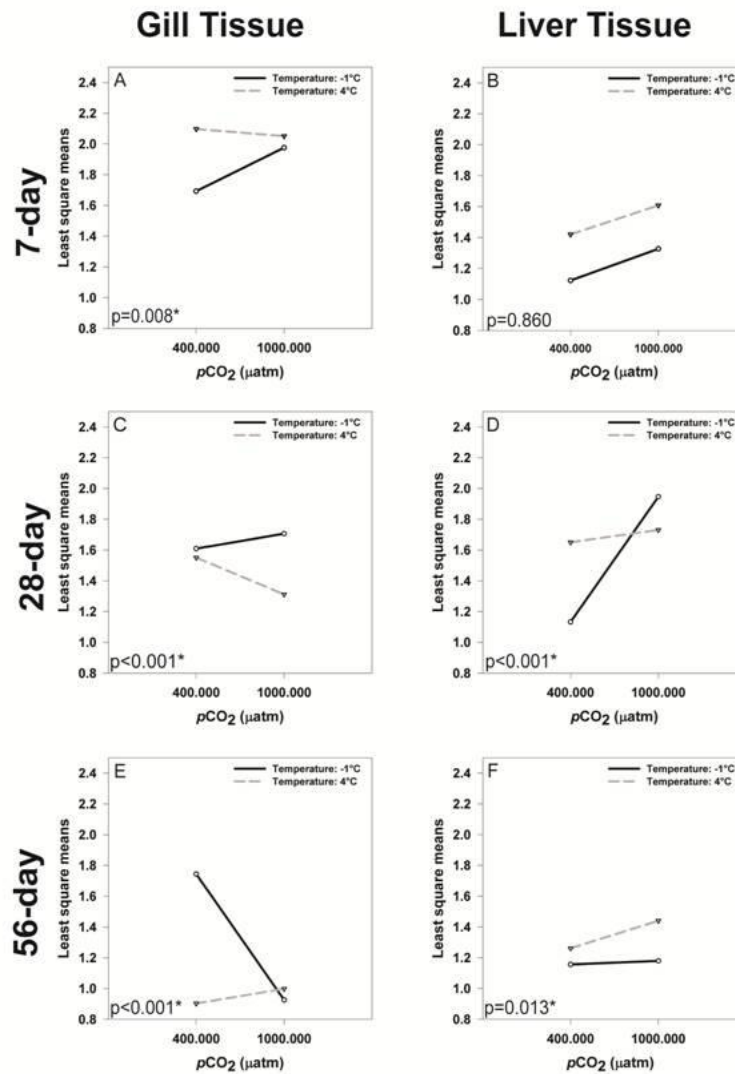


Figure 2.2. Protein Carbonyl interaction plots of *T. bernacchii* gill and liver tissues acclimated for 7-days (gill: A, liver: B), 28-days (gill: C, liver: D), and 56-days (gill: E, liver: F)

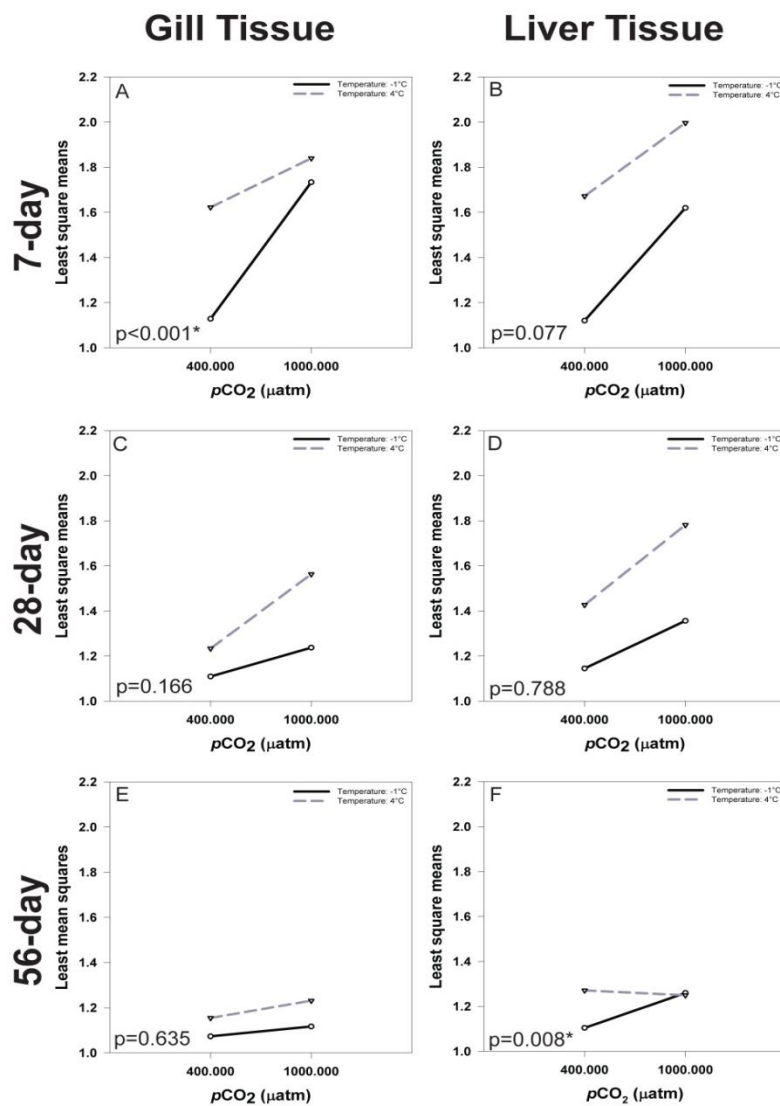


Figure 2.3. Protein Carbonyl interaction plots of *P. borchgrevinki* gill and liver tissues acclimated for 7-days (gill: A, liver: B), 28-days (gill: C, liver: D), and 56-days (gill: E, liver: F)

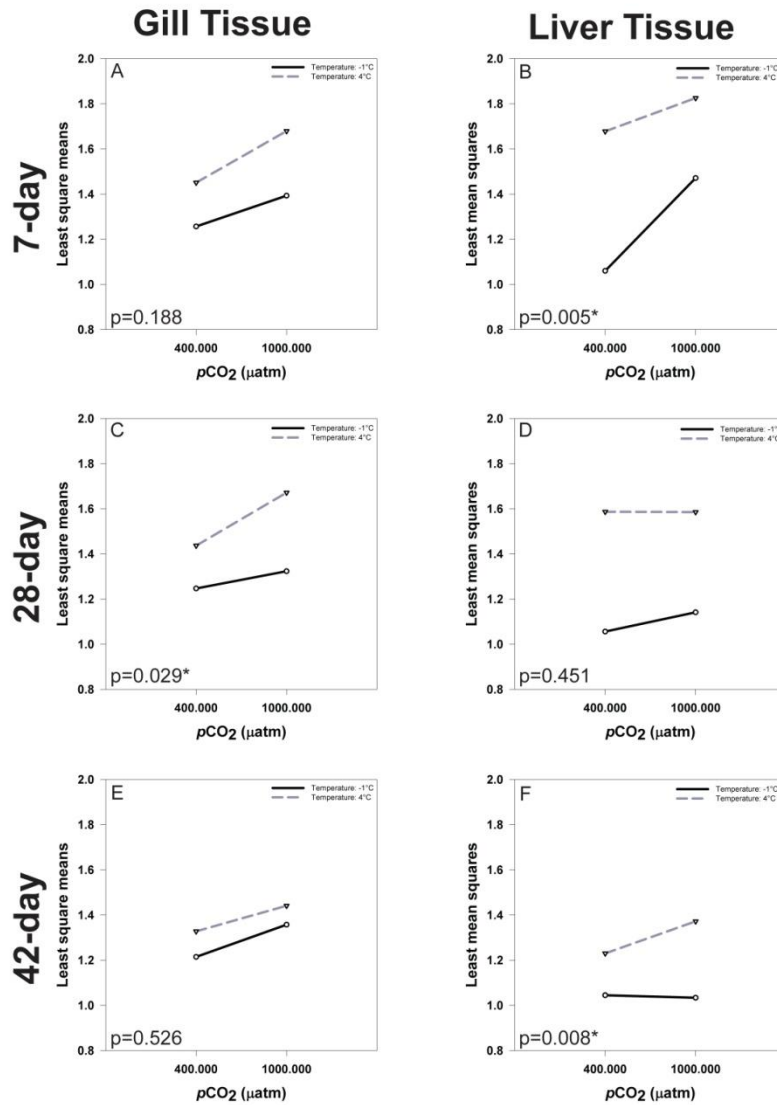


Figure 2.4. Protein Carbonyl interaction plots of *T. newnesi* gill and liver tissues acclimated for 7-days (gill: A, liver: B), 28-days (gill: C, liver: D), and 42-days (gill: E, liver: F)

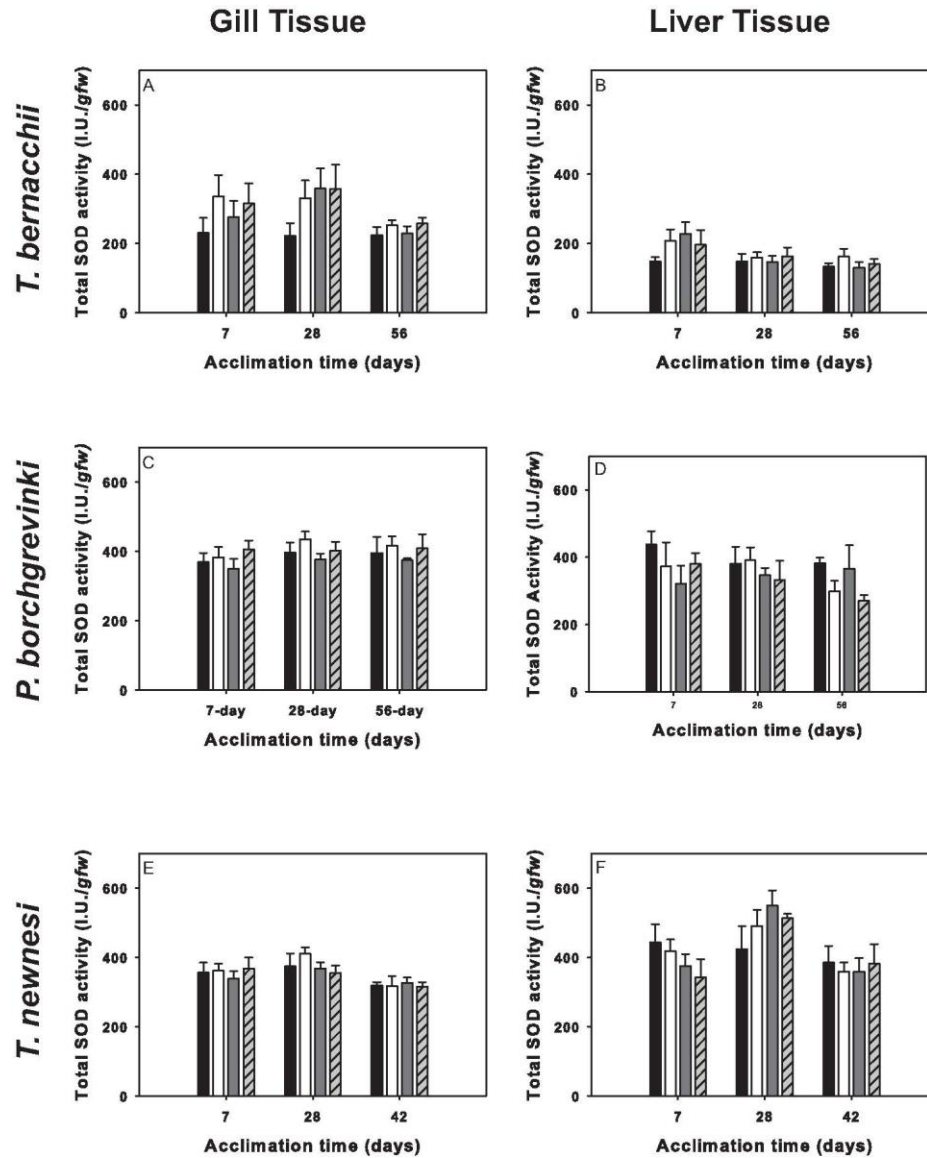


Figure 2.5. Superoxide Dismutase Enzyme Activity ( $\pm\text{SE}$ ) of *T. bernacchii* gill and liver tissues (A and B), *P. borchgrevinki* gill and liver tissues (C and D) and *T. newnesi* gill and liver tissues (E and F) acclimated at 7, 28 and 42/56 days to treatments of ambient conditions, (Low Temperature + Low  $p\text{CO}_2$ ; black bars), Low Temperature + High  $p\text{CO}_2$  (white bars), High Temperature + Low  $p\text{CO}_2$  (dark grey bars) and High Temperature + High  $p\text{CO}_2$  (light grey bars with crosshatch's)

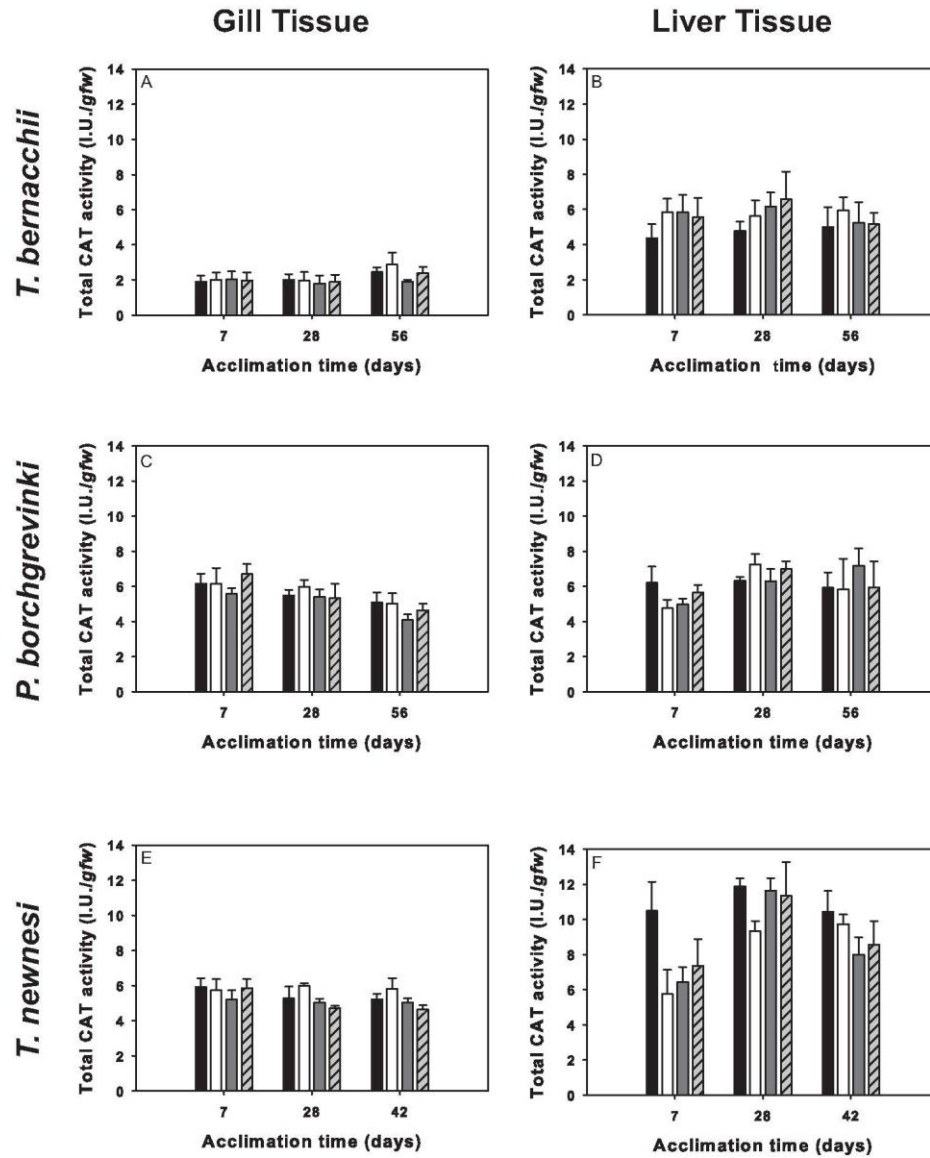


Figure 2.6. Catalase Enzyme Activity ( $\pm$ SE) of *T. bernacchii* gill and liver tissues (A and B), *P. borchgrevinki* gill and liver tissues (C and D) and *T. newnesi* gill and liver tissues (E and F) acclimated at 7, 28 and 42/56 days to treatments of ambient conditions, (Low Temperature + Low  $p\text{CO}_2$ ; black bars), Low Temperature + High  $p\text{CO}_2$  (white bars), High Temperature + Low  $p\text{CO}_2$  (dark grey bars) and High Temperature + High  $p\text{CO}_2$  (light grey bars with crosshatch's)

Table 2.1. Mean measurements of  $p\text{CO}_2$  and Temperature  $\pm$  SD over the course of the 2011 and 2012 field seasons

2011 Season	$p\text{CO}_2$ ( $\mu\text{atm}$ )	Temperature ( $^{\circ}\text{C}$ )	2012 Season	$p\text{CO}_2$ ( $\mu\text{atm}$ )	Temperature ( $^{\circ}\text{C}$ )
Incoming Seawater	$417.15 \pm 12.26$	$-1.24 \pm 0.08$	Incoming Seawater	$427.66 \pm 23.97$	$-1.03 \pm 0.152$
Low Temperature + Low $p\text{CO}_2$	$438.82 \pm 16.08$	$-0.61 \pm 0.17$	Low Temperature + Low $p\text{CO}_2$	$432.04 \pm 22.50$	$-0.707 \pm 0.153$
Low Temperature + High $p\text{CO}_2$	$953.89 \pm 50.38$	$-0.45 \pm 0.16$	Low Temperature + High $p\text{CO}_2$	$1024.76 \pm 94.20$	$-0.578 \pm 0.150$
High Temperature + Low $p\text{CO}_2$	$525.11 \pm 21.07$	$4.02 \pm 0.44$	High Temperature + Low $p\text{CO}_2$	$525.16 \pm 22.41$	$3.86 \pm 0.484$
High Temperature + High $p\text{CO}_2$	$1026.66 \pm 9.03$	$4.22 \pm 0.56$	High Temperature + High $p\text{CO}_2$	$1053.44 \pm 71.87$	$4.03 \pm 0.316$



## CHAPTER 3

# INTERACTIVE EFFECTS OF OCEAN ACIDIFICATION AND ELEVATED TEMPERATURE DIFFERENTIALLY IMPACT ACID-BASE BALANCE IN ANTARCTIC FISH<sup>3</sup>

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<sup>3</sup> Enzor, L.A. and S.P. Place. Submitted to, *Comparative Biochemistry and Physiology, Part A*, 9/18/2014

### 3.1 ABSTRACT

Numerous studies have identified a host of physiological processes that display interspecific variation in their sensitivity to ocean acidification and increased sea surface temperatures. However, we still have a poor understanding of the interactive effects of climate change and how those interactions may alter our understanding of physiological responses. Concurrent changes in sea surface temperature and pH are likely to generate significant challenges to marine organisms and this is especially true for polar oceans. Antarctic fish occupy a unique position in stress physiology and have been considered potential indicator species for rapid changes in the Southern Ocean. Recent studies suggest these fish have retained some capacity to acclimate to future ocean conditions and may even benefit at some level from slight temperature increases. Here we report on the capacity of Antarctic fish to maintain cellular homeostasis after long-term acclimation to simultaneous increases in seawater temperature and  $p\text{CO}_2$ . Our results indicated some species are able to robustly defend acid-base balance under these conditions; however, this response was not ubiquitous among notothenioids. Furthermore, when combined with recent studies measuring metabolic changes and cellular damage, our data point to potential energetic-tradeoffs in these fish when acclimated to simultaneous changes in temperature and pH.

### 3.2 INTRODUCTION

Opening of the Drake Passage approximately 25 million years ago created an extremely cold, stable environment in which the dominant fish fauna of the Southern Ocean, the notothenioids, have evolved (Eastman, 1993). The evolutionary pressures exerted by this extreme cold environment have led to several distinctive adaptations in these fish (DeVries, 1969; Lin et al., 1974; Clarke et al., 1984; Hofmann et al., 2000; O'Brien and Sidell, 2000; Place et al., 2004). Among these cellular-level adaptations is the unique physiological response of these fish when confronted with the challenge of osmoregulation. Antarctic fishes have serum osmolalities that are nearly double that of temperate and tropical marine teleosts (O'Grady and DeVries, 1982; Gonzalez-Cabrera et al., 1995; Guynn et al., 2002; Brauer et al., 2005). This hyperosmolality is attributed to an increase in NaCl concentration within the blood serum, which in turn decreases the osmotic gradient between the extracellular fluid (approximately 600 mosmol/kg) and the seawater in which they live (approximately 1000 mosmol/kg; Gonzalez-Cabrera et al., 1995; Brauer et al., 2005). Increasing serum osmolality is believed to provide two benefits to notothenioid fishes. First and foremost, it lowers the freezing point of fluids, providing freeze-resistance to fish (Somero and DeVries, 1967; O'Grady and DeVries, 1982). Second, as a higher serum osmolality decreases the ionic gradient between blood and seawater, less energy is needed to maintain the osmotic gradient, providing a potential energy-saving adaptation (Prosser et al., 1970).

In teleost fish, ionic regulation and acid-base balance are invariably linked; however, unlike terrestrial organisms, which can alter plasma pH by increasing or decreasing ventilation rates, acid-base balance in fish requires direct exchange of ions

with the external environment via specialized chloride cells within the gill epithelium (Karnacky, 1986). As such, when confronted with an acid-base imbalance, fish cannot “off-gas” CO<sub>2</sub> by increasing ventilation rates. The compensation for imbalance requires the direct transfer of an acid (H<sup>+</sup>) and base (HCO<sub>3</sub><sup>-</sup>) for Na<sup>+</sup> and Cl<sup>-</sup> respectively, across the gills, kidneys and/or intestine (Claiborne et al., 2002; Gilmour and Perry, 2009). This transfer is achieved primarily through the use of Na<sup>+</sup>/K<sup>+</sup> ATPase pumps found in the chloride cell (Claiborne et al., 2002). The chloride cells and Na<sup>+</sup>/K<sup>+</sup> ATPase pumps work in concert with carbonic anhydrase, a ubiquitous enzyme found in vertebrates, which catalyzes the hydrolysis reaction of CO<sub>2</sub>, enhancing the exchange of Na<sup>+</sup> and Cl<sup>-</sup> for H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> (Gilmour and Perry, 2009; Perry and Gilmour, 2006).

A handful of studies have examined how increases in temperature could affect the osmoregulation of various members of the notothenioids (Gonzalez-Cabrera et al., 1995; Guynn et al., 2002; Brauer et al., 2005). The role of ocean acidification in osmoregulation and acid-base balance in teleost fish has also been heavily investigated as of late, (Melzer et al., 2009; Munday et al., 2009a,b; Munday et al., 2010; Munday et al., 2011; Esbaugh et al., 2012; Heuer et al., 2012; Nowicki et al., 2012; Bignami et al., 2013; Chivers et al., 2014; Munday et al., 2014; Pope et al., 2014), however no studies have examined how these changes will specifically affect Antarctic fish. Overall, the number of studies which examine how the interacting effects of ecologically relevant changes in temperature and *p*CO<sub>2</sub> affect an organisms’ physiology are small in number (Reid et al., 1997; Munday et al., 2009a; Nowicki et al., 2012; Hurst et al., 2012), and even fewer have focused on notothenioid species (Strobel et al., 2012; Enzor et al., 2013; Strobel et al., 2013a, b).

Currently, there are no studies that examine how the combined effect of temperature and  $p\text{CO}_2$  could affect the osmoregulatory abilities and acid-base balance of Antarctic fish.

To this end, we set out to determine how predicted increases in temperature and  $p\text{CO}_2$  affect the osmoregulation and acid-base balance of three species of Antarctic fish, *Trematomus bernacchii* (Boulenger, 1902), *Trematomus newnesi* (Boulenger, 1902), and *Pagothenia borchgrevinki* (Boulenger, 1902). We analyzed changes in the osmoregulatory capacity of these fish by quantifying the activity and concentration of the primary pump involved in maintaining acid base balance,  $\text{Na}^+/\text{K}^+$  ATPase (NKA). To characterize a second measure of acid-base regulatory mechanisms in the gill tissue, as well as investigate if treatments resulted in an increase of  $\text{Na}^+$  and  $\text{Cl}^-$  excretion rate, we measured the changes in protein levels for the enzyme carbonic anhydrase II (CAII).

### 3.3 MATERIALS AND METHODS

#### Fish Collection and Experimental Design

*Trematomus bernacchii*, *Trematomus newnesi*, and *Pagothenia borchgrevinki* were collected from McMurdo Sound, Antarctica from October through December in 2011 and September through December in 2012. Fish were caught using hook and line through 10-inch holes drilled in the sea ice and transported back to McMurdo Station in aerated coolers. Fish were acclimated for one week in a flow-through aquarium in ambient seawater temperatures ( $-1.5^\circ\text{C}$ ), after which they were placed in experimental treatment tanks.

We used four experimental tanks (1240 L each) in order to assess the combined effects of temperature and  $p\text{CO}_2$  on *T. bernacchii*, *T. newnesi*, and *P. borchgrevinki*. Our

four treatments consisted of an experimental tank held at near ambient conditions (control; -1°C and 430µatm), a low temperature + high  $p\text{CO}_2$  treatment (-1°C and 1000µatm), a high temperature + low  $p\text{CO}_2$  treatment (4°C and 430µatm), and a high temperature + high  $p\text{CO}_2$  treatment (4°C and 1000µatm). Fish were placed in experimental treatment tanks for a period of 7 days (n=9 per treatment), 28 days (n=9 per treatment), 42 days (n=5 per treatment, *T. newnesi* only) or 56 days (n=5 per treatment, *T. bernacchii* and *P. borchgrevinki* only). Once the experimental endpoint was reached, fish were sacrificed and tissues were collected and flash-frozen in liquid nitrogen. Tissues were transported on dry-ice back to our home institution where they were housed at -80°C until analyzed.

#### Seawater manipulation

We used thermostated titanium heaters combined with an experimental  $p\text{CO}_2$  manipulation system first described by Fangue et al. (2010; adapted for large-scale use) to create our four experimental treatments. Briefly, atmospheric air was pumped through columns filled with drierite to remove moisture, and then columns filled with Sodasorb to scrub air of  $\text{CO}_2$ . Atmospheric air was then blended with  $\text{CO}_2$ -free air using digital mass-flow controllers and bubbled into flow-through header tanks, which in turn supplied experimental tanks with  $\text{CO}_2$ -infused water via Venturri injectors. Daily measurements of salinity, temperature, total alkalinity and pH were taken from both experimental tanks and incoming seawater (mean values  $\pm$ SD are reported in Table 3.1). Additionally, experimental tanks were sampled daily for the presence of ammonia, nitrites and nitrates (no discernible levels detected, data not shown)

#### Enzyme activity measurements; $\text{Na}^+/\text{K}^+$ ATPase

Approximately 30 mg of gill tissue was homogenized on ice in an imidazole buffer (50 mM Imidazole HCl, pH=7.3, 20mM  $\text{Na}_2\text{EDTA}$ , 300mM sucrose, 0.2mM phenylmethylsulfonylfluoride, and 5mM beta mercaptoethanol). Briefly, samples were run in duplicate either uninhibited, or inhibited with an 11.25mM ouabain solution. Enzyme activity was measured at 4°C (maintained by temperature-controlled cells connected to a circulating water bath which contained a 50% glycol solution), at 340nm, over a period of 12 minutes. A reference cuvette was placed in the spectrophotometer (Shimadzu 1800 UV/Vis) with approximately 2.0mL of assay buffer (148mM NaCl, 23.7mM KCl, 7.74mM  $\text{MgCl}_2$ , 35.5mM imidazole HCl, 0.59mM EGTA, and 0.47mM KCN, pH=7.25) to measure background rate. Sample cuvettes contained 25uL of crude homogenate combined with 2.0mL of NKA cocktail (19 parts assay buffer, combined with one part substrate stock solution; [22.5mM  $\text{Na}_2\text{ATP}$ , 6.75mM  $\text{Na}_2\text{NADH}$ , 45mM phosphoenolpyruvate], 100uL pyruvate kinase enzyme [10mg/mL stock, Roche Diagnostics] and 100uL lactate dehydrogenase enzyme [10mg/2mL stock, Roche Diagnostics]). Ouabain solution (200uL) was added to the inhibited samples, and 200uL of milli Q water was added to the non-inhibited samples. The slopes of the inhibited sample rates were subtracted from the slopes of the non-inhibited rates to determine the NKA activity rate. The calculated activity was reported as IU per gram fresh tissue weight (gfw).

#### Western Blotting

The specificity of the primary antibodies for  $\text{Na}^+/\text{K}^+$  ATPase (NKA; monoclonal  $\alpha 5$  developed by Dr. Douglas Fambrough, Developmental Studies Hybridoma Bank,

University of Iowa) and carbonic anhydrase II (CAII; sc-25596, Santa Cruz Biotechnology) were confirmed by SDS-PAGE of 10 µg of gill protein extract followed by Western blot analysis to ensure a single band of appropriate size (NKA:~100 kDa and CAII: ~29kDa ) was detected.

NKA: Proteins from gill tissue were extracted by homogenizing approximately 20mg tissue in 250µL of imidazole buffer (described above). For immunoblotting, protein extracts (5µg) were diluted in 100µL of 1x phosphate buffered saline (PBS; 8mM NaH<sub>2</sub>PO<sub>4</sub>, 2.7mM KCl, 137mM NaCl, 1.5mM KH<sub>2</sub>PO<sub>4</sub>) and immobilized directly onto a hydrated nitrocellulose (NC) membrane using a BioRad DotBlot apparatus. All samples, including a non-experimental gill tissue sample used as a protein standard in order to normalize spot density across blots were randomly loaded in duplicate. Samples were allowed to gravity-feed through the apparatus for 20 min after which remaining sample in the wells was gently removed using a light vacuum. With the vacuum still running, the NC membrane was removed from the apparatus and immediately washed in 1x PBS for 5 min. Membranes were placed in blocking solution (5% non-fat dry milk in 1x PBS with 0.1% tween; 1xPBST) for 1 hour at room temperature (approximately 22°C). After which, membranes were then incubated in primary antibody (1:3000 dilution; diluted in blocking solution) for 1.5 hours at room temperature and secondary antibody (goat anti-mouse, Santa Cruz Biotechnology, sc-2005, 1:5000 dilution in blocking solution) for 1 hour at room temperature. Membranes were incubated for 5 min in West Pico Super Signal and exposed on x-ray film. DotBlot images were scanned using a Fotodyne imaging system and Image J software used to perform densitometry. The mean density



for each sample was normalized across blots by dividing the mean density of each sample by that of the protein standard.

CAII: Proteins from gill tissues were extracted by homogenizing approximately 50mg of tissue in 500 $\mu$ L of SDS-homogenization buffer (50mM Tris-HCl, pH=6.8, 4% SDS, 1mM EDTA). Samples and a non-experimental protein standard were randomly loaded in duplicate and immobilized on NC membrane as described above. Membranes were placed in blocking solution (described above) for 1 hour at room temperature, followed by incubation of primary antibody (1:3000 dilution in blocking solution) for 1.5 hours at room temperature and secondary antibody (1:2000 dilution in blocking solution) for 1 hour at room temperature. Membranes were incubated for 5 min in West Pico Super Signal and exposed on x-ray film. Densitometry was performed as described above.

#### Statistical analysis

A three-way Analysis of Variance (ANOVA) was used to evaluate differences between the independent variables of time (7, 28 and 42/56-days of acclimation), temperature (-1 and 4°C) and  $p\text{CO}_2$  treatment level (400 or 1000 $\mu$ atm). When a significant interaction was found ( $p < 0.05$ ), a Holm-Sidak Multiple Comparison test was run, and interaction profiles generated by plotting the least square means for temperature x  $p\text{CO}_2$  at each experimental end point in order to visualize the nature of the interaction.

### 3.4 RESULTS

#### $\text{Na}^+/\text{K}^+$ ATPase Enzyme Activity

*T. bernacchii* - Increased temperature and increased  $p\text{CO}_2$  had a significant effect on the levels of NKA activity in gill tissues from *T. bernacchii*. Both single-stress

treatments showed an increase in enzyme activity over time, peaking after 56-days of acclimation (Figure 3.1A). Gill tissues from this species also showed a significant main effect of time ( $p < 0.001$ ), with single-stress treatment enzyme activity levels from 28 and 56-day acclimated tissues being significantly higher than 7-day acclimated tissues (Figure 3.1A). Additionally, there was a three-way interaction between time, temperature and  $p\text{CO}_2$  in this species ( $p = 0.012$ ). Interaction plots showed the nature of this interaction to be antagonistic across time (Figure 3.1B); which is illustrated by the fact that NKA enzyme activity levels in the multi-stress treatment did not exhibit the increases seen in the single stressor treatment levels. Instead, the overall effect of the combined treatments appears to be no different than control treatments (Figure 3.1A).

*P. borchgrevinki* - Overall, gill tissues from *P. borchgrevinki* displayed higher basal NKA activity, nearly double that of *T. bernacchii*. In addition, gill tissue from this species appeared relatively insensitive to the treatments, unlike the response of *T. bernacchii*. In the single stress treatments, NKA activity levels showed only a moderate increase from 7 to 28-days of acclimation in the high  $p\text{CO}_2$  treatment (Figure 3.1C). Furthermore, no significance was found when analyzed for the main effects of temperature,  $p\text{CO}_2$  or time. Additionally, no significant interaction between these factors was observed. Lastly, unlike the response observed for *T. bernacchii*, the moderate increases in NKA activity levels in the high  $p\text{CO}_2$  treatments returned to basal levels by 56-days of acclimation. The interaction plots between variables do not change between 7 and 28-days of acclimation but appeared to become potentially antagonistic in nature after 56-days of acclimation (Figure 3.1D).

*T. newnesi* - NKA activity from *T. newnesi* displayed a similar response to that of *P. borchgrevinki*. Activity levels in the single stress treatments displayed only moderate changes from 7 to 28-days of acclimation and similar to *P. borchgrevinki*, but these increases were not significant (Figure 3.1E). We also found no statistically significant interactions among any of the fixed effects.

#### *Na<sup>+</sup>/K<sup>+</sup> ATPase Protein Concentration*

*T. bernacchii* - Overall, protein levels of NKA mirrored the trends seen in NKA activity levels in tissues from *T. bernacchii*.  $\text{Na}^+/\text{K}^+$  ATPase protein concentrations increased continuously from 7 through 56-days of acclimation time in both the high temperature and high  $p\text{CO}_2$  treatments (Figure 3.2A). Gill tissues showed significant main level effects of temperature,  $p\text{CO}_2$  and time ( $p < 0.001$ ), as well as an interaction between these three variables ( $p < 0.001$ ). Interaction plots showed this is relationship to be antagonistic in nature (Figure 3.2B), further illustrated by the un-changing protein concentration in the multi-stress treatment (Figure 3.2A).

*P. borchgrevinki* -  $\text{Na}^+/\text{K}^+$  ATPase protein concentrations from *P. borchgrevinki* also followed the same trends seen in NKA activity measurements, with both the high temperature and high  $p\text{CO}_2$  treatments displaying only moderate changes in protein level after 28-days of acclimation. However, unlike the small changes in activity observed in this species, there was a significant main effect of temperature ( $p = 0.025$ ),  $p\text{CO}_2$  ( $p < 0.001$ ) and time ( $p < 0.001$ ) on protein levels. We also found a significant interaction between temperature,  $p\text{CO}_2$  level and time ( $p < 0.001$ ). Matching the observed NKA activity changes in *P. borchgrevinki*, we also found protein levels declined after 56-days

of acclimation (Figure 3.2C). The nature of the interaction between factors in this species appeared again to be antagonistic (Figure 3.2D).

*T. newnesi* - Gill tissues from this species showed a different trend when compared with gill tissues from *T. bernacchii* and *P. borchgrevinki*. In this species we observed a rapid increase in NKA protein concentration at 7-days of acclimation, with the high  $p\text{CO}_2$  treatment showing the largest increase above control levels (Figure 3.2E). When analyzed by 3-way ANOVA, main level effects of time and  $p\text{CO}_2$  were found ( $p < 0.001$ ), as well as a significant interaction between time, temperature and  $p\text{CO}_2$  ( $p = 0.002$ ). NKA protein levels started to decrease at 28-days of acclimation in all treatments, and by 42-days of acclimation, the two high temperature treatments were no longer distinguishable from control levels (Figure 3.2E). The interaction plots generated for this species suggested an additive relationship was present throughout the entire acclimation period (Figure 3.2F).

#### Carbonic Anhydrase II Protein Concentration

*T. bernacchii* – In general, CAII protein levels were found to be highest in tissues from *T. bernacchii*, which also displayed the largest magnitude of response to the treatments (Figure 3.3). Similar to  $\text{Na}^+/\text{K}^+$  ATPase protein concentrations in *T. bernacchii*, CAII protein levels increased over time in both the single stress treatments. Unlike NKA though, CAII protein levels also increased significantly in the multi-stress treatment (Figure 3.3A). After 7-days of acclimation, protein concentrations were elevated above control levels in all treatments, with the high  $p\text{CO}_2$  treatment eliciting the strongest effect. CAII protein levels remained elevated in the 28-day acclimation group and displayed further increases in the 56-day acclimated fish. We again observed fish in

the high  $p\text{CO}_2$  treatment exhibited a larger magnitude of change compared to the two high temperature treatments (Figure 3.3A). Three-way ANOVA showed there was a main effect of temperature ( $p=0.001$ ),  $p\text{CO}_2$  ( $p<0.001$ ), and time ( $p<0.001$ ) in *T. bernacchii* gill. Additionally, a significant interaction between these three factors ( $p=0.030$ ) was found. The interaction plots generated for *T. bernacchii* CAII protein levels suggested the nature of this interaction was antagonistic across all acclimation time points despite the overall increase in CAII protein levels compared to control fish (Figure 3.3B).

*P. borchgrevinki*: Carbonic anhydrase protein levels in gill tissues from *P. borchgrevinki* differed slightly than those of *T. bernacchii*. While CAII protein levels remained elevated above control values across the entire experimental period, CAII protein concentrations in *P. borchgrevinki* gill tissues did not show the same pattern of response to individual treatments. A significant main effect of temperature and  $p\text{CO}_2$  was found ( $p<0.001$ ), as well as a significant three-way interaction between temperature,  $p\text{CO}_2$  and time ( $p=0.008$ ). After 7-days of acclimation, CAII protein concentrations increased in both the single stress treatments as well as the multi-stress treatment (Figure 3.3C). Unlike *T. bernacchii*, the high temperature + high  $p\text{CO}_2$  treatment resulted in significantly elevated CAII protein levels after 28 days of acclimation and the interaction appeared to be synergistic in nature. After 56-days of acclimation CAII protein concentrations remained significantly elevated in all three treatments, however the interaction no longer appeared synergistic (Figure 3.3C, D).

*T. newnesi*: Carbonic anhydrase levels in gill tissues from *T. newnesi* followed a similar trend when compared with tissues from *T. bernacchii*, although temperature appeared to be a more significant driver of the observed increase in CAII protein. Protein

concentration in the high  $p\text{CO}_2$  treatment and the high temperature + high  $p\text{CO}_2$  remained near-control levels after 7-days of acclimation in this fish. However, the high temperature treatment did increase above control levels. After 28-days of acclimation, the high temperature treatment continued to increase, while the high temperature + high  $p\text{CO}_2$  treatment increased only slightly. After 42-days of acclimation, all treatments were elevated when compared to control values; with protein concentrations in the single stress treatments displaying a larger magnitude of change when compared to the multi-stressor treatment (Figure 3.3E). Significant main effects of temperature and time were found ( $p < 0.001$ ) as well as a significant interaction between temperature, time and  $p\text{CO}_2$  ( $p < 0.001$ ). Similar to *T. bernacchii*, interaction plots suggest the nature of the interaction was largely antagonistic in *T. newnesi* (Figure 3.3F).

### 3.5 DISCUSSION

The current study represents the first data set to examine the interacting effects of temperature and  $p\text{CO}_2$  on the osmoregulatory abilities of three species of Antarctic notothenioid fishes, *Trematomus bernacchii*, *Pagothenia borchgrevinki*, and *Trematomus newnesi*. Overall, our data from single stress treatments follow a similar trend as previously gathered measurements; and suggest that Antarctic notothenioids have the capacity to restore acid-base balance over an extended acclimation period when confronted with either an increase in temperature or an increase in  $p\text{CO}_2$ . The data from this study also provide novel insight into the mechanisms notothenioid fish use to re-establish acid-base balance when confronted with a long-term exposure to a multi-stress scenario.

### Response to increases in temperature

When confronted with an increase in temperature, all three species appeared to compensate at some level for extracellular changes, illustrated by an increase in  $\text{Na}^+/\text{K}^+$  ATPase as well as carbonic anhydrase II capacity. These data concur with those presented by Gonzalez-Cabrera et al. (1995) and Guynn et al. (2002) which illustrated that increasing temperature resulted in an increase in NKA activity in notothenioid fish. Increases in temperature present two challenges to Antarctic fish, both of which require an increase in ion excretion in order to re-establish osmotic balance. First, an increase in temperature results in a decline of serum osmolality, which increases the osmotic gradient between fish blood serum and the external environment (Gonzalez-Cabrera et al., 1995; Guynn et al., 2002; Brauer et al., 2005). In order to maintain a balanced osmotic gradient, fish need to increase the number of ions shunted across gill epithelium in order to re-establish acid-base balance. Second, an increase in temperature causes metabolic acidosis, brought about by an increase in oxygen consumption, and hence, an accumulation of  $\text{H}^+$  ions (Enzor et al., in prep). This latter scenario requires fish to shunt hydrogen ions in addition to sodium ions across gill epithelia in order to maintain acid-base balance. In these Antarctic species, the increased ion excretion is accomplished with an increase in either NKA capacity, CAII capacity, or both.  $\text{Na}^+/\text{K}^+$  ATPase capacity declined at 56 and 42-days of acclimation in *P. borchgrevinki* and *T. newnesi*, respectively, indicating an increased capacity for ion excretion is no longer required. Alternatively in *T. bernacchii*,  $\text{Na}^+/\text{K}^+$  ATPase capacity continue to increase, indicated by changes in both enzyme activity and protein concentration. Given that we did not observe a similar drop in NKA capacity in this species when compared to

*P. borchgrevinki* and *T. newnesi* (28-days versus 7-days), it is possible that *T. bernacchii* requires a longer acclimation period to re-establish acid-base balance when confronted with an increase in temperature.

#### Response to increases in $p\text{CO}_2$

Increases in  $p\text{CO}_2$  caused markedly different effects in all three species of fish examined. Recent work by Strobel et al. shows that when confronted with an acidic environment, notothenioid fish will attempt to accumulate  $\text{HCO}_3^-$  to compensate for the drop in  $p\text{CO}_2$  levels (2013a). Accumulating these ions allows for an increase in buffering capacity, as well as increases acid excretion. The noted increase in  $\text{Na}^+/\text{K}^+$  ATPase capacity seen in *T. bernacchii* and *P. borchgrevinki* at 7-days of acclimation is indicative of an attempt to enhance the shunting  $\text{Na}^+$  ions out of the gill, taking protons with it. This would allow bicarbonate ions to remain within the blood plasma, effectively decreasing metabolic acidosis, and re-establishing acid-base balance. After 56-days of acclimation to high  $p\text{CO}_2$ , we again observed a delayed response in gill tissues from *T. bernacchii*. While NKA activity levels are declining or remaining unchanged in the other two species, the concentration of NKA protein is increasing, as is the concentration of CAII protein, indicating that these fish are still attempting to re-establish acid-base balance, even after 8-weeks of acclimation.

*Trematomus newnesi* is unique in the notothenioids when examined in an increased  $p\text{CO}_2$  environment. Unlike the other species used in this study, oxygen consumption rates from *T. newnesi* in a high  $p\text{CO}_2$  environment do not significantly elevate above control values regardless of acclimation time (Enzor et al., 2013; Enzor et al., in prep). While activity levels and protein concentrations of NKA are elevated above



control values at 7 and 28-days of acclimation in this species, by 42-days, they have both declined, indicating these fish could be acclimating to an increased  $p\text{CO}_2$  environment. Interestingly, there is no change in CAII protein levels until 42-days of acclimation. This could be explained by an increase in excretion of both  $\text{Na}^+$  and  $\text{Cl}^-$  ions as acid-base balance is again achieved.

#### The Effect of Multiple stressors

While it seems that all three species from this study are capable of re-establishing acid-base balance when confronted with an increase in temperature or an increase in  $p\text{CO}_2$ , when combined, it does not appear that *T. bernacchii* or *P. borchgrevinki* prioritize defense of acid-base balance under simultaneous environmental changes. No significant changes were noted in NKA activity or protein concentration levels from either of these species in the multi-stressor treatment. Therefore, it would seem that the additional oxygen gained from the initial increases in metabolic rate previously observed in these fish under similar treatments is being shunted to a pathway other than increasing capacity for osmoregulation or acid-base balance (Enzor et al., 2013; Enzor and Place, 2014). Our previous work on *T. bernacchii* and *P. borchgrevinki* illustrated that over time, these species also experience a significant drop in oxidative damage when acclimated to the same multi-stressor conditions (Enzor and Place, 2014). This significant drop occurred in the absence of a significant increase in antioxidant defense mechanisms and was coupled with a return of resting metabolic rates to basal levels. Taken all together, these data suggest at least some species of Antarctic fish are potentially employing energetic trade-offs. For instance, these fish may be sacrificing acid-base balance capacity for protein homeostasis, in an effort to mitigate the metabolic costs associated with acclimation to a

warmer, more acidic ocean. Alternatively, these fish may be balancing increasing energetic demands associated with defending cellular homeostasis by a whole-sale reduction in cellular processes.

Hypercapnic acidosis has been shown to elicit a significant drop in metabolic rates (Pörtner et al., 2004; Reipschläger and Pörtner, 1996; Langenbuch and Pörtner, 2003). If these fish are not actively defending acid base balance, the drop in extracellular pH may elicit metabolic depression in these fish which would, in turn, lead to a drop in cellular energy demands from costly processes such as protein synthesis and maintenance of  $\text{Na}^+$  ion gradients (Langenbuch and Pörtner, 2003). This would be followed by a concomitant drop in oxygen consumption and the production of reactive oxygen species, potentially explaining the reduction in oxidative damage our lab group previously reported in these fish (Enzor and Place, 2014).

*Trematomus newnesi*, on the other hand, appears capable of re-establishing and maintaining long-term acid-base balance under the multi-stressor scenario. The capacity of  $\text{Na}^+/\text{K}^+$  ATPase activity increases above control levels at 7-days of acclimation, along with the single stress treatments, and declines throughout the rest of the experimental time period suggesting a rapid attempt to re-establish osmotic and acid-base equilibrium may have occurred. Furthermore, this species does not show a similar drop in oxidatively damages proteins as was observed in the other notothenioid species (Enzor and Place, 2014), possibly signaling that *T. newnesi* are prioritizing the re-establishment of acid-base balance over other cellular processes. Lastly, these notothenioid fish do not display the same rapid decline in metabolic rates after long-term acclimation to high  $p\text{CO}_2$  and high temperature conditions that was previously observed in *T. bernacchii* and

*P. borchgrevinki* (Enzor et al., 2013; Enzor et al., in prep). These data suggest *T. newnesi* may be attempting to re-establish acid-base balance and defend cellular homeostasis over significantly longer acclimation periods compared to closely related species. However, it remains unclear if this extended acclimation period comes at significant cost to other cellular processes such as growth and reproduction.

Overall, the data presented here suggest that Antarctic notothenioids attempt to re-establish acid-base balance when confronted with either an increase in temperature or an increase in  $p\text{CO}_2$ . Our data also illustrate that despite being closely related and adapted to the same environment over evolutionary time-scales, not all notothenioid fish will respond the same to global climate change. *Trematomus newnesi*, in particular appears to continually display variant physiological responses compared to other notothenioid fishes. Lastly, it appears that when faced with an environment where multiple variables change co-comittently, at least some notothenioid species may experience energetic trade-offs or whole organism metabolic depression, and thus, may not have the capacity to re-establish long-term cellular homeostasis. Metabolic depression has already been shown to impact protein synthesis in Antarctic fish (Langenbuch and Pörtner, 2003), and it is likely any energetic trade-off would also impact protein synthesis rates to some degree. Therefore, for these highly endemic species, extended periods of exposure to environmental conditions that may reduce protein synthesis (potentially including the environmental conditions used in this study) can be postulated to have long-term physiological consequences; affecting both growth and reproduction and negatively impacting current population levels.

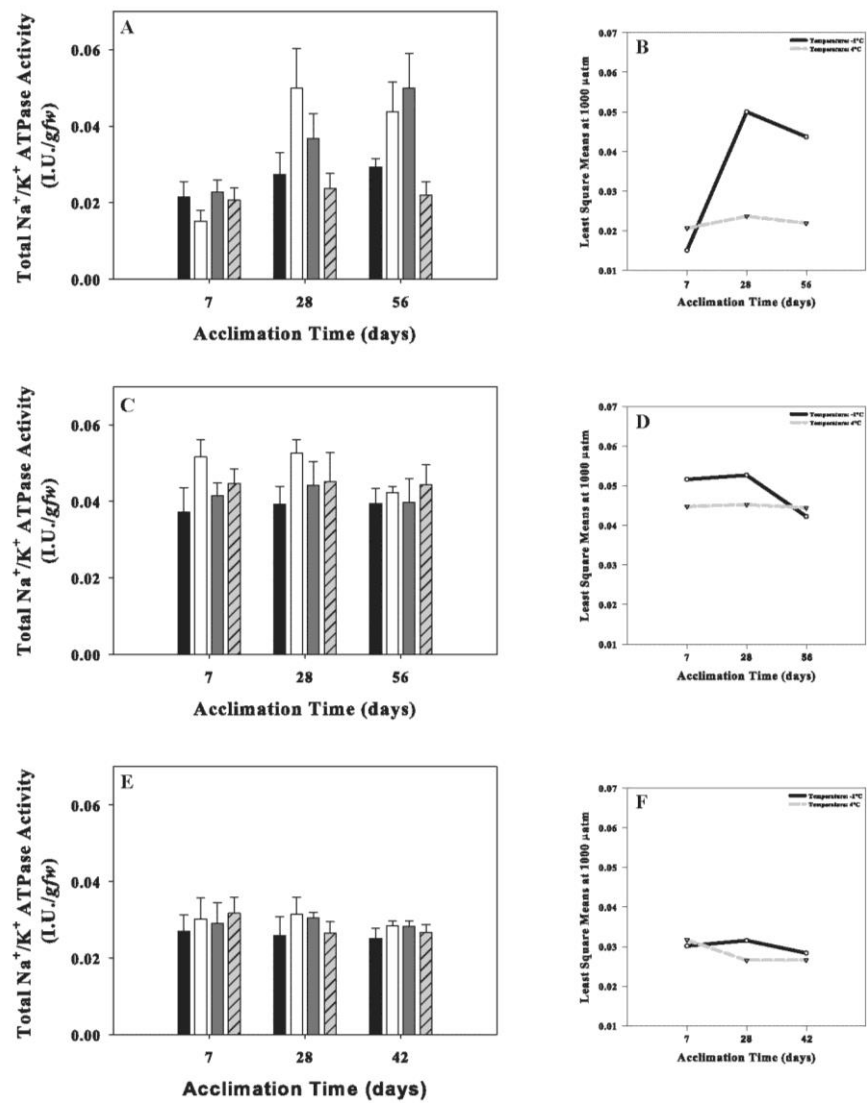


Figure 3.1. Total Na<sup>+</sup>/K<sup>+</sup> ATPase activity ( $\pm$ SE) of gill tissues acclimated at 7, 28 and 42/56 days to treatments of ambient conditions, (Low Temperature + Low pCO<sub>2</sub>; black bars), Low Temperature + High pCO<sub>2</sub> (white bars), High Temperature + Low pCO<sub>2</sub> (dark grey bars) and High Temperature + High pCO<sub>2</sub> (light grey bars with crosshatch's) as well as interaction plots of each dataset. Individual panels represent data gathered from *Trematomus bernacchii* (A and B), *Pagothenia borchgrevinki* (C and D), and *Trematomus newnesi* (E and F)

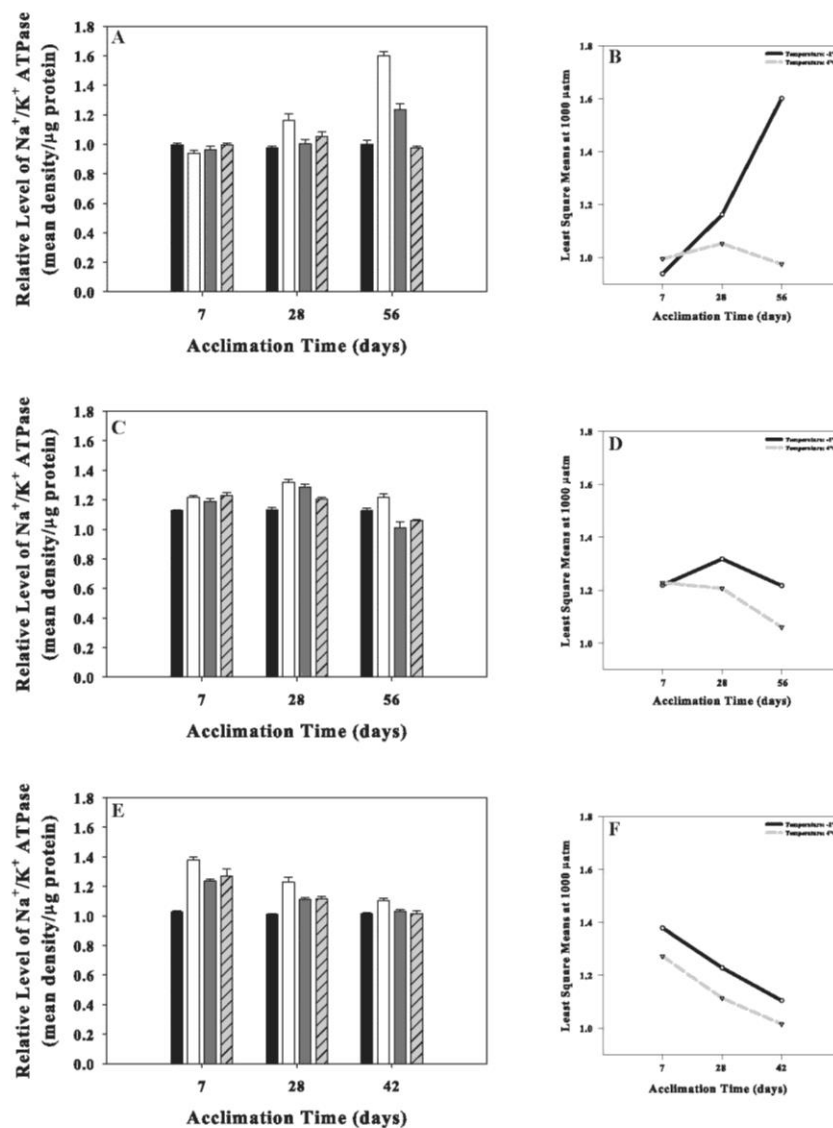


Figure 3.2. Na<sup>+</sup>/K<sup>+</sup> ATPase protein concentrations ( $\pm$ SE) of gill tissues acclimated at 7, 28 and 42/56 days to treatments of ambient conditions, (Low Temperature + Low pCO<sub>2</sub>; black bars), Low Temperature + High pCO<sub>2</sub> (white bars), High Temperature + Low pCO<sub>2</sub> (dark grey bars) and High Temperature + High pCO<sub>2</sub> (light grey bars with crosshatch's) as well as interaction plots of each dataset. Individual panels represent data gathered from *Trematomus bernacchii* (A and B), *Pagothenia borchgrevinki* (C and D), and *Trematomus newnesi* (E and F)

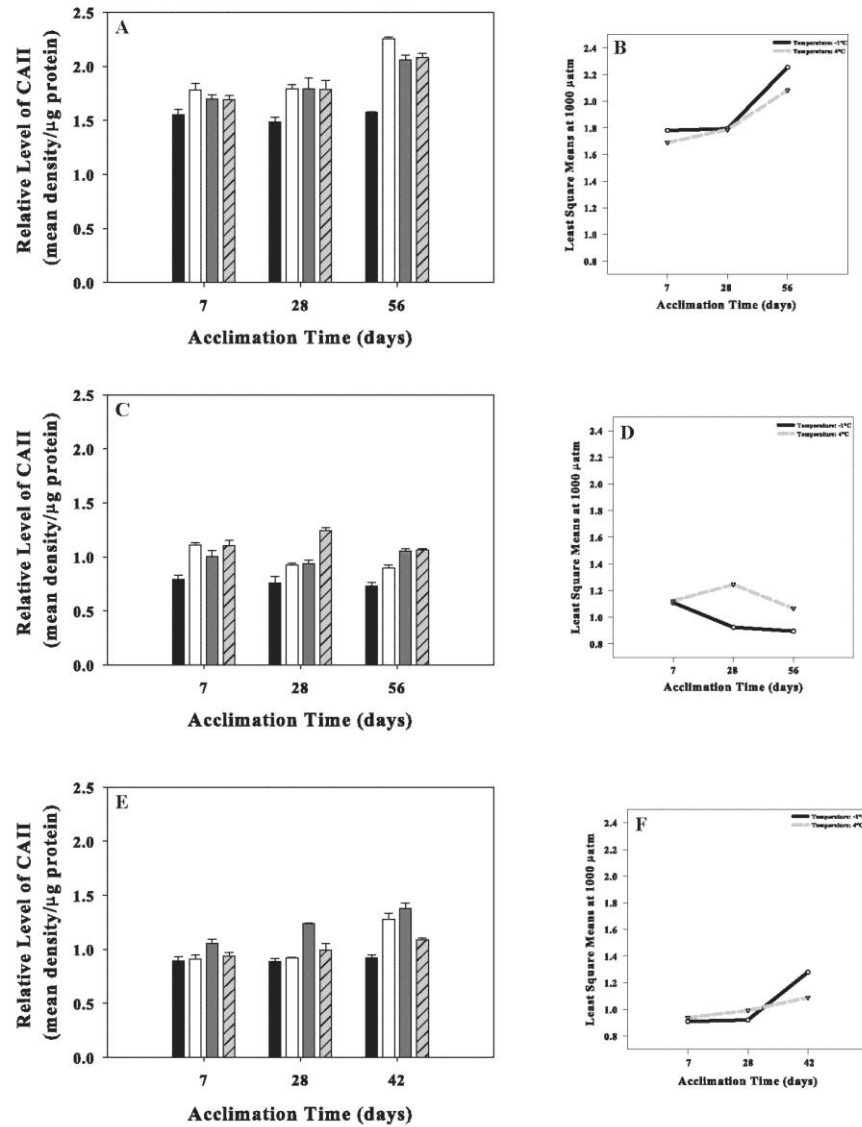


Figure 3.3. Carbonic anhydrase II protein concentrations ( $\pm$ SE) of gill tissues acclimated at 7, 28 and 42/56 days to treatments of ambient conditions, (Low Temperature + Low  $p\text{CO}_2$ ; black bars), Low Temperature + High  $p\text{CO}_2$  (white bars), High Temperature + Low  $p\text{CO}_2$  (dark grey bars) and High Temperature + High  $p\text{CO}_2$  (light grey bars with crosshatch's) as well as interaction plots of each dataset. Individual panels represent data gathered from *Trematomus bernacchii* (A and B), *Pagothenia borchgrevinki* (C and D), and *Trematomus newnesi* (E and F)

Table 3.1. Mean measurements of  $p\text{CO}_2$  and Temperature  $\pm$  SD over the course of the 2011 and 2012 field seasons

2011 Season	$p\text{CO}_2$ ( $\mu\text{atm}$ )	Temperature ( $^{\circ}\text{C}$ )	2012 Season	$p\text{CO}_2$ ( $\mu\text{atm}$ )	Temperature ( $^{\circ}\text{C}$ )
Incoming Seawater	$417.15 \pm 12.26$	$-1.24 \pm 0.08$	Incoming Seawater	$427.66 \pm 23.97$	$-1.03 \pm 0.152$
Low Temperature + Low $p\text{CO}_2$	$438.82 \pm 16.08$	$-0.61 \pm 0.17$	Low Temperature + Low $p\text{CO}_2$	$432.04 \pm 22.50$	$-0.707 \pm 0.153$
Low Temperature + High $p\text{CO}_2$	$953.89 \pm 50.38$	$-0.45 \pm 0.16$	Low Temperature + High $p\text{CO}_2$	$1024.76 \pm 94.20$	$-0.578 \pm 0.150$
High Temperature + Low $p\text{CO}_2$	$525.11 \pm 21.07$	$4.02 \pm 0.44$	High Temperature + Low $p\text{CO}_2$	$525.16 \pm 22.41$	$3.86 \pm 0.484$
High Temperature + High $p\text{CO}_2$	$1026.66 \pm 9.03$	$4.22 \pm 0.56$	High Temperature + High $p\text{CO}_2$	$1053.44 \pm 71.87$	$4.03 \pm 0.316$

## CHAPTER 4

### THE EFFECTS OF ELEVATED TEMPERATURE AND OCEAN ACIDIFICATION ON THE METABOLIC PATHWAYS OF NOTOTHENIROID FISH<sup>4</sup>

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#### 4.1 ABSTRACT

Notothenioid fish of the Southern Ocean have evolved several unique adaptations to deal with extreme cold. However, these adaptations may have left these fish poorly poised to deal with a changing environment. As such, the expected environmental perturbations brought on by global climate change have the potential to significantly affect the oxygen consumption and subsequent cellular processes necessary for survival. Previous studies have illustrated the narrow thermal window in which the notothenioids can survive; however more recent studies have demonstrated the ability to acclimate to temperature is not lost. Despite these lines of evidence, the cellular level effects of temperature acclimation in these fish remain largely unknown. Also, little information exists on the capacity of Antarctic fish to respond to changes in multiple environmental variables. We have examined the effects of increased temperature and  $p\text{CO}_2$  on the rate of oxygen consumption in three notothenioid species, *Trematomus bernacchii*, *Pagothenia borchgrevinki*, and *Trematomus newnesi*. We have combined these measurements with enzyme activities of citrate synthase (an oxidative enzyme), lactate dehydrogenase (a glycolytic enzyme), and measurements of total triglyceride and fish condition factor. Our findings indicate that temperature is the major driver of oxygen consumption in these fish, yet the cellular-level responses differed across species. Overall, we illustrate that acclimation to predicted environmental changes of the Southern Ocean were likely accomplished using energetic trade-offs and may not be sustainable over long time scales.

## 4.2 INTRODUCTION

Evolution in extremely cold, stable waters has necessitated the dominant fish fauna of the Southern Ocean, the notothenioids, to evolve a suite of adaptations to combat life in extreme cold. Physiological adaptations such as the constant expression of heat shock proteins (Hofmann et al., 2000; Place et al., 2004), the functional loss of heme proteins (O'Brien and Sidell, 2000; Sidell and O'Brien, 2006), the presence of antifreeze glyco-proteins (DeVries, 1969; Cheng et al., 2006), the use of lipid peroxidation as an energy source (Lin et al., 1974; Johnston et al., 1998), and increased mitochondrial density (Clarke et al., 1984, Pörtner et al., 2005) are some ways the notothenioids deal with extreme temperature. Increasing mitochondrial density is thought to be the reason why aerobic capacity is enhanced in these fishes (Johnston et al., 1998; van Dijk et al., 1998). However, this increase in aerobic capacity makes the notothenioids more vulnerable to changes in temperature (van Dijk et al., 1998). Indeed, previous studies on these fish have illustrated the narrow thermal window in which the notothenioids can exist, with upper limits of 6°C being common (Somero and DeVries, 1967). Also contributing to their limited thermal tolerance is a limited anaerobic capacity (Pörtner et al., 2005).

It has been postulated that in the face of global climate change, the synergistic effects of increased temperature and increased  $p\text{CO}_2$  adversely affect marine ectotherms by decreasing their aerobic scope (Munday et al., 2009; Strobel et al., 2012; 2013a,b). Additionally, it is assumed that this decrease in aerobic scope will determine how an organism will physiologically respond to climate change (Pörtner and Knust, 2007; Pörtner and Farrell, 2008). In the case of fish, the amount of oxygen available to tissues is

dependent upon the circulatory system being able to meet oxygen demand. When temperature is increased, oxygen demand also increases, and hence, additional burden is placed upon the circulatory system to deliver needed oxygen. When this demand cannot be met, aerobic scope declines, and the fish is no longer performing at peak level (i.e. the Oxygen Limited Thermal Tolerance Hypothesis, OLTTH; Pörtner, 2010). Additionally, it has been shown that hypercapnia can narrow the optimum thermal window for an organism, and in turn, narrow the performance window (Pörtner, 2010).

Given the narrow thermal window in which the notothenioids exist, it can be assumed that these ectotherms will be no exception to suffering a decreased aerobic scope when confronted with an increase in temperature and/or  $p\text{CO}_2$ . Previous work on polar fishes have focused on temperature as a stress alone (Davison et al., 1990; Seebacher et al., 2005, Pörtner, 2008; Robinson and Davison, 2008a,b), or if the effect of multiple stressors were examined, a focus was placed on aerobic metabolism, leaving anaerobic pathways largely unexplored (Strobel et al., 2012, Strobel et al., 2013a, b). To this end, we set out to determine the energetic response and the possible use of anaerobic pathways as compensation in three species of notothenioid fish, *Trematomus bernacchii*, *Pagothenia borchgrevinki*, and *Trematomus newnesi* to predicted increases of temperature and  $p\text{CO}_2$ . In addition to gathering whole fish oxygen consumption rates, we measured total triglyceride content of fish tissues combined with calculations of Fulton's Index, a measure of overall fish condition (Fulton, 1902; Peig and Green, 2010), in an effort to determine if fish were depleting energy stores necessary to maintain strong growth potential. Lastly, we monitored metabolic changes by measuring the activity of a

biochemical marker of aerobic capacity, citrate synthase (Cai and Adelman, 1990), and anaerobic capacity, lactate dehydrogenase (Hochachka and Somero, 2002).

#### 4.3 MATERIALS AND METHODS

##### Fish Collection and Experimental Design

*Trematomus bernacchii* (Boulenger, 1902), *Trematomus newnesi* (Boulenger, 1902), and *Pagothenia borchgrevinki* (Boulenger, 1902) were collected from McMurdo Sound, Antarctica using hook and line through 10-inch holes drilled in the sea ice. Fish were collected from October through December, 2011 and September through December, 2012. Once collected, fish were transported back to McMurdo Station in aerated coolers, where they were acclimated for one week in a flow-through aquarium in ambient seawater (-1.5°C and ~430 $\mu$ atm CO<sub>2</sub>).

After the initial acclimation period, fish were randomly placed into one of four experimental treatment tanks (1240 each) in order to assess the response to increased temperature, increased  $p$ CO<sub>2</sub> or a combination of increased temperature and increased  $p$ CO<sub>2</sub>. The treatment tanks consisted of tank held at ambient conditions (control treatment; -1°C and 430 $\mu$ atm), a low temperature + high  $p$ CO<sub>2</sub> tank (-1°C and 1000 $\mu$ atm), a high temperature + low  $p$ CO<sub>2</sub> tank (4°C and 430 $\mu$ atm), and a high temperature + high  $p$ CO<sub>2</sub> tank (4°C and 1000 $\mu$ atm). Fish were exposed to experimental treatments for a period of 7 days (n=9 per treatment), 28 days (n=9 per treatment), 42 days (*T. newnesi* only; n=5 per treatment), or 56 days (*T. bernacchii* and *P. borchgrevinki*; n=5 per treatment). While in experimental treatment tanks, fish were fed frozen anchovy once every 3-4 days. At each experimental endpoint, fish were removed from experimental treatment tanks and placed in a respirometer to evaluate resting

metabolic rates, after which, fish were returned to the appropriate treatment tank. Food was withheld from these fish for at least 48 hours prior to being run in the respirometer. Concurrently, a sub-set of experimental fish (the same species and sample sizes as described above) were removed at each experimental endpoint, sacrificed, and tissues were collected and flash-frozen in liquid nitrogen. Tissues were transported back to the University of South Carolina on dry ice where they were housed at -80°C until used.

#### Seawater manipulation

We used an experimental  $p\text{CO}_2$  manipulation system first described by Fangue et al. (2010; adapted for large-scale use) combined with thermostated titanium heaters to create our four experimental treatments. Briefly, atmospheric air was pumped through columns filled with drierite to remove moisture, and then columns filled with Sodasorb to scrub air of  $\text{CO}_2$ . Atmospheric air was then blended with  $\text{CO}_2$ -free air using digital mass-flow controllers and bubbled into flow-through header tanks, which in turn supplied experimental tanks with  $\text{CO}_2$ -infused water via Venturri injectors. Daily measurements of salinity, temperature, total alkalinity and pH were taken from both experimental tanks and incoming seawater (mean values  $\pm\text{SD}$  are reported in Table 4.1). Additionally, experimental tanks were sampled daily for the presence of waste products (no discernible levels detected, data not shown).

#### Resting Metabolic Rates

Resting Metabolic Rates (RMR's) were determined using an automated intermittent respirometry system from Loligo. Respirometry chambers were housed in covered 99-L tanks which received a continuous flow of treatment tank water. All tanks were submerged within an 850 L sea table with a continuous flow of ambient seawater in

order to maintain temperature of cold water treatments. Tanks which were used with warm-acclimated fish were fitted with glass aquarium heaters to maintain 4°C water consistently. The entire respirometry set-up was surrounded with a tarp to minimize outside disturbance while respirometry was running. Additionally, all respirometry measurements were recorded over-night to minimize possible disturbances to fish.

Fish were placed in respirometry chambers with the flush pumps running, and chambers were sealed. Fish were acclimated to the respirometry chamber with the same measurement cycle used for actual respirometry measurements (20 min measure followed by a 5 min flush) for a period of 1-2 hours. Once the acclimation period was over, the program was re-started and fish were left in the respirometer for 8-10 hours. Mean  $\dot{M}O_2$  values were calculated by averaging five sequential measurements whose values had an  $R^2$  value >0.95 for the slope describing the rate of oxygen consumption. Oxygen consumption rates were standardized to a 100-g fish (Steffensen 2005; Robinson and Davison, 2008 a,b), using a mass-exponent of -0.25 (Schmidt-Nielsen, 1984).

#### Fulton's Index

In order to determine overall fish condition over the course of the experiment, we gathered standard length (in mm) and weight (in g) measures for each fish before being placed in respirometry chambers. Data were calculated from the following equation:

$$K = \frac{100,000 \times W}{L^3}$$

Where:

W= Weight in grams

L= Standard length in mm

Fish values from each treatment were averaged in order to give one value per treatment per time-point.

### Total Triglycerides

Infinity™ Triglycerides Reagent was used in order to quantify the total triglycerides present in white muscle from fish at each experimental endpoint. Approximately 50mg of white muscle was homogenized in ice-cold 1x Phosphate Buffer Solution with 1% Triton-X. A standard curve was run with each plate using a Stan-Bio Triglyceride Standard (200mg/dL). All samples and standards were kept on ice and run in duplicate, with wells filled with DI water as a blank. Six  $\mu\text{L}$  of each standard was combined with 294 $\mu\text{L}$  of Infinity Reagent, and 10 $\mu\text{L}$  of each sample was combined with 290 $\mu\text{L}$  of Infinity Reagent. Plates were incubated in the dark on an orbital shaker, after which they were immediately run on a 96-well plate reader (Bio-Tek) at 500nm using a pathway correction. Total triglycerides were calculated using slope of the standard curve line and was reported as total triglyceride per gram fresh weight.

### Citrate Synthase Activity

We used a spectrophotometric method to quantify total Citrate Synthase (CS) activity in both liver and gill tissues from all three fish species. Approximately 20 mg of gill tissue was homogenized on ice in a 50mM Potassium phosphate buffer (pH=6.8). Once extracted, supernatant was stored at -20°C until ready for use.

All samples were run in duplicate and enzyme activity was measured at 4°C (maintained by temperature-controlled cells within the spectrophotometer), at 412nm, over a period of five minutes. A reference cuvette was placed in the spectrophotometer (Shimadzu 1800 UV/Vis) with approximately 2.0mL of CS Cocktail (50mM Imidazole-HCl, pH=8.2, 15mM  $\text{MgCl}_2$ , 0.8 mg/mL DTNB, 3mg Acetyl CoA) to measure background rate. Sample cuvettes contained 25 $\mu\text{L}$  of supernatant combined with 2.0mL of CS Cocktail. Reactions were started by adding 25 $\mu\text{L}$  of oxaloacetate to sample

cuvettes. The slopes of the background rates were subtracted from the slopes of the oxaloacetate-dependent rates to determine the CS activity rate. The calculated activity was reported as International Units (IU) per gram fresh tissue weight.

#### Lactate Dehydrogenase Activity

We also used a spectrophotometric method to quantify total Lactate Dehydrogenase (LDH) activity from gill and liver tissues. Tissue extracts were prepared as described above. Samples were run at 340nm for three minutes. A small amount of tissue supernatant (5 $\mu$ L) was combined with 2.00mL LDH cocktail (0.20 M Imidazole-HCl buffer, pH=7.0, 5.50 mM NADH, 2.00mM sodium pyruvate) and placed in the spectrophotometer. The slope of the absorbance change was used to calculate the LDH activity rate, and was reported as IU per gram fresh weight. Once we ran tissues from *T. bernacchii* and realized there were no significant changes until the last experimental endpoint, we opted to eliminate the middle time-point (28-days of acclimation) from the *P. borchgrevinki* and *T. newnesi* analyses.

#### Statistical Analysis

A three way ANOVA was used to evaluate the relationship between time (7, 28 or 42/56 days of acclimation), temperature (-1 and 4°C) and  $p\text{CO}_2$  level (400 or 1000 $\mu$ atm). A Holm-Sidak Multiple Comparison test was run when a significant interaction was found ( $p < 0.05$ ), and interaction profiles generated by plotting the least square means for temperature x  $p\text{CO}_2$  at each experimental end point in order to visualize the nature of the interaction. Additionally, fish from time point 0 (i.e. measured before being added into experimental treatment tanks) were included in the ANOVA for Fulton's Index.



## 4.4 RESULTS

### Resting Metabolic Rates

*T. bernacchii*: Oxygen consumption rates from *T. bernacchii* showed significant main effects of temperature ( $p < 0.001$ ) as well as  $p\text{CO}_2$  level ( $p = 0.042$ ), with no three-way interactions shown. Overall, temperature had a pronounced effect across time, significantly elevating RMR's in both high temperature treatments at all three acclimation times (Fig. 4.1A; 7-day:  $p < 0.001$ , 28-day:  $p < 0.001$ , 56-day:  $p = 0.007$ ). While a significant elevation in RMR's was noted at 7-days of acclimation in the high  $p\text{CO}_2$  treatment ( $p = 0.010$ ), by 28-days, these levels dropped and were no longer significant (Fig. 4.1A).

*P. borchgrevinki*: RMR's from *P. borchgrevinki* also showed a significant main effect of temperature ( $p < 0.001$ ), however no significant effects of  $p\text{CO}_2$  were found ( $p = 0.396$ ), and no significant interactions were found in this species. While temperature also had a significant effect on RMR's in *P. borchgrevinki* over shorter acclimation periods, oxygen consumption in both high temperature treatments did decline over time (Fig 4.1B) and RMR's in the two high temperature treatments were not found to be statistically different from control values at 56-days of acclimation ( $p = 0.206$ ).

*T. newnesi*: Overall, oxygen consumption rates from *T. newnesi* were much higher than that of *T. bernacchii* or *P. borchgrevinki*. RMR's from *T. newnesi* showed significant main level effects of temperature ( $p < 0.001$ ), and acclimation time ( $p = 0.004$ ), however no significant effect of  $p\text{CO}_2$  was noted ( $p = 0.555$ ). The high  $p\text{CO}_2$  treatment didn't seem to have the same effect on oxygen consumption in *T. newnesi* when compared to the other species, as the RMR's in this treatment were only slightly elevated

above control values, if at all (Fig. 4.1C). Temperature, on the other hand, had a pronounced effect, and oxygen consumption levels were elevated significantly above control levels across all acclimation time-points (Fig. 4.1C;  $p < 0.001$  at all time-points).

#### Fulton's Index

*T. bernacchii*: There was a significant effect of temperature on the condition factor of individuals of *T. bernacchii* ( $p < 0.001$ ). Overall, *T. bernacchii* experienced a decline in condition factor over time, despite having access to food (Fig. 4.2A). Within each treatment (apart from control), K declined by a factor of at least 0.1 from day 0 to day 56.

*P. borchgrevinki*: Overall, *P. borchgrevinki* had lower condition factors than either *T. bernacchii* or *T. newnesi*, and 3-way ANOVA revealed a significant effect of  $p\text{CO}_2$  in these fish ( $p = 0.023$ ). Individuals of *P. borchgrevinki* appear to use energy reserves quickly, illustrated by the rapid drop in condition factor from day 0 to day 7 (Figure 4.2B). However, these fish do seem to recover at later time periods, as K-values increased from day 28 to day 56 in both high temperature treatments (Fig. 4.2B).

*T. newnesi*: As with *T. bernacchii*, a significant effect of temperature was seen in *T. newnesi* ( $p = 0.001$ ), and condition factor did decrease steadily over time (Fig. 4.2C). These fish also displayed a decline in K-factor in the experimental treatments of 0.1 from day 0 to day 56.

#### Lipid Analysis

Only small changes were noted across all three species in regards to total triglycerides present in white muscle over time (Fig. 4.3). A main effect of acclimation

time was found in tissues from *T. bernacchii* ( $p=0.034$ ). Overall, it appears that *T. bernacchii* may become less reliant on fatty acid metabolism under these conditions as total triglyceride levels increased across treatments over the course of the experimental acclimation (Fig. 4.3A). Alternatively, *Pagothenia borchgrevinki* appear to use energy reserves in the multi-stressor treatment, as levels of total triglyceride were lowest at 56-days of acclimation (Fig. 4.3B). *Trematomus newnesi*, on the other hand, appear to utilize energy reserves during shorter acclimation times (7-days), and then attempt to re-establish the reserves, indicated by an increase in total triglycerides at 28-days (Fig. 4.3C).

#### Citrate Synthase Activity

*T. bernacchii*: Both gill and liver tissues from *T. bernacchii* responded similarly when analyzed for citrate synthase activity. A main effect of time ( $p<0.001$ ), and  $p\text{CO}_2$  (Gill:  $p=0.040$ , Liver:  $p=0.003$ ) were found, and in liver tissues, a main effect of temperature ( $p=0.020$ ) and a significant interaction between time, temperature and  $p\text{CO}_2$  was found ( $p=0.002$ ). In both gill and liver tissues, there was a large increase in CS activity across all treatments relative to control values at 7-days of acclimation. This increase continued until 28-days of acclimation, after which, a rapid drop in CS activity was seen at 56-days of acclimation (Fig. 4.4A and B). The interaction found in the liver tissues between time, temperature and  $p\text{CO}_2$  appears to be largely antagonistic in nature (Fig. 4.5 B, D and F).

*P. borchgrevinki*: We noted a tissue-specific response in CS activity in *P. borchgrevinki*. Gill tissues showed a main effect of time ( $p<0.001$ ) and temperature ( $p=0.020$ ), while liver tissues only showed a main effect of time ( $p=0.006$ ). Gill tissues in

all treatments except control showed an increase in CS activity from 7 to 28-days of acclimation, followed by a decrease in activity at 56-days (Fig. 4.4C). Alternatively, CS activity in liver tissues remained relatively stable, with a slight decline in activity over the course of the experiment in all treatments when compared to control values (Fig. 4.4D).

*T. newnesi*: Only small changes in CS activity were noted in tissues from *T. newnesi*. While liver tissues remained around control levels, there was a small increase in activity in the high temperature treatments at 28 days of acclimation, and in the high  $p\text{CO}_2$  treatment at 56-days of acclimation, although these changes were not statistically significant. Tissues from *T. newnesi* also displayed the lowest values of CS activity when compared to *T. bernacchii* and *P. borchgrevinki* (Fig. 4.4E and F).

#### Lactate Dehydrogenase Activity

*T. bernacchii*: Overall, tissues from *T. bernacchii* showed the lowest levels of LDH activity. Both gill and liver tissues showed a main effect of time ( $p < 0.001$ ), and liver tissues showed a main effect of temperature ( $p = 0.017$ ). In both tissues, activity in treatments stayed right at control levels, until 56-days of acclimation, where they increased significantly, with the multi-stressor treatment showing the greatest increase in activity level (Fig. 4.6).

*P. borchgrevinki*: We noted a tissue-specific response in *P. borchgrevinki*. Gill tissues showed a significant main effect of acclimation time ( $p = 0.038$ ), while no significance was found in liver tissues. Gill tissues from *P. borchgrevinki* displayed a similar response to those from *T. bernacchii*, with all treatment activity levels increasing compared to control values at 56-days of acclimation. However, unlike the response

noted from *T. bernacchii*, the high  $p\text{CO}_2$  treatment activity increased almost as much as the multi-stressor treatment. The LDH activity levels in gill tissues were the highest seen across fish species in this study (Fig. 4.6A and B). In liver tissues, activity levels remained stable, even at 56-days of acclimation, with only a minor increase in LDH activity noted in the high temperature treatment (Fig. 4.6C and D).

*T. newnesi*: A main effect of temperature was seen in gill tissues from *T. newnesi* ( $p=0.030$ ). LDH levels in gill tissues increased from 7 to 42-days of acclimation in all treatments relative to control, with the high temperature treatments increasing the most (Fig. 4.6A and B). Levels of LDH activity did increase in the multi-stressor treatment in liver tissues, but this increase was not statistically significant (Fig. 4.6C and D).

#### 4.5 DISCUSSION

In an era of research devoted to Global Climate Change, one of the main questions put forth by scientists is whether or not species can acclimate and in turn adapt to predicted changes of oceanic waters. In the case of three notothenioid fish, *Trematomus bernacchii*, *Pagothenia borchgrevinki*, and *Trematomus newnesi*, the answer is complex. In this study, we attempted to discern the acclimation capacity of these three species to an increase in temperature and/or an increase in  $p\text{CO}_2$  level. While *T. bernacchii*, *P. borchgrevinki*, and *T. newnesi* have evolved in the same stable, cold environment, these fishes do not physiologically respond to factors of Global Climate Change in the same manner. Below, we offer some insights into both the whole-organism response as well as a biochemical response of three species of notothenioid to both single and multiple stressors. We believe that these data provide support to the idea that while it may appear some species are capable of acclimating to predicted changes on a whole-

organismal scale, examination of cellular-level processes illustrate a different picture, and the perceived acclimation is really the result of energetic trade-offs.

#### Whole organism response

Any change in the demand for ATP at the tissue-level is reflected in the rate of organismal oxygen consumption. In *T. bernacchii*, and *T. newnesi*, Resting Metabolic Rates in both high temperature treatments were drastically elevated above control values across all experimental endpoints, signaling a need for energy, as biochemical reaction rates increase with temperature (Hochachka and Somero, 2002). Previous work on *P. borchgrevinki* has shown that these fish are capable of acclimating to 4°C (Franklin et al., 2007; Robinson and Davison, 2008a, b). Our data follow a similar trend, showing that although elevated at 56-days of acclimation, RMR's in the two high temperature treatments were not significantly different from control values. Fish acclimated to high  $p\text{CO}_2$  levels increased oxygen consumption over short time scales, but values quickly returned to control levels. These findings concur with previous studies which have shown that when placed in a hypercapnic environment, metabolic depression occurs, signaled by a decrease in oxygen consumption rate and hence, a decrease in aerobic scope (Langenbuch and Pörtner, 2003; Strobel et al., 2012). It has also been illustrated that placing fish into a hypercapnic environment stimulates a shift in metabolic pathways from aerobic to anaerobic (Michaelidis et al., 2007), decreasing oxygen consumption. The RMR's from *T. newnesi* in this study do not concur with previous findings from our lab (Enzor et al., 2013). In these previous findings, RMR values from *T. newnesi* were elevated above control values in fish acclimated to a high  $p\text{CO}_2$  environment at 28-days. While the RMR's from this study at 28-days of acclimation in the high  $p\text{CO}_2$  treatment

are elevated, they are not statistically significant, which is likely a result of the overall higher RMR's we noted in fish from our 2012 season when compared to our 2011 season.

Temperature also played a significant role in the condition factor of both *T. bernacchii* and *T. newnesi*, decreasing K-values in both fish over time. In *P. borchgrevinki*, a change in  $p\text{CO}_2$  appeared to be the driving force behind a decline in condition factor. As fish in our study were fed to eliminate the possibility that food was a limiting factor, this decrease is likely due to a decrease in aerobic scope for these species.

#### Cellular-level response

While previous studies have demonstrated notothenioid fishes can increase heat tolerance during warm acclimation (Bilyk and DeVries, 2011; Bilyk et al., 2012), these studies examined a whole organismal response, leaving the cellular level effects largely unexplored. The previous work on *P. borchgrevinki* which illustrated that these fish have the capacity to acclimate to increases in temperature specifically by upregulating energy producing metabolic processes, shown by increased Cytochrome C Oxidase activity and Lactate Dehydrogenase activity (Seebacher et al., 2005). Alternatively, Weinstein and Somero have demonstrated that *T. bernacchii* cannot acclimate to increases in temperature as there was no mitochondrial compensation (1998) when fish were placed in 4°C for 14 days. This was confirmed in another species of notothenioid, *Notothenia rossii*, by Strobel et al. (2013b), where fish were placed in elevated temperature and  $p\text{CO}_2$  treatments for 4-6 weeks.

In the notothenioid species in this study, it appears the capacity to acclimate to increases in temperature and/or  $p\text{CO}_2$  are present, but limited. Individuals of *T.*

*bernacchii* showed a rapid increase of CS activity to 28-days of acclimation, followed by a swift decline in CS activity coupled with an increase in LDH activity at 56-days. This increase in CS and LDH activities correlates with a decline in protein carbonyl formation (Enzor and Place, 2014), lending evidence to the idea that *T. bernacchii* are combating reactive oxygen species formation brought about by increased oxygen consumption by switching over to an anaerobic energy pathway. As reactive oxygen species are primarily formed in the third mitochondrial complex of the electron transport chain (Murphy, 2009), switching over to an anaerobic pathway would decrease reactive oxygen species formation and subsequent oxidative damage. A similar trend was also seen in gill tissues from *P. borchgrevinki*. Liver tissues from this fish showed a different response when analyzed for citrate synthase and lactate dehydrogenase activity, illustrating the idea that different metabolic regulation processes exist for different tissues, and energetic trade-offs likely exist across tissue types (Strobel et al., 2013a,b). The use of lipid stores seen in tissues from the multi-stressor treatment also points towards aerobic metabolism being maintained in liver tissues from *P. borchgrevinki*, versus an anaerobic pathway being utilized. Additionally, work by Torres and Somero found that LDH levels increased with activity level in Antarctic fishes (1988). As *P. borchgrevinki* are cryo-pelagic compared to the benthic *T. bernacchii*, this could explain the differences in LDH activity levels seen in this study.

A rapid increase in LDH activity was also noted by Jayasundara et al. (2013), and demonstrate that while the anaerobic capacity of notothenioid fishes has always thought of as limited (Pörtner et al., 2005), it appears this surge in lactate dehydrogenase is likely due to a shift in metabolic fuel preference versus an increase in anaerobic capacity. This



supposition is further supported by the findings that Antarctic fish will shift from using a lipid-fueled metabolic pathway, to an anaerobic pathway as the primary ATP generating mechanism when placed under thermal stress (Windisch et al., 2011) or stress induced by hypercapnia (Michaelidis et al., 2007). This switch may explain the small decrease in RMR's noted over time as carbohydrate oxidation via glycolysis, overall, consumes less energy than a lipid-reliant pathway (Windisch et al., 2011). This line of reasoning also explains the minor changes in total triglycerides seen over time in this study as small increases in lipid content noted here may be further indication of a change in metabolic substrate preference.

In *T. newnesi*, the lack of citrate synthase activity seen can possibly be explained by an increase in bicarbonate ions. Our previous results have shown that *T. newnesi* will prioritize the re-establishment of acid-base equilibrium above other cellular processes such as repairing oxidative damage (Enzor and Place, 2014; Enzor and Place, in review). It has been shown that Antarctic fish will accumulate  $\text{HCO}_3^-$  ions in an attempt to re-establish acid-base balance (Strobel et al., 2012), and high bicarbonate levels have been shown to inhibit citrate synthase function (Simpson, 1967). Previous work by Strobel and colleagues has also illustrated that when placed into a high temperature and/or high  $p\text{CO}_2$  environment, a lack of increase in CS activity was explained by a shift in TCA-cycle intermediates away from the electron transport chain and into gluconeogenesis (2013a,b). The lack of lactate dehydrogenase activity seen in tissues from *T. newnesi* supports the idea that these fish maintain aerobic energy pathways and do not utilize the same energetic trade-offs as *T. bernacchii* and *P. borchgrevinki*.

In summary, it appears that *Trematomus bernacchii*, *Pagothenia borchgrevinki*, and *Trematomus newnesi* have a limited ability to compensate for increases in temperature and  $p\text{CO}_2$ . The sharp increase in resting metabolic rates seen in this study over short acclimation times (i.e. up to 4 weeks) point towards a rapid restructuring on the cellular level as fish attempt to re-establish homeostasis. Over longer time scales, it appears that fish utilize energetic trade-offs in order to compensate for rising basal maintenance costs and additional energy requirements associated with acclimating to a changing environment. The trade-offs likely come at the expense of long-term processes such as growth, and may indeed have a negative effect on reproduction as well.

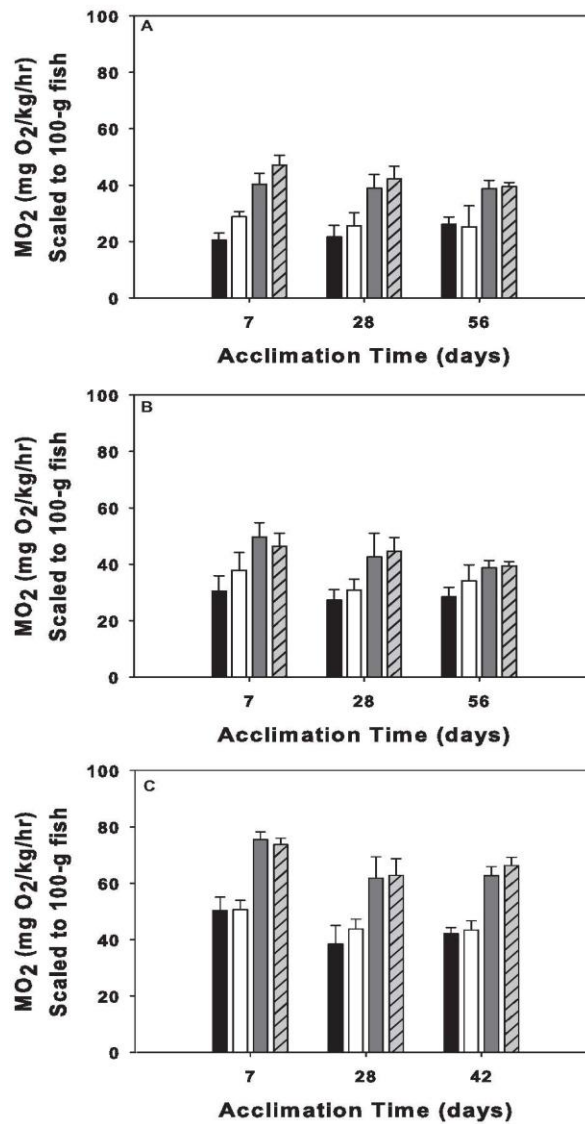


Figure 4.1. Resting Metabolic Rates ( $\pm$ SE) of *T. bernacchii* (A), *P. borchgrevinki* (B) and *T. newnesi* (C) acclimated at 7, 28 and 42/56 days to treatments of ambient conditions, (Low Temperature + Low pCO<sub>2</sub>; black bars), Low Temperature + High pCO<sub>2</sub> (white bars), High Temperature + Low pCO<sub>2</sub> (dark grey bars) and High Temperature + High pCO<sub>2</sub> (light grey bars with crosshatch's)

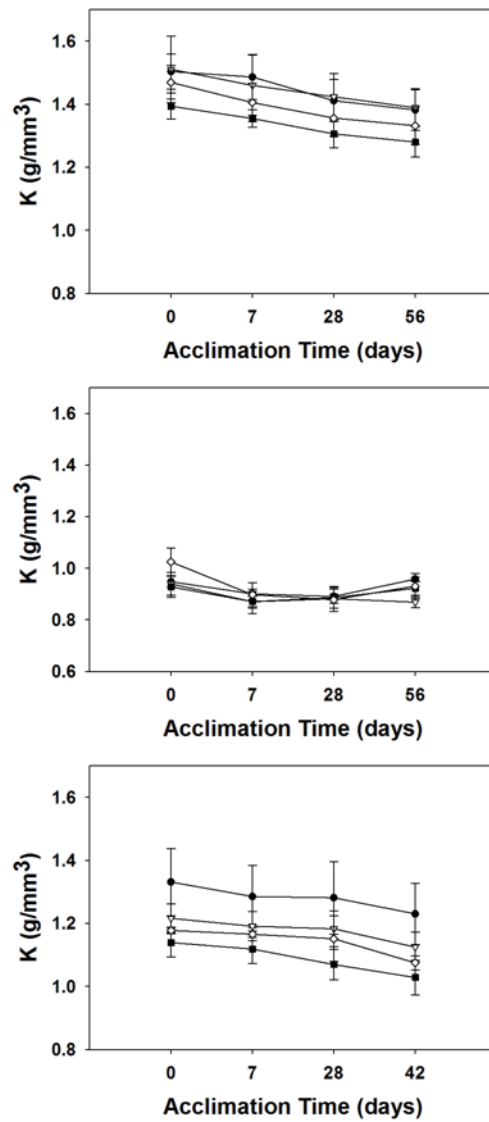


Figure 4.2. Fulton's Index ( $\pm$ SE) of *T. bernacchii* (A), *P. borchgrevinki* (B) and *T. newnesi* (C) acclimated at 7, 28 and 42/56 days to treatments of ambient conditions, (Filled circles), Low Temperature + High  $p\text{CO}_2$  (filled triangles), High Temperature + Low  $p\text{CO}_2$  (filled squares) and High Temperature + High  $p\text{CO}_2$  (open diamonds)

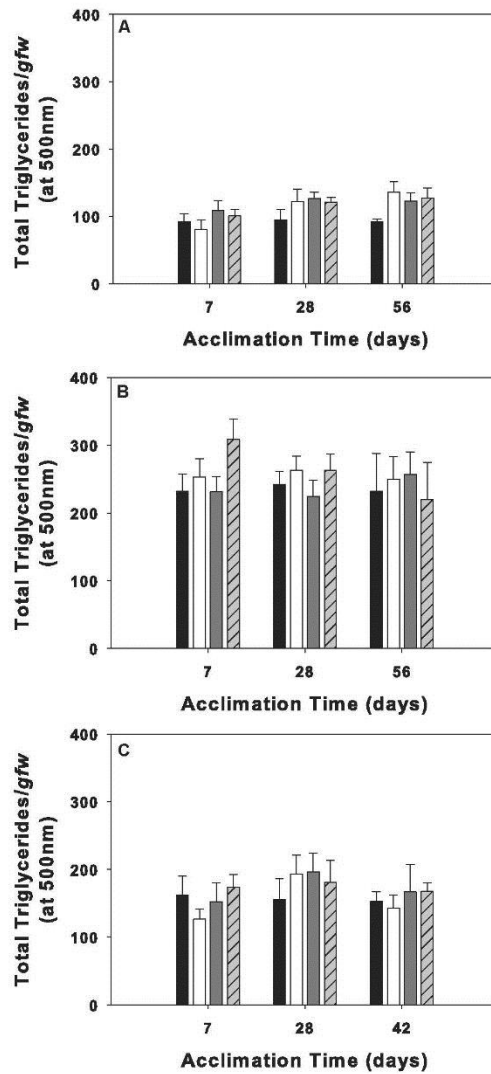


Figure 4.3. Total triglycerides ( $\pm$ SE) from white muscle of *T. bernacchii* (A), *P. borchgrevinki* (B) and *T. newnesi* (C) acclimated at 7, 28 and 42/56 days to treatments of ambient conditions, (Low Temperature + Low  $p\text{CO}_2$ ; black bars), Low Temperature + High  $p\text{CO}_2$  (white bars), High Temperature + Low  $p\text{CO}_2$  (dark grey bars) and High Temperature + High  $p\text{CO}_2$  (light grey bars with crosshatch's)

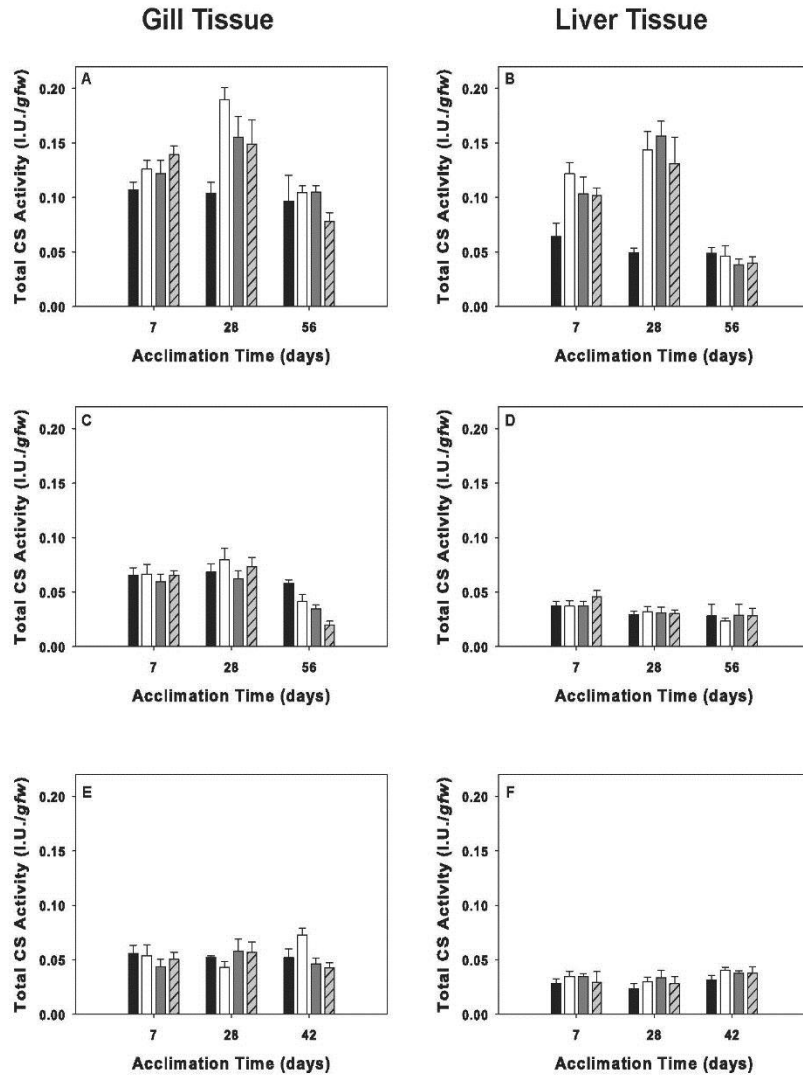


Figure 4.4. Citrate Synthase Enzyme Activity ( $\pm$ SE) of *T. bernacchii* gill and liver tissues (A and B), *P. borchgrevinki* gill and liver tissues (C and D) and *T. newnesi* gill and liver tissues (E and F) acclimated at 7, 28 and 42/56 days to treatments of ambient conditions, (Low Temperature + Low  $p\text{CO}_2$ ; black bars), Low Temperature + High  $p\text{CO}_2$  (white bars), High Temperature + Low  $p\text{CO}_2$  (dark grey bars) and High Temperature + High  $p\text{CO}_2$  (light grey bars with crosshatch's)

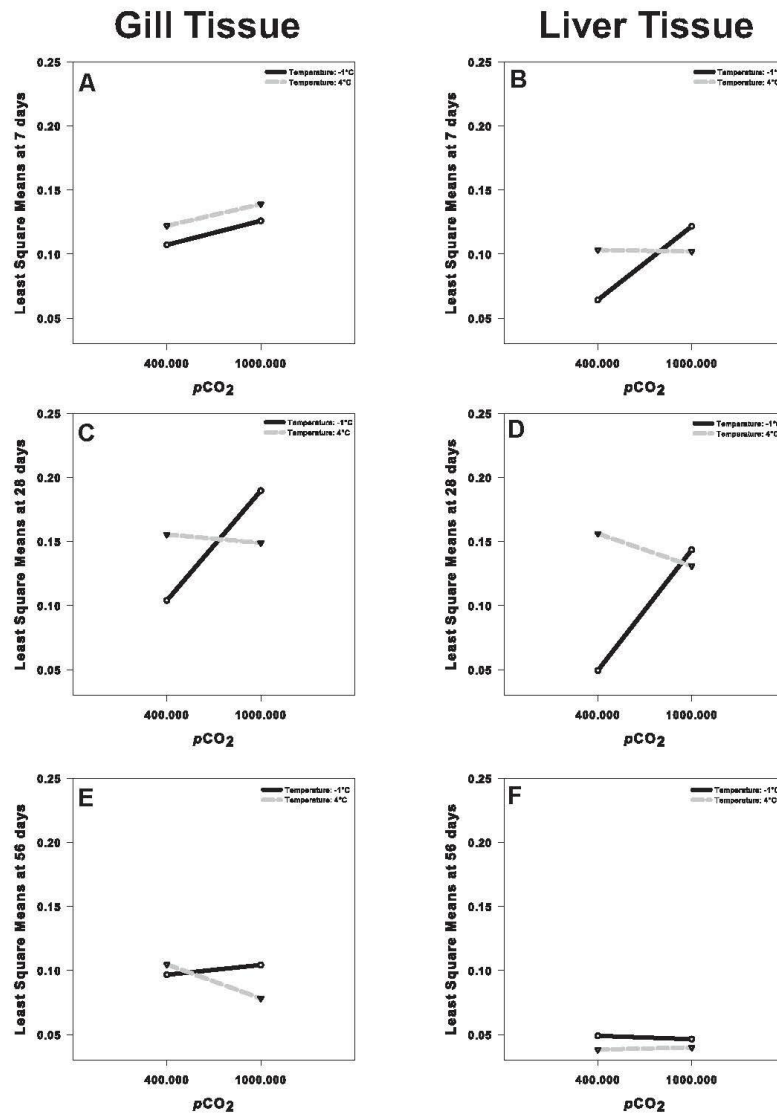


Figure 4.5. Citrate Synthase Enzyme Activity interaction plots of *T. bernacchii* gill and liver tissues acclimated for 7-days (gill: A, liver: B), 28-days (gill: C, liver: D), and 56-days (gill: E, liver: F)

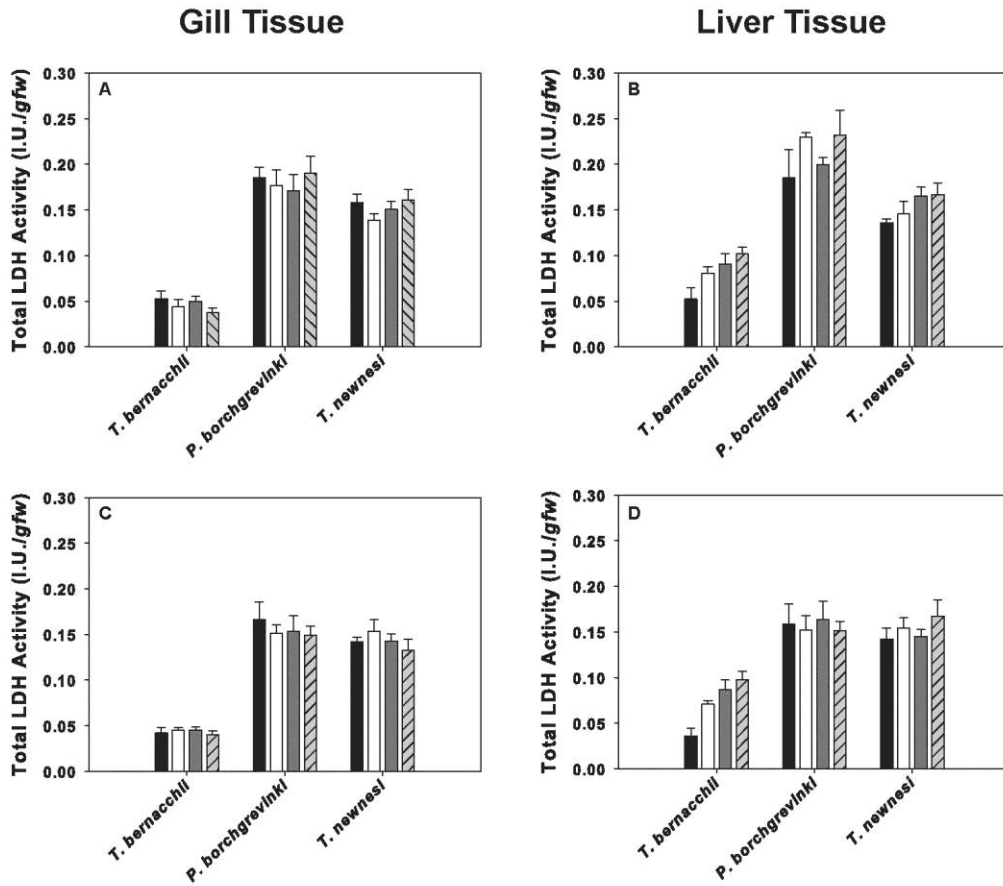


Figure 4.6. Lactate Dehydrogenase Enzyme Activity ( $\pm$ SE) of *T. bernacchii*, *P. borchgrevinki*, and *T. newnesi* gill and liver tissues (A and B), *P. borchgrevinki* gill and liver tissues (C and D) and *T. newnesi* gill (A and C) and liver tissues (B and D) acclimated at 7 (A and B) and 42/56 (C and D) days to treatments of ambient conditions, (Low Temperature + Low  $p\text{CO}_2$ ; black bars), Low Temperature + High  $p\text{CO}_2$  (white bars), High Temperature + Low  $p\text{CO}_2$  (dark grey bars) and High Temperature + High  $p\text{CO}_2$  (light grey bars with crosshatch's)



Table 4.1. Mean measurements of  $p\text{CO}_2$  and Temperature  $\pm$  SD over the course of the 2011 and 2012 field seasons

2011 Season	$p\text{CO}_2$ ( $\mu\text{atm}$ )	Temperature ( $^{\circ}\text{C}$ )	2012 Season	$p\text{CO}_2$ ( $\mu\text{atm}$ )	Temperature ( $^{\circ}\text{C}$ )
Incoming Seawater	$417.15 \pm 12.26$	$-1.24 \pm 0.08$	Incoming Seawater	$427.66 \pm 23.97$	$-1.03 \pm 0.152$
Low Temperature + Low $p\text{CO}_2$	$438.82 \pm 16.08$	$-0.61 \pm 0.17$	Low Temperature + Low $p\text{CO}_2$	$432.04 \pm 22.50$	$-0.707 \pm 0.153$
Low Temperature + High $p\text{CO}_2$	$953.89 \pm 50.38$	$-0.45 \pm 0.16$	Low Temperature + High $p\text{CO}_2$	$1024.76 \pm 94.20$	$-0.578 \pm 0.150$
High Temperature + Low $p\text{CO}_2$	$525.11 \pm 21.07$	$4.02 \pm 0.44$	High Temperature + Low $p\text{CO}_2$	$525.16 \pm 22.41$	$3.86 \pm 0.484$
High Temperature + High $p\text{CO}_2$	$1026.66 \pm 9.03$	$4.22 \pm 0.56$	High Temperature + High $p\text{CO}_2$	$1053.44 \pm 71.87$	$4.03 \pm 0.316$

## CONCLUSION

While the notothenioid species examined in this research all evolved during the same time frame and under the same environmental conditions, it has become clear that not all species have the same physiological response when placed into an increased temperature and/or increased  $p\text{CO}_2$  environment (Kroeker et al., 2010; 2013). Not only were there distinct differences between fish species in the biochemical pathway defended first, there were also differences in the timing of response. Regardless of these differences, it appears that these fish employ energetic trade-offs to physiologically cope with a changing environment. While it does appear that these fish can acclimate to environmental perturbations to some degree on the cellular level, the costs of acclimation will likely have whole-organism impacts, such as decreases in growth, reproduction, and overall population numbers for notothenioids of the Southern Ocean. In order to ascertain exactly what the long-term impacts on the notothenioids will be, studies on multiple generations will need to be performed.

Below, I offer some general conclusions and possible rationales for the responses seen in *Trematomus bernacchii*, *Pagothenia borchgrevinki*, and *Trematomus newnesi* in the face of a multi-stress scenario.

Resting Metabolic Rates and Fulton's Index: Oxygen consumption rates were significantly elevated above control levels in all species at 7 and 28-days of acclimation. These elevated RMR's indicate increased energy expenditure to cope with increased

temperature which results in an increased need for energy production; a universal response (Fry, 1947). While oxygen consumption did decrease slightly over the course of the experiment in *T. bernacchii* and *T. newnesi*, at 42/56-days of acclimation, these levels were still significantly elevated above control values. Fulton's index in these species, while not statistically significant, did slightly decrease over time. In *P. borchgrevinki*, RMR's were also elevated after 56-days of acclimation; however they are no longer significantly elevated above control values, indicating these fish may not need as much energy at this time point. This is also supported by the slight increase of Fulton's condition factor at 56-days. These findings fit with previous data, which have illustrated that *P. borchgrevinki* is capable of acclimating to 4°C (Seebacher et al., 2005; Robinson and Davison, 2008a,b), however *T. bernacchii* cannot acclimate to increases in temperature (Weinstein and Somero, 1998). These findings for *T. bernacchii* and *T. newnesi* also concur with other studies of notothenioid fish, which show that acclimation to an increase in temperature and  $p\text{CO}_2$  results in an increase in RMR, regardless of acclimation time (Strobel et al., 2012).

Lipid Use: The slight decrease in body condition noted in *T. bernacchii* in conjunction with the fact these fish seem to be depositing lipid stores over time versus using them lend evidence to the idea these fish are utilizing a metabolic pathway shift away from aerobic processes. Interestingly, it appears *P. borchgrevinki* may continue to use lipid peroxidation to some degree in the multi-stress treatment as triglyceride levels declined over time. *Trematomus newnesi* triglyceride levels did not change over time, indicating these fish were not utilizing lipid stores to combat energy needs.

Energetic Enzymes: In *T. bernacchii*, a large increase in citrate synthase activity was seen at the 7 and 28-day time-points (indicating fish are attempting to compensate for an increased need for energy), and at 56-days, there was a sharp decline in citrate synthase activity with a concomitant increase in lactate dehydrogenase activity; pointing towards a switch from an aerobic to an anaerobic energy pathway. In *P. borchgrevinki*, the same response in citrate synthase activity was noted, but the response was muted; enzyme activity levels were not as high in these fish. However, the same decline in CS activity was seen at 56-days, coupled with a rise in lactate dehydrogenase activity, again signaling a change from an aerobic to an anaerobic energy pathway. This pathway switch has also been noted in previous studies on both polar and non-polar fish in response to an increase in temperature (Strobel et al., 2013a; Windisch et al., 2011) or an increase in  $p\text{CO}_2$  (Michaelidis et al., 2007). There was a markedly different response noted in *T. newnesi*. Citrate synthase activity increased over time with no comparable increase in lactate dehydrogenase activity, indicating these fish retain use of an aerobic pathway for energy versus switching to an anaerobic pathway.

Acid-base Balance: *Trematomus bernacchii* showed a large increase in CAII protein levels over time, indicative of an attempt to decrease energy use, yet no increased  $\text{Na}^+/\text{K}^+$  ATPase capacity was seen, lending evidence that these fish were putting forth energy into re-establishing acid-base balance, but were not accomplishing it. Levels of CA II were most notable at 28 days of acclimation in *P. borchgrevinki*, illustrating individuals from this species do not put forth as much energy into re-establishing acid-base balance, shown by the lack of increase in  $\text{Na}^+/\text{K}^+$  ATPase capacity. Unlike *T. bernacchii* and *P. borchgrevinki*, *T. newnesi* appear to actively defend acid-base balance,

illustrated by the increase in  $\text{Na}^+/\text{K}^+$  ATPase capacity noted across time. There was also not the rapid increase in CAII protein levels during shorter acclimation times noted in *T. newnesi*. Previous findings have shown that notothenioid fish defend acid-base balance when confronted with an increase in temperature by increasing  $\text{Na}^+/\text{K}^+$  ATPase capacity (Gonzalez-Cabrera et al., 1995; Guynn et al., 2002). It would seem that the additional stress of increased  $p\text{CO}_2$  alters the ability of *T. bernacchii* and *P. borchgrevinki* to defend acid-base balance efficiently.

**Oxidative Damage and Antioxidant Capacity:** In *T. bernacchii*, there was a decrease in oxidative damage over time with no increase in antioxidant capacity, potentially indicating that despite an increased potential for ROS production due to elevated oxygen consumption, these fish are less susceptible to cellular damage under these predicated changes. Additionally, the increase in ROS production due to increased oxygen consumption may be partially off-set as protein turnover would decrease at higher temperatures, effectively helping to lower energy expenditure. Also similar to *T. bernacchii*, *P. borchgrevinki* showed a rapid decline in oxidatively damaged protein over time, with no increase in antioxidant capacity. These trends fit with previous studies, which have illustrated that an increase in temperature does not result in an increase in antioxidant capacity in notothenioid fishes (Mueller et al., 2011; 2012). Tissues from *T. newnesi* did not show the same decrease in oxidative damage as seen in the other species, and the antioxidant response while not significant, was much more variable.

Overall, it appears that all three species are utilizing energetic trade-offs as part of the acclamatory response. When placed in a multi-stressor scenario, the basal maintenance costs of *T. bernacchii*, *P. borchgrevinki*, and *T. newnesi* are increased, and

combining the evidence of increased RMR's with cellular level effects illustrates these fishes show a markedly different response to an increase in temperature and  $p\text{CO}_2$ . For *T. bernacchii*, changing from an aerobic pathway to a glycolytic or possible anaerobic pathway in conjunction with no use of lipid stores points towards a metabolic pathway shift. The sharp decrease in oxidative damage with no increase in antioxidant capacity, combined with a small amount of energy being put forth to re-establishing acid-base equilibrium, illustrate that this species appears to put energy into defending oxidative damage first. While it appears that *P. borchgrevinki* has the largest capacity of the three notothenioid species examined to acclimate to a new environment, shown by the decline in RMR's, these fish are also repairing oxidatively damaged proteins first, sacrificing cellular homeostasis. Like *T. bernacchii*, it appears *P. borchgrevinki* may also be utilizing a metabolic pathway shift. Unlike the other two species studied, *Trematomus newnesi* appears to put more emphasis on re-establishing acid-base balance versus combating reactive oxygen species when confronted with a changing environment. While there was a slight decline in body condition over time, there was no evidence of a metabolic pathway shift in this species, shown by no change in citrate synthase or lactate dehydrogenase activities. Regardless of these differences, the energetic trade-offs noted in these fish may shunt energy away from processes such as growth and reproduction, which may have long-term effects on population numbers.

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