

INDUCTION OF HEAT SHOCK PROTEINS IN COLD- ADAPTED AND COLD-
ACCLIMATED FISHES


By

Laura Elizabeth Teigen

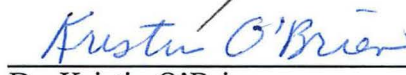
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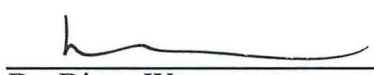
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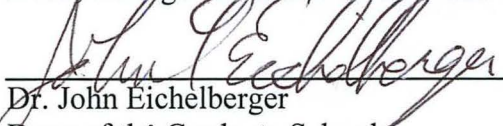


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INDUCTION OF HEAT SHOCK PROTEINS IN COLD- ADAPTED AND COLD-
ACCLIMATED FISHES

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THESIS

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Abstract

I examined the effects of oxidative stress and changes in temperature on heat shock protein (Hsp) levels in cold-adapted and cold-acclimated fishes. Adaptation of Antarctic notothenioids to cold temperature is correlated with high levels of Hsps, thought to minimize cold-induced protein denaturation. Hsp70 levels were measured in red- and white-blooded Antarctic notothenioid fishes exposed to their critical thermal maximum (CTMax), 4°C warm acclimated, and notothenioids from different latitudes. I determined the effect of cold acclimation on Hsp levels and the role of sirtuins in regulating Hsp expression and changes in metabolism in threespine stickleback, *Gasterosteus aculeatus*, cold-acclimated to 8°C. Levels of Hsps do not increase in Antarctic notothenioids exposed to their CTMax, and warm acclimation reduced levels of Hsp70. Hsp70 levels were higher in Antarctic notothenioids compared to a temperate notothenioid and higher in white-blooded notothenioids compared to red-blooded notothenioids, despite higher oxidative stress levels in red-blooded fish, suggesting Hsp70 does not mitigate oxidative stress. Cold acclimation of stickleback resulted in tissue-specific increases in some Hsps and sirtuins. My research indicates that cold acclimation increases Hsp levels, and moderate increases in temperature reduce Hsp levels in cold-adapted fishes. Together, these data lend support to the hypothesis that cold denatures proteins.

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Introduction

Temperature can have profound effects on ectotherms. Specifically, decreases in temperature can lead to decreases in levels of ATP (Hochachka and Somero, 2002), warranting metabolic remodeling to maintain ATP levels. This occurs through increases in the concentration of aerobic metabolic enzymes and/or mitochondrial biogenesis (Egginton and Sidell, 1989; Johnston and Maitland, 1980; Orczewska et al., 2010).

Additionally, studies comparing activities of antioxidants between cold-adapted and temperate ectotherms suggest higher levels of oxidative stress in animals inhabiting colder environments. Oxidative stress results from an imbalance between rates of reactive oxygen species (ROS) production and the cell's capacity to defend against the damaging effects of ROS, leading to an accumulation of damaged macromolecules (Berlett and Stadtman 1997). The maximal activity of the antioxidant superoxide dismutase (SOD) is higher in the polar molluscs *Nacella concinna*, *Tonicella marmoreal*, *Margarites helycinus*, and *Yoldia eightsi* compared to the temperate molluscs *Cerastoderma edule*, *Mya arenaria*, and *Scrobicularia plana* (Abele and Puntarulo 2004). Likewise, maximal activity of the enzyme catalase (CAT) is higher in the Antarctic scallop *Adamussium colbecki* compared to molluscs from the Mediterranean sea (*Mytilus galloprovincialis* and *Pecten jacobaeus*) (Regoli et al. 1997).

Cold acclimation in ectothermic organisms is also correlated with increases in oxidative stress (Heise et al. 2007; Ibarz et al. 2010; Tseng et al. 2011). Levels of protein carbonyls, an indirect measure of ROS, and the maximal activity of SOD is higher in

liver and pectoral muscle of threespine stickleback (*Gasterosteus aculeatus*) cold acclimated to 8°C compared to animals at 20°C (Kammer et al. 2011). Similarly, the eelpout *Zoarces viviparus* has higher levels of peroxidized lipids and carbonyls, and higher SOD activity when acclimated to 6°C compared to at 12°C (Heise et al. 2007). A decline in temperature can also alter levels of heat shock proteins (Hsps) in ectotherms.

Hsps are conserved among prokaryotes and eukaryotes, and are found in two forms: Hsp, which is stress inducible, and Hsc, which is thought to be constitutively expressed at a constant level. The heat shock response (HSR) results in rapid synthesis of Hsps following heat stress (Lindquist 1986). The different families of Hsps are categorized according to molecular mass and include Hsp100, Hsp90, Hsp70, Hsp60, and the small Hsps (Mogk et al. 2001). Hsps have multiple functions, including protein folding, transmembrane transfer of proteins, and assembly of protein complexes (Feder and Hofmann 1999).

Studies in cold-adapted Antarctic fishes suggest that cold denatures proteins, warranting higher levels of Hsps compared to fishes inhabiting warmer waters. Levels of ubiquitin-conjugated proteins are higher in Antarctic fishes, including the emerald rockcod (*Trematomus bernacchii*), sharp-spined notothenia (*Trematomus pennellii*), and the bald notothen (*Pagothenia borchgrevinki*) compared to the closely-related temperate thornfish (*Bovichtus variegatus*) and Maori chief (*Notothenia angustata*) inhabiting New Zealand (Place et al. 2004; Todgham et al. 2007). Levels of Hsps are also higher in Antarctic species compared to temperate ones. Transcript levels of *HSP70* are higher in *T. bernacchii* compared to both *B. variegatus* and *N. angustata* (Place et al. 2004).

Similarly, the Antarctic limpet *N. concinna* has higher expression of *HSC70* compared to related South American limpets *Nacella magellanica* and *Nacella deaurata* (Koenigstein et al. 2013).

While Antarctic fishes maintain high constitutive levels of Hsps, they are incapable of inducing a HSR, once thought to be ubiquitous among all organisms. *T. bernacchii* was the first Antarctic notothenioid documented to have lost the HSR when a 2h heat shock at 10°C failed to detect increased synthesis of Hsps in brain, gill, heart, liver, or spleen (Hofmann et al. 2000). Further experiments revealed that related New Zealand species (*B. variegatus* and *N. angustata*) possess a HSR, indicating that the loss of a HSR occurred in *T. bernacchii* during their evolution in the icy cold Antarctic waters (Place et al. 2004).

To date, the HSR has only been examined in notothenioids inhabiting the Ross Sea, where temperatures are stable at -1.9°C (Littlepage 1965). The Western Antarctic Peninsula (WAP) region experiences temperatures that fluctuate seasonally from +1.5°C in the summer to -1.8°C in the winter (DeWitt 1971), and sea surface temperatures in the WAP have increased by 1.5°C since 1950, 1°C higher than the global mean (Folland et al. 2001). Warmer temperatures in the WAP are correlated with a greater heat tolerance in notothenioids from the WAP compared to notothenioids from the Ross Sea (Bilyk and DeVries 2011), which may be due to their ability to induce a HSR.

The HSR has also yet to be characterized in Antarctic fishes lacking the oxygen binding protein hemoglobin (Hb). Evidence in the literature suggests that +Hb (red-blooded) species have higher levels of oxidative stress and greater rates of protein

synthesis compared to icefishes lacking Hb (-Hb) (Mueller et al. 2012; Haschemeyer 1983), and may warrant higher levels of Hsps. Oxidative stress can lead to an accumulation of damaged proteins (Berlett and Stadtman 1997). These damaged proteins need to be replaced by newly synthesized ones that are folded by Hsps, potentially leading to increased levels of Hsps. For example, exposure to cyanobacteria leads to increases in malondialdehyde, a marker for lipid peroxidation and oxidative stress, and increased protein levels of Hsp70 in juvenile bighead carp (*Hypophthalmichthys nobilis*) (Sun et al. 2013). Together, this suggests that the higher levels of oxidative stress and greater rates of protein synthesis found in red-blooded notothenioids may be correlated with higher levels of Hsps compared to notothenioids lacking Hb.

The high levels of Hsps in cold-adapted fishes such as the *T. bernacchii* suggests that Hsps might also increase in temperate fishes during acclimation to cold temperature. Elevations in Hsps in response to cold stress have been documented in a number of ectothermic organisms, but few studies have investigated changes in Hsp levels in fishes during cold acclimation. Cold shock of 4-5°C for 1-5h in carp (*Cyprinus carpio* and *Ctenopharyngodon idella*) results in increased expression of *HSP90* in blood, brain, intestine, liver, skin, and spleen, and increased expression of *HSC70* in blood, muscle, and skin (Ferencz et al. 2012; Ali et al. 2003; Wu et al. 2012). Additionally, *HSP101*, *HSP70*, *HSP60*, and *HSP17* are induced during cold exposure in both plants and insects (Rinehart et al. 2007; Lopez-Matas et al. 2004; Janska et al. 2011). Based on these findings, I hypothesized that levels of Hsps may increase during cold acclimation of fishes.

The expression of Hsps is regulated by heat shock factor 1 (Hsf1). Hsf1 is an inducible transcription factor that binds to the promoter region of *HSP* genes to activate transcription (Satyal et al. 1998). Sirtuin 1 (Sirt1) regulates expression of Hsps by deacetylating lysine residue K80 within the DNA binding domain of Hsf1, promoting Hsf1 binding to the promoter region of *HSP* genes (Westerheide et al. 2009).

Sirtuins are a family of NAD-dependent deacetylases and ADP-ribosyltransferases that, in addition to mediating oxidative stress, regulate metabolism (Ahn et al. 2008; Osborne et al. 2013). In mammals, Sirt1 regulates mitochondrial biogenesis (Lagouge et al. 2006) and Sirt1 activity is positively correlated with citrate synthase (CS) activity and cytochrome c oxidase (COX) protein levels in hearts and muscle of rats (Gurd et al. 2011). Cold acclimation in fishes also alters metabolism to offset the depressive effects of cold temperature on enzyme activity (Egginton and Sidell 1989; Johnston and Maitland 1980). For example, mitochondrial volume density increases in the muscle tissue of striped bass (*Morone saxatilis*) and threespine stickleback during cold acclimation (Egginton and Sidell 1989; Orczewska et al. 2010). Activity of aerobic metabolic enzymes CS and COX also increase in killifish (*Fundulus heteroclitus*) and stickleback in response to cold acclimation (Fangue et al. 2009; Orczewska et al. 2010). The molecular mechanisms driving metabolic changes in fishes are largely unknown, but may be mediated by sirtuins.

I investigated the interrelationship between cold temperature and heat shock proteins in both cold-adapted and cold-acclimated fishes and tested the following hypotheses:

(1). Evolution in the warm and temperature-variable Western Antarctic Peninsula Region maintained a heat shock response in notothenioid fishes inhabiting this region. To test this hypothesis, *Notothenia coriiceps* (+Hb) and *Chaenocephalus aceratus* (-Hb) were exposed to an acute heat stress (critical thermal maxima, CTMax), and *N. coriiceps* and *Chionodraco rastrospinosus* (-Hb) were warm acclimated to 4°C for 1 wk. Protein and mRNA levels of Hsp70 were quantified in heart ventricle tissue of all species.

(2). Higher levels of oxidative stress in hearts of red-blooded notothenioids will be correlated with higher levels of Hsps compared to -Hb (white-blooded) fishes. To test this hypothesis, protein and mRNA levels of Hsp70 were quantified in heart ventricle of both red- and white-blooded fishes from the WAP (*C. aceratus*, *C. rastrospinosus*, *Champscephalus gunnari* (-Hb), *Gobionotothen gibberifrons* (+Hb), *N. coriiceps*), *Pagothenia borchgrevinki* (+Hb) from the Ross Sea, and *Eleginops maclovinus* (+Hb) from Patagonia.

(3). Levels of Hsps will increase during cold acclimation of the temperate threespine stickleback and increased levels of Hsps will correlate with increased levels of sirtuins. To test these hypotheses, threespine stickleback were either warm acclimated (20°C) or cold acclimated (8°C) for 9 wk. Transcript levels of *HSP90*, *HSP70*, *HSP60*, *HSC70*, and *SIRT1-4* were quantified in liver and pectoral muscle, along with protein levels of Hsp70 and Hsc70.

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Chapter 1: Heat shock proteins in hearts of red- and white-blooded notothenioids¹

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1.1 Abstract

Levels of oxidized proteins are higher in red-blooded Antarctic notothenioids compared to hemoglobinless (-Hb) icefishes. We hypothesized that heat shock proteins (Hsps) would be higher in red-blooded notothenioids to enhance folding of newly synthesized proteins replacing oxidatively damaged ones. Additionally, we hypothesized that notothenioids in the Western Antarctic Peninsula region (WAP), a temperature-variable region of the Southern Ocean, might display a heat shock response. Protein levels of Hsp70/Hsc70 and mRNA levels of *HSP70* were quantified in heart ventricles of *Notothenia coriiceps* (+Hb) and *Chaenocephalus aceratus* (-Hb) exposed to their critical thermal maxima (CTMax) and *N. coriiceps* and *Chionodraco rastrispinosus* (-Hb) warm acclimated to 4°C for 1 week. Levels of Hsp70/Hsc70 proteins and mRNA levels of *HSP70* were also quantified in hearts of the red-blooded notothenioids *Gobionotothen gibberifrons*, *Pagothenia borchgrevinki*, *Eleginops maclovinus*, and in the icefish *Champscephalus gunnari*. When pooled, red-blooded notothenioids displayed lower *HSP70* transcript levels than icefishes, but Hsp protein levels did not differ. *HSP70* transcript levels were lower in *E. maclovinus* than other notothenioids but Hsp protein levels were similar among all species. Neither *HSP70* mRNA nor protein levels of Hsps increased in response to exposure to CTMax and transcript levels of *HSP70* declined in response to warm acclimation. Our data suggest that, while the loss of Hb reduces levels of oxidatively damaged proteins, it does not result in a concomitant decrease in Hsps.

Additionally, while notothenioids from the WAP do not induce Hsps in response to acute elevations in temperature, moderate increases in temperature may reduce Hsp levels.

Keywords: Antarctic fishes, heat stress, heat shock proteins, hemoglobin

1.2 Introduction

The suborder Notothenioidei dominates the Antarctic fish fauna, with its six families composing 54.5% of the benthic species and over 90% of the biomass on the continental shelf (Eastman 1993). Notothenioids originated on the Gondwanan shelf and radiated following the separation of Antarctica from Gondwana and the formation of the Antarctic circumpolar current, approximately 25 MYA (Eastman 1993). The cooling of the Southern Ocean, and the presence of ice, shaped their evolution. Water temperatures are estimated to have been less than 5°C for at least 12 MY (Clark 1980). Today, water temperatures on the shelf are -1.0°C to -1.86°C and vary even less in McMurdo Sound, between -1.7°C and -1.86°C (Knox 2006). Notothenioids are well adapted to life in the cold with high levels of circulating antifreeze glycoproteins (DeVries 1971; Chen et al. 1997), membranes rich in polyunsaturated phospholipids (Logue et al. 2000), cold-adapted enzymes (Fields and Somero 1998), cold-stable microtubules (Detrich et al. 1987), and large diameter muscle fibers to minimize energetic demands for maintaining ion gradients (O'Brien et al. 2003; Jimenez et al. 2013).

Several traits have also been lost during the evolution of notothenioids at cold temperature. Members of the family Channichthyidae (suborder Notothenioidei), all lack the oxygen-binding protein hemoglobin (Hb), and six do not express the intracellular oxygen-binding protein, myoglobin (Mb) in their heart ventricle (Sidell et al. 1997; Grove et al. 2004). The loss of Hb results in a decrement in blood-oxygen-carrying capacity to only 10% that of red-blooded species (Holeton 1970). Associated with the loss of Hb is an extensive remodeling of the icefish cardiovascular system to improve

oxygen delivery, suggesting that the loss of Hb is a disaptation (Montgomery and Clements 2000). Icefishes have enlarged hearts, 0.3% of their body weight compared to 0.06-0.1% in red-blooded notothenioids; three-fold greater blood volumes and capillary diameters, 1.5 to 4-fold higher vascular densities in the retina, and larger diameter vasculature with greater branching in the brain compared to red-blooded fishes (Hemmingsen and Douglas 1970; Holeton 1970; Twelves 1972; Kilarski et al. 1982; Fitch et al. 1984; Eastman and Lannoo 2004; Sidell and O'Brien 2006; Wujcik et al. 2007).

Antarctic notothenioids have also lost the capacity to mount a heat shock response (HSR), once thought to be ubiquitous among all organisms (Hofmann et al. 2000; Place et al. 2004; Place and Hofmann 2005). The HSR results in a rapid increase in the synthesis of heat shock proteins (Hsps), also called molecular chaperones, following heat stress (Lindquist 1986). Hsps assist in protein folding, transmembrane transfer of proteins, and assembly of protein complexes (Feder and Hofmann 1999). The different families of Hsps are conserved among prokaryotes and eukaryotes and categorized according to molecular mass. These include Hsp100, Hsp90, Hsp70, Hsp60, and the small Hsps (Mogk et al. 2001).

The loss of a HSR has been documented in several red-blooded notothenioids from McMurdo Sound, where temperatures hover near -1.9°C (Littlepage 1965; Hofmann et al. 2000; Place et al. 2004; Place and Hofmann 2005). The loss of the HSR was first described in *Trematomus bernacchii* where metabolic labeling experiments failed to detect an increase in the synthesis of any size class of Hsps in spleen, liver, gill,

brain, or heart after a 2 h heat shock at 10°C followed by 6 h of recovery at -1.5°C (Hofmann et al. 2000). A comparison of molecular chaperones from the 70 kDa family of Hsps (Hsp70) in gill and liver between Antarctic (*T. bernacchii*) and New Zealand notothenioids (*Bovichtus variegatus* and *Notothenia angustata*) showed that all three expressed transcripts of *HSP70*, but only the New Zealand fishes induce expression of *HSP70* after exposure to an increase in temperature (Place et al. 2004). Levels of *HSP70* mRNA do not change within a 12 h heat shock for either *Pagothenia borchgrevinki* or *T. bernacchii*, however levels increase in zoarcid *Lycodichthys dearborni*, suggesting that HSR has not been lost in all Antarctic fishes (Place and Hofmann 2005).

Levels of Hsps have not yet been quantified in icefishes and some evidence suggests that Hsp expression may differ compared to red-blooded fishes. Both Mb and Hb contain an iron-centered heme that when bound to oxygen, can be oxidized to ferric iron (Fe^{III}), producing the superoxide radical (O_2^-) (Reeder and Wilson 2005). Increased levels of reactive oxygen species (ROS), such as O_2^- , and/or diminished levels of antioxidants that protect against ROS, lead to oxidative stress and an increase in levels of oxidized lipids, DNA, and proteins (Berlett and Stadtman 1997). Correlated with the presence of heme-centered proteins, hearts of the red-blooded notothenioids *Notothenia coriiceps* and *Gobionotothen gibberifrons*, and the +Mb icefish *Chionodraco rastrispinosus* have higher levels of protein carbonyls than hearts of the -Mb icefish *Chaenocephalus aceratus* (Mueller et al. 2012). Activities of the antioxidants superoxide dismutase and catalase are also higher in blood, liver, heart, gill, and muscle tissue of red-blooded notothenioids compared to icefishes (Witas et al. 1984; Cassini et al. 1993;

Ansaldo et al. 2000; Mueller et al. 2012). Moreover, in the brain, gill, heart, pectoral muscle, epaxial muscle, kidney, spleen, and testes of the red-blooded notothenioid *N. coriiceps* rates of protein synthesis are higher than the icefish *C. aceratus*, which may be crucial for replacing oxidatively damaged proteins (Haschemeyer 1983). Higher rates of protein synthesis, levels of oxidized proteins, and antioxidant activities compared to icefishes suggest that red-blooded notothenioids may have higher levels of chaperones to assist in folding new or damaged proteins.

Temperatures are higher and more variable in the Western Antarctic Peninsula region (WAP) compared to the Ross Sea. Sea temperatures in the WAP range from +1.5°C in the summer to -1.8°C in the winter (DeWitt 1971). Moreover, sea surface temperatures in the WAP are rising and have increased by more than 1.5°C since 1950 (Vaughan et al. 2003; Meredith and King 2005; Clarke et al. 2007). Correlated with differences in habitat temperature are differences in thermal tolerances between notothenioids from the WAP and Ross Sea. The critical thermal maximum (CTMax) is the temperature at which a fish can no longer right itself, and is obtained by a constant rate of temperature increase. The CTMax of notothenioids from the WAP are approximately 0.5-4°C higher than notothenioids from McMurdo Sound (Bilyk and DeVries 2011). The underlying physiological and biochemical processes responsible for differences in thermal tolerance are unknown but may be due to differences in the HSR.

We hypothesized that red-blooded notothenioids would have higher levels of Hsps compared to icefishes and that both red-blooded notothenioids and icefish from the WAP might display a HSR. To test these hypotheses, *N. coriiceps* (+Hb) and *C. aceratus*

(-Hb) were exposed to their CTMax and *N. coriiceps* and *C. rastrospinosus* (-Hb) were warm acclimated to 4°C for 1 week. Hsp70/Hsc70 protein and *HSP70* mRNA transcript levels were quantified in heart ventricles. Additionally, levels of Hsp70/Hsc70 protein and *HSP70* mRNA in heart ventricles were compared among fishes from the WAP (*C. aceratus*, *Champscephalus gunnari*, *C. rastrospinosus*, *G. gibberifrons*, and *N. coriiceps*), McMurdo Sound (*P. borchgrevinki*), and Patagonia (*Eleginops maclovinus*).

1.3 Materials and Methods

1.3.1 Tissue collection

C. aceratus (Lönnerberg 1906), *C. gunnari* (Lönnerberg 1906), *C. rastrospinosus* (DeWitt and Hureau 1979), *G. gibberifrons* (Lönnerberg 1905), and *N. coriiceps* (Richardson 1844) were captured in Dallmann Bay (64°08'S, 62°40'W) and off the southwest shore of Low Island (63°28'S 62°41'W), Antarctica in 2005, 2009, and 2011. Heart ventricles from *E. maclovinus* (Cuvier in Cuvier and Valenciennes 1830) and *P. borchgrevinki* (Boulenger 1902) were collected in 2004 and provided by the late Dr. Bruce Sidell (University of Maine). *E. maclovinus* is from the Patagonian region, whereas *P. borchgrevinki* is from McMurdo Sound. All protocols were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (134774-2).

1.3.2 Experimental design

CTMax was measured in *C. aceratus* and *N. coriiceps* in 2011. For each trial, 3 fish were transferred into a 700 L insulated Xactics tank equipped with circulating seawater at $0.3 \pm 0.04^\circ\text{C}$ and allowed to recover from the stress of handling for approximately 12h. Tanks were covered to minimize stress. To measure CTMax, incoming seawater was turned off and seawater recirculated through a 3 kV titanium inline heater (AquaLogic, San Diego, CA, USA). Temperature was monitored using a HOBO Water Temp Pro V2 temperature logger (Onset Computer, Pocasset, MA, USA) and increased at a rate of 4°C hr^{-1} until fish lost the ability to right themselves. Tanks were surrounded by black visquene and fish monitored using red lights to minimize stress. One of the three fish was harvested prior to increasing the temperature (at $0.3 \pm 0.04^\circ\text{C}$), the second when water temperature reached 8°C and the third and final animal when it reached its CTMax. Fish were harvested and euthanized with an overdose of MS-222 diluted 1:7500 in seawater at the same temperature at the time of harvest. The spinal cord was transected once the fish was anesthetized. Tissues were excised, frozen in liquid nitrogen, and stored at -80°C until further use. CTMax for *C. aceratus* and *N. coriiceps* was $13.1 \pm 0.4^\circ\text{C}$ and $16.8^\circ\text{C} \pm 0.2^\circ\text{C}$, respectively.

C. rastrosipinosus and *N. coriiceps* were acclimated $4 \pm 0.5^\circ\text{C}$ for 1 wk in 2009 as described in Beers and Sidell (2011). Tank temperatures was increased at a rate of $1.3^\circ\text{C hr}^{-1}$ to 4°C and maintained at $4 \pm 0.5^\circ\text{C}$ using a 3 kV titanium inline heater. Fresh seawater was circulated to prevent buildup of nitrogenous wastes. Control animals were

held in the same tanks with circulating seawater at $0 \pm 0.5^\circ\text{C}$ for 1 wk. Following the acclimation or control period, fish were harvested as described above.

1.3.3 Hsp70/Hsc70 protein levels

Heart ventricles were homogenized in 4 volumes of 10 mM Tris·HCl, 10 mM NaCl, and 0.1 mM EDTA, pH 7.5 at 4°C . Protein concentration was determined using a Bradford assay (Bradford 1976). Samples were prepared by diluting 100 μg of protein in SDS sample buffer (20 mM NaPO_4 , 8% glycerol, 40 mM dithiothreitol, 0.025% Bromophenol Blue, 4% SDS, pH 6.8). Homogenates from both control (animals at ambient temperature) and experimental (animals exposed to their CTMax or acclimated to 4°C) were always loaded on the same gel. Similarly, when comparing Hsps among different species of notothenioids, samples from each species were loaded on every gel. Proteins were separated on 12% SDS-polyacrylamide gels and then transferred to nitrocellulose membrane using a semi-dry transfer apparatus (GE Healthcare, Pittsburgh, PA, USA) set at 0.8 mA cm^{-1} of mask opening for 1h. The transfer buffer contained 25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol.

Membranes were blocked overnight in Blotto (5% nonfat dry milk in PBS with 0.1% Tween-20) at 4°C and washed as follows: 2 quick rinses, once for 15 min, and twice for 5 min each in PBS-0.1% Tween. To detect levels of Hsp70 and Hsc70, membranes were incubated with mouse anti-Hsp70/Hsc70 primary antibody (Pierce MA1-10891, Thermo Fisher Scientific, Rockford, IL, USA) diluted 1:2000 in Blotto for 3h at room temperature. Membranes were then washed as described above and incubated

in donkey anti-mouse horseradish peroxidase-linked secondary antibody (Pierce PA1-28748, Thermo Fisher Scientific, Rockford, IL, USA) diluted 1:2500 in Blotto for 1h at room temperature. After a final wash as described above, membranes were developed using Amersham ECL Prime Western Blotting Detection Reagent kit (GE Healthcare, Pittsburgh, PA, USA) following manufacturer's instructions and visualized using an AlphaImager 3400 CCD camera and AlphaEaseFC software v4.0.1 (ProteinSimple, Santa Clara, CA, USA). Following visualization, membranes were stripped in 0.65% β -mercaptoethanol, 1.3 mM Tris·HCl, pH 6.7, and 2% SDS at 50°C for 30 min with shaking. Membranes were then washed in PBS-0.1% Tween twice for 10 min each and blocked in Blotto overnight. Actin was detected using a goat anti-actin primary antibody diluted 1:500 in Blotto (Santa Cruz sc1615, Santa Cruz Biotechnology, Dallas, TX, USA) followed by a donkey anti-goat horseradish peroxidase-linked secondary antibody, diluted 1:5000 in Blotto (Santa Cruz sc2020, Santa Cruz Biotechnology, Dallas, TX, USA) as described above. Protein levels were quantified using ImageQuant TL v.8.1 (GE Healthcare, Pittsburgh, PA, USA). Levels of Hsp70/Hsc70 were normalized to levels of actin. All measurements were made in duplicate in 3-6 individuals per species and treatment.

1.3.4 RNA extraction

RNA was isolated using the RNeasy Fibrous Tissue Mini-kit (Qiagen, Germantown, MD, USA), with minor modifications to increase yield. Samples were treated with DNase I once for 25 min and again for 20 min. Complimentary DNA

(cDNA) was synthesized using TaqMan reverse transcription reagents (Applied Biosystems, Grand Island, NY, USA) as described previously (Orczewska et al. 2010; Kammer et al. 2011).

1.3.5 Cloning and sequencing of *HSP70* and *EF-1 α*

Gene-specific primers were designed for amplifying, cloning and sequencing 304 bp of *HSP70* in all species, except *N. coriiceps*, which was previously sequenced (GenBank, EU588987). Degenerate primers were designed for amplifying, cloning and sequencing 561 bp of Elongation factor 1 α (*EF-1 α*) in *C. gunnari*, *E. maclovinus*, and *P. borchgrevinki* (Table 1). Partial sequences of *EF-1 α* were obtained previously for *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons*, and *N. coriiceps* (GenBank, EU857824, EU857825, EU857826) (Urschel and O'Brien 2008; Mueller et al. 2012). *HSP70* and *EF-1 α* sequences were amplified using an iCycler (Bio-Rad, Hercules, CA, USA) with annealing temperatures between 56 and 65°C. PCR products were separated on a 2% agarose gel stained with ethidium bromide and bands of the appropriate length excised. cDNA sequences were purified using QIAquick Gel Extraction Kit (Qiagen, Germantown, MD, USA) and cloned into *Escherichia coli* TOP10 chemically competent cells using TOPO TA Cloning Kit with a pCR2.1-TOPO vector (Invitrogen, Grand Island, NY, USA). Transformed colonies were selected using ampicillin resistance and blue/white screening on LB plates (10 mg ml⁻¹ bactotryptone, 5 mg ml⁻¹ yeast extract, 5 mg ml⁻¹ NaCl, 1 mg ml⁻¹ glucose, 15 mg ml⁻¹ agar, and 50 μ g ml⁻¹ ampicillin)

supplemented with $64 \mu\text{g ml}^{-1}$ 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal). Transformed colonies were grown in LB media (10 mg ml^{-1} bactotryptone, 5 mg ml^{-1} yeast extract, 5 mg ml^{-1} NaCl, 1 mg ml^{-1} glucose) supplemented with $50 \mu\text{g ml}^{-1}$ ampicillin at 37°C in a shaking water bath overnight. Plasmids were purified with QIAprep Spin Miniprep Kit (Qiagen, Germantown, MD, USA) and labeled for sequencing with the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Grand Island, NY, USA). Products were purified using Centri-Sep columns (Princeton Separations, Adelphia, NJ, USA) packed with Sephadex G-50 gel (Sigma Aldrich, St. Louis, MO, USA) and sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Grand Island, NY, USA). Sequences were compared with *HSP70* in other species using the NCBI nucleotide database (<http://blast.ncbi.nlm.nih.gov>) to confirm cDNA sequences. *HSP70* sequences in all species and *EF-1 α* sequences in *C. gunnari*, *E. maclovinus*, and *P. borchgrevinki* were deposited into GenBank (Accession KF942411-KF942419).

1.3.6 Quantitative real-time PCR

Gene-specific primers were designed using Primer Express v3.0 software (Applied Biosystems, Grand Island, NY, USA) with either the forward or reverse primer of each set annealing over a splice site whenever possible to ensure genomic DNA was not amplified (Table 1). One set of primers was designed for measuring *HSP70* and *EF-1 α* levels in *C. aceratus*, *C. rastrospinosus*, and *N. coriiceps*. Subsequent comparisons

among red-blooded notothenioids and icefishes required designing a second set of *HSP70* and *EF-1 α* gene-specific primers that were conserved among all species (Table 1.1).

Transcript levels of *HSP70* and *EF-1 α* were measured using quantitative real-time PCR (qRT-PCR) and an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Grand Island, NY, USA). Each reaction contained 1X Power SYBR Green PCR Master Mix, 0.3 μ M of each forward and reverse primer, and 5 ng cDNA, except 15 ng cDNA was used when measuring gene expression in animals acclimated to 4°C and corresponding control animals because *HSP70* mRNA levels were low in fishes acclimated to 4°C. A standard curve was created by serially diluting pooled cDNA from all species and treatments to quantify relative transcript levels of *HSP70* and *EF-1 α* .

Relative transcript levels of *HSP70* were normalized to transcript levels of *EF-1 α* , which was previously determined to be a suitable housekeeping gene in both red- and white-blooded notothenioids after exposure to CTMax and acclimation to 4°C (Urschel and O'Brien 2008; Mueller et al. 2012; Mueller et al. in press). All measurements were made in triplicate in 3-12 individuals per species and treatment.

1.3.7 Statistical analyses

All statistical analyses were completed using SigmaPlot v11.2 (Systat Software, San Jose, CA, USA). Significant differences in Hsp levels between species and temperature treatments were determined using a two-way ANOVA followed by a multiple comparisons test with a Bonferroni correction. Significant differences in levels of Hsps among species were determined using a one-way ANOVA followed by a

multiple comparisons test with a Bonferroni correction. Levels of Hsps were compared between pooled samples of red- and white-blooded notothenioids using a Student's t-test. Data were log transformed when necessary to meet assumptions of equal variance and normality. Normality was determined by a Shapiro-Wilk's test. Significance was set at $\alpha < 0.05$.

1.4 Results

1.4.1 Hsp70 levels in fishes exposed to their CTMax

Transcript levels of *HSP70* were significantly higher in hearts of *C. aceratus* compared to *N. coriiceps* at both 0°C and CTMax, but did not increase in either species in response to exposure to CTMax (Fig. 1.1a). Protein levels of Hsp70/Hsc70 did not differ between species, nor did they change in response to exposure to CTMax ($P > 0.05$; Fig. 1.2a and 1.2b).

1.4.2 Hsp70 levels after 1 wk acclimation to 4°C

Transcript levels of *HSP70* decreased in response to acclimation at 4°C in hearts of both *N. coriiceps* and *C. rastrorpinosus* ($P < 0.05$; Fig. 1.1b). In addition, levels of *HSP70* mRNA were significantly higher in *C. rastrorpinosus* compared to *N. coriiceps* at 0°C. There was no difference in Hsp70/Hsc70 protein levels between the two species, nor did levels change with acclimation to 4°C ($P > 0.05$; Fig. 1.2c and 1.2d). When

temperature treatments were pooled, levels of Hsp70/Hsc70 were higher in *C. rastrorpinosus* than *N. coriiceps* ($P < 0.05$).

1.4.3 Hsp70 levels in icefishes and red-blooded notothenioids

Consistent with previous studies, levels of *HSP70* mRNA were lower in the temperate notothenioid *E. maclovinus* compared to Antarctic notothenioids ($P < 0.05$, Fig. 1.3a). When pooled together, icefishes had 3.3-fold higher levels of *HSP70* transcripts compared to red-blooded Antarctic notothenioids (excluding *E. maclovinus*) ($P < 0.001$). However, levels of Hsp70/Hsc70 proteins were not significantly different among the species, nor were they different between red-blooded notothenioids and icefishes ($P > 0.05$, Fig. 1.3b and 1.3c).

1.5 Discussion

Our results indicate that despite higher levels of oxidative stress, red-blooded notothenioids do not have concomitantly higher levels of Hsp70 proteins compared to icefishes. Rather, levels of *HSP70* mRNA are higher in hearts of icefishes compared to red-blooded species. Moreover, despite living in a warmer and more temperature variable environment compared to notothenioids in McMurdo Sound, neither icefishes nor red-blooded species in the WAP induce Hsp expression in response to exposure to CTMax, an acute heat stress. Conversely, mRNA levels of *HSP70* decline in response to warm acclimation, suggesting moderate elevations in temperature may minimize protein denaturation.

1.5.1 Levels of Hsps in hearts of red- blooded notothenioids and icefishes

Despite having higher levels of oxidized proteins and greater rates of protein synthesis (Haschemeyer 1983; Mueller et al. 2011; Mueller et al. 2012), hearts of red-blooded notothenioids have lower levels of *HSP70* mRNA and equivalent or lower levels of Hsp70/Hsc70 proteins compared to icefishes. These data indicate that high levels of oxidatively-modified proteins do not warrant high levels of Hsp70s. We can not rule out the possibility that other Hsps may be elevated in red-blooded notothenioids. For example, transcriptome analysis of *Dissostichus mawsoni* revealed that *HSP90 β* , along with *HSP70* and *HSC70* were among the top 12 most highly expressed transcripts in brain, ovary, liver, and kidney (Chen et al. 2008). Levels of other Hsps, such as Hsp90, Hsp60, and Hsp40, have not yet been quantified in Antarctic fishes at ambient temperature or following heat stress, and levels of some of these Hsps increase in response to heat stress in other fishes. For example, *HSP90* mRNA levels significantly increase in the liver, spleen, kidney, brain, and intestine of the grass carp (*Ctenopharyngodon idella*) after a heat shock of 34°C for 2h (Wu et al. 2012) and *HSP47* mRNA increases in zebrafish (*Danio rerio*) exposed to a 37°C heat shock for 2h (Murtha and Keller 2003).

Contrary to our expectations, *HSP70* mRNA levels were higher in hearts of icefishes compared to red-blooded notothenioids. Differences in Hsp70 protein levels may have gone undetected because the antibody we used recognizes both Hsc70 and Hsp70, and differing levels of Hsc70 may have masked differences in Hsp70.

The higher level of *HSP70* mRNA in hearts of icefishes may be due to differences in mitochondrial densities between red- and white-blooded notothenioids. The loss of Hb and Mb is correlated with an increase in the percent cell volume displaced by mitochondria (O'Brien and Mueller 2010), with hearts of icefishes having up to 2-fold higher mitochondrial densities compared to red-blooded fishes (O'Brien and Sidell 2000; O'Brien and Mueller 2010). Mammalian mitochondria possess 600-1,400 proteins, of which only 13 are encoded within the mitochondrial genome; the vast majority are encoded in the nuclear genome, synthesized on free ribosomes, and imported into the mitochondrion (Taylor et al. 2003; Baker et al. 2012; Lotz et al. 2013). Protein import occurs post-translationally. Proteins are unfolded in the cytosol by chaperones, and Hsp90, Hsp70, and Hsc70 bind to prevent aggregation prior to transfer through the TOM and TIM translocase complexes (Craig et al. 1989; Kiebler et al. 1990; Scherer et al. 1990; Scherer et al. 1992; Terada et al. 1996; Fan et al. 2006). Hsp70 is also located within the mitochondrial matrix, where along with Hsp60, they assist with protein translocation and folding (Cheng et al. 1989; Kang et al. 1990; Ostermann et al. 1990). Thus, high levels of Hsp70 may be crucial for maintaining high mitochondrial densities in cardiac myocytes of icefishes.

1.5.2 Hsp protein levels do not differ among notothenioids from different regions

Levels of *HSP70* mRNA and Hsp70/Hsc70 proteins were similar between notothenioids from the WAP and *P. borchgrevinki* from McMurdo Sound, indicating that

minor fluctuations in temperature do not impact levels of Hsp70. Our data are consistent with previous studies showing that Antarctic fishes display higher levels of *HSP70* mRNA than temperate notothenioids. Levels of *HSP70* mRNA were 9- to 56-fold greater in the Antarctic species compared to *E. maclovinus*. However, this was not reflected in protein levels of Hsp70/Hsc70, where there were no significant differences among the species. Similarly, constitutive levels of *HSP70* mRNA are higher in the Antarctic notothenioid *T. bernacchii* compared to the New Zealand species *N. angustata* and *B. variegatus* (Place et al. 2004). However, this trend is not always reflected in levels of Hsp70/Hsc70 proteins. Comparison of Hsp70/Hsc70 protein levels in *N. angustata*, *T. bernacchii*, *T. hansonii*, and *T. pennellii* reveal similar levels in gill tissue and higher levels only in white muscle tissue of *N. angustata* compared to the three *Trematomus* species (Carpenter and Hofmann 2000).

1.5.3 *HSP70* mRNA levels decline in response to moderate warming

Despite living in waters with minor seasonal fluctuations in temperatures, notothenioids from the WAP do not exhibit the classic heat shock response (HSR). Neither transcripts nor protein levels of Hsps increased in response to exposure to CTMax in either red- or white-blooded notothenioids. These results align with previous studies showing a lack of a HSR in other red-blooded notothenioids inhabiting the Southern Ocean (Hofmann et al. 2000; Buckley et al. 2004; Place and Hofmann 2005; Clark et al. 2008).

The mechanism behind the loss of a HSR in Antarctic notothenioids is not fully understood. Studies in *T. bernacchii* show that heat shock factor 1 (Hsf1), an inducible transcription factor that binds to the promoter region of inducible HSP genes to enhance transcription, does not increase binding activity in response to heat shock, indicating Hsf1 is only important for constitutive expression of Hsps in notothenioids (Satyal et al. 1998; Buckley et al. 2004). Heat shock binding protein 1 (Hsbp1) is also involved in regulating the HSR by binding to Hsf1 in its active state and converting it to its inactive state, thereby preventing Hsf1 from transactivating HSP expression (Satyal et al. 1998). Indeed, overexpression of *HSBP1* represses Hsf1 activity and blocks the HSR (Satyal et al. 1998). Interestingly, the icefish *Chionodraco hamatus* displays a high proportion of duplicated genes, including *HSBP1*, which may inhibit the HSR (Coppe et al. 2013).

Somewhat surprisingly, *HSP70* mRNA decreased in hearts of both red- and white-blooded notothenioids acclimated to 4°C for 1 week. Similarly, levels of *HSP70* and *HSC70* mRNA tended to decrease in liver tissue of the Antarctic notothenioid *Harpagifer antarcticus* after exposure to 6°C for 2-48 hours (Clark et al. 2008; Thorne et al. 2010). The higher levels of ubiquitin-conjugated proteins in gill, liver, heart, and spleen of Antarctic *T. bernacchii*, *T. pennellii*, *P. borchgrevinki*, and *L. dearborni* compared to the temperate species *N. angustata*, *B. variegatus*, and *Bellapiscis medius* suggests that cold temperature denatures proteins (Todgham et al. 2007). Transcriptome studies have also supported this. The Antarctic notothenioids *P. borchgrevinki*, *N. coriiceps*, *C. aceratus*, *Pleuragramma antarcticum*, and *Dissostichus mawsoni* have higher mRNA levels of genes involved in protein ubiquitination compared to species

from warmer waters (Chen et al. 2008; Shin et al. 2012; Bilyk and Cheng 2013). Additionally, a genomic study in *D. mawsoni*, *P. borchgrevinki*, and *C. aceratus* revealed gene duplication of several genes involved in protein ubiquitination compared to non-Antarctic *B. variegatus* and *E. maclovinus* (Chen et al. 2008). If cold denatures proteins, then warm acclimation to 4°C may reduce levels of cold-denatured proteins, resulting in a decrease in *HSP70* transcript levels. Additionally, increases in temperature will enhance rates of protein folding, potentially minimizing demand for chaperones (Scalley and Baker 1997). Given that Hsf1 effectively binds DNA in notothenioids (Buckley et al. 2004), it is conceivable that it can also dissociate and return to its inactive state, bound to Hsp90, as levels of denatured proteins decline, effectively attenuating transcription of HSPs (Vabulas et al. 2012).

The decline in *HSP70* transcripts with warm acclimation is consistent with previous finding that mRNA levels of the antioxidants superoxide dismutase and catalase decline in heart ventricles of *N. coriiceps* and *C. rastrospinosus* in response to acclimation at 4°C (Mueller et al. 2012; Mueller et al. in press). This and other studies have shown that despite their extreme stenothermy, Antarctic fishes possess a limited capacity to adjust to warming (Seebacher et al. 2005; Franklin et al. 2007; Strobel et al. 2012). Warm acclimation of *P. borchgrevinki* to 4°C for 4-5 wk increased cardiac output compared to animals held at ambient temperature (Franklin et al. 2007). Maximal activities of cytochrome c oxidase and lactate dehydrogenase (LDH) also increased in muscle of *P. borchgrevinki* in response to warm acclimation to 4°C (Seebacher et al. 2005). While maximum activity of LDH also increased in skeletal muscle of *T.*

bernacchii acclimated to 4.5°C for 14 days, activities of citrate synthase declined in skeletal and cardiac muscle, as did LDH in cardiac muscle (Jayasundara et al. 2013). Additionally, acclimation to 4°C over 7-21 days was sufficient to increase the CT_{Max} by 1-3°C in several Antarctic notothenioids (Bilyk and DeVries 2011). The underlying molecular basis of this acclimatory response is unknown, but our data suggest that elevations in Hsp70s do not contribute to enhanced thermal tolerance following warm acclimation in notothenioids. Together, these data suggest that not only do notothenioids possess some thermal plasticity but, also, moderate warming may reduce stress in notothenioids.

1.6 Figures

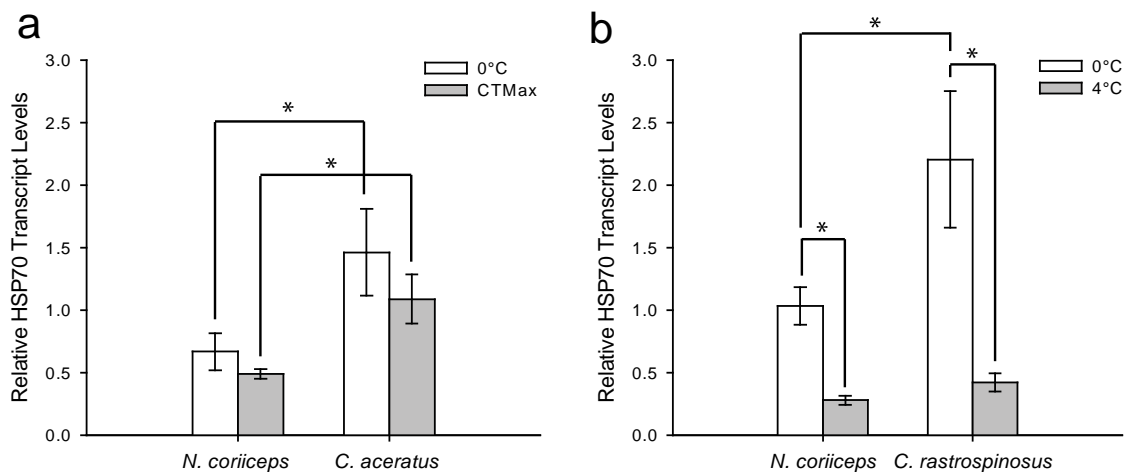


Fig. 1.1 *HSP70* transcript levels in ventricle of heat stressed Antarctic fishes. *N. coriiceps* and *C. aceratus* exposed to their CTMax or held at 0°C (a). *N. coriiceps* and *C. rastrospinosus* acclimated to 4°C or held at 0°C (b). Transcript levels of *HSP70* were normalized to transcript levels of the housekeeping gene *EF-1 α* . Significant differences are indicated with an asterisk ($P < 0.05$). Values are mean \pm SEM, N = 6-7 for (a) and 8-12 for (b)

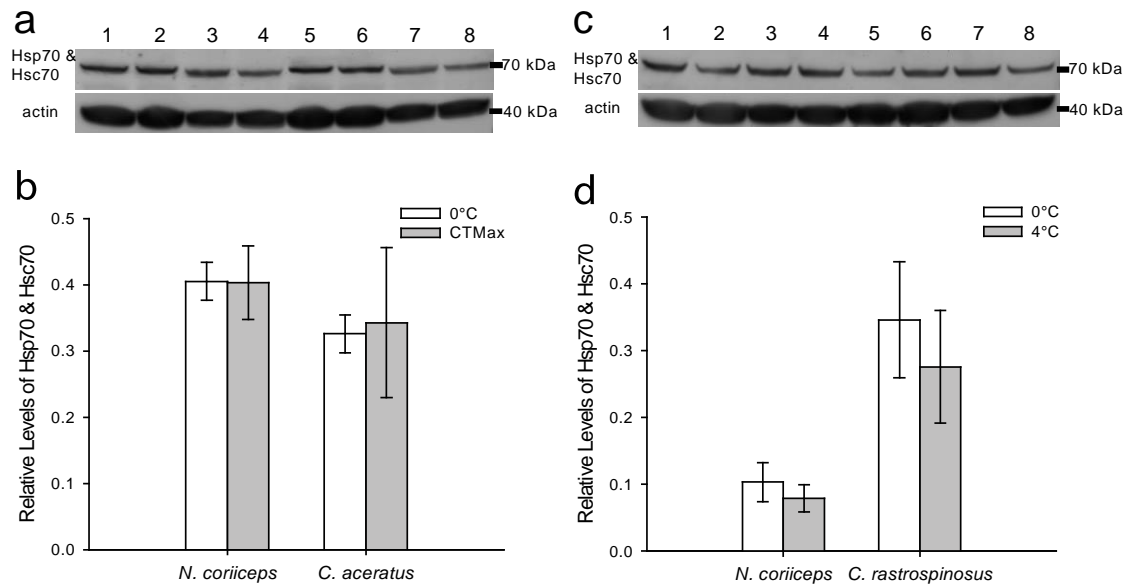


Fig. 1.2 Hsp70/Hsc70 protein levels in ventricle of heat stressed Antarctic fishes. *N. coriiceps* and *C. aceratus* exposed to their CTMax or held at 0°C (a & b). Representative Western blot; lanes 1 and 5, *N. coriiceps* 0°C; lanes 2 and 6, *N. coriiceps* CTMax; lanes 3 and 7, *C. aceratus* 0°C; lanes 4 and 8, *C. aceratus* CTMax (a). *N. coriiceps* and *C. rastrispinosus* acclimated to 4°C or held at 0°C (c & d). Representative Western blot; lanes 1-2, *N. coriiceps* 0°C; lanes 3-4, *C. rastrispinosus* 0°C; lanes 5-6, *N. coriiceps* 4°C; lanes 7-8, *C. rastrispinosus* 4°C (b). Levels of Hsp70 and Hsc70 in hearts of *N. coriiceps*, *C. aceratus*, and *C. rastrispinosus* were normalized to actin. Values are mean \pm SEM, N = 4-6

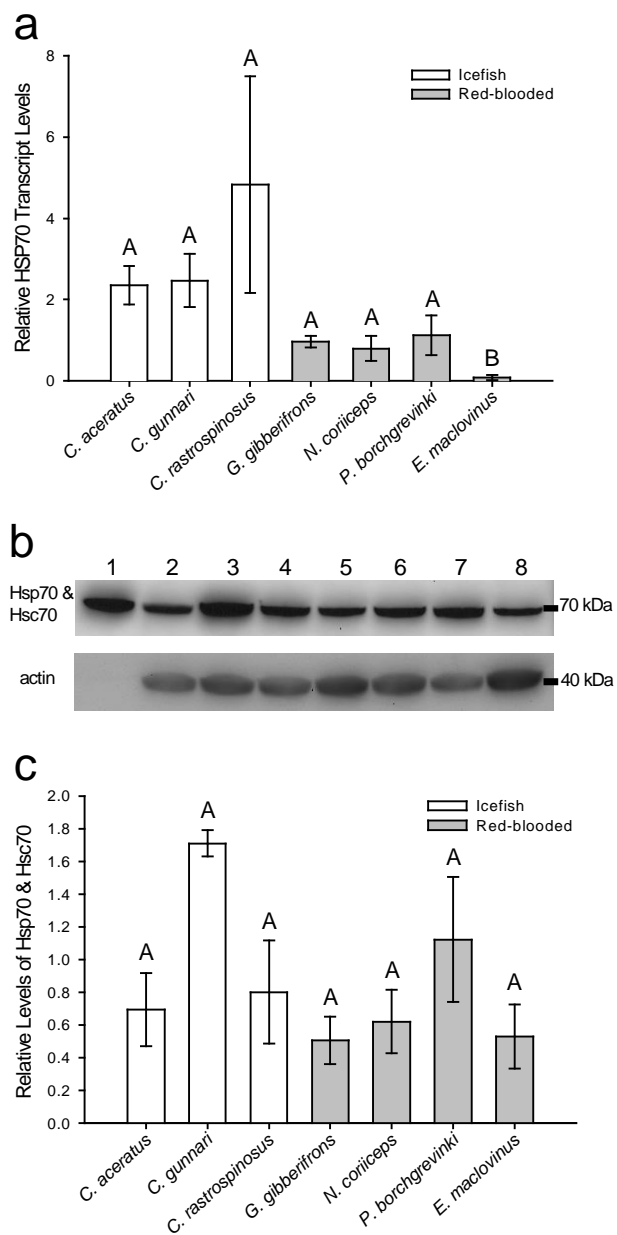


Fig. 1.3 *HSP70* transcript and Hsp70/Hsc70 protein levels in ventricle of Antarctic fishes. *HSP70* mRNA levels in hearts of notothenioids (a). Representative Western blot of Hsp70 and Hsc70 protein levels; lane 1, rat liver; lane 2, *C. aceratus*; lane 3, *C. gunnari*; lane 4, *C. rastrospinosus*; lane 5, *G. gibberifrons*; lane 6, *N. coriiceps*; lane 7, *P. borchgrevinki*; lane 8, *E. maclovinus* (b). Levels of Hsp70 and Hsc70 normalized to actin (c). Different letters denote statistically homogeneous subsets ($P < 0.05$). Values are mean \pm SEM, $N = 3-8$ for (a) and 3-6 for (b & c)

1.7 Tables

1.1 Primers used for cloning and quantitative real-time PCR in Antarctic fishes. EF-1 α degenerate primers used for cloning and gene specific primers used for qRT-PCR in the CTMax and acclimation experiments were previously published by Urschel and O'Brien (2008)

Gene	Use	Primer	Amplicon
HSP70	Cloning & Sequencing	F 5' CGTTCACTGACACCGAGAGG 3' R 5' ACGACTGCATTGGACACAGT 3'	304 bp
HSP70	qRT-PCR CTMax & Acclimation	F 5' ACCAGGTGGCTTTGAACCCT 3' R 5' CCACCACTGGATCATCAATCTTT 3'	81 bp
HSP70	qRT-PCR Species Comparison	F 5' TTCACTGACACCGAGAGGCTC 3' R 5' GCCACCTGGTTCTTTGCTG 3'	50 bp
EF-1 α	Cloning ^a & Sequencing	F 5' CGACATCGCCCTGTGGAARTTYGARAC 3' R 5' GATGGCCGCCGATCTTGTANACRTCYTG 3'	561 bp
EF-1 α	qRT-PCR CTMax & Acclimation	F 5' CTGGAAGCCAGTGAAAAGATGAC 3' R 5' ACGCTCAACCTTCCATCCC 3'	51 bp
EF-1 α	qRT-PCR Species Comparison	F 5' CCTCGGTGTGAAGCAGCTC 3' R 5' GTTGTCTCCGTGCCATCCA 3'	175 bp

qRT-PCR, quantitative real-time PCR
^aDegenerate nucleotides are indicated by N, R, and Y (N=A or C or G or T; R=A or G; Y=C or T).

1.8 Acknowledgements

We thank the Masters and Crew of the ARSV *Laurence M. Gould* and the Raytheon support staff at Palmer Station, Antarctica. We gratefully acknowledge the outstanding assistance from Ololade Olawale and Isabel Raymundo of Lindblom Math and Science Academy, and the University of Alaska Fairbanks Fall 2012 BIOL 261 class who all contributed to preliminary experiments. Funding for this work was provided by the National Science Foundation (grant no. ANT-071301) to K.M.O.

1.9 Appendix A: *HSP70* and *EF-1 α* sequencing in Antarctic Fishes

Methods

For full description of the methods, see Sections 1.3.4 and 1.3.5. cDNA sequences were amplified using an iCycler with annealing temperatures between 56 and 65°C. PCR products were separated on a 2% agarose gel stained and purified. Plasmids were cloned into *Escherichia coli* TOP10 chemically competent cells. Transformed colonies were selected using ampicillin resistance and blue/white screening on LB plates supplemented with X-Gal. Transformed colonies were grown in LB media supplemented with ampicillin at 37°C in a shaking water bath overnight. Plasmids were purified and labeled for sequencing with the BigDye Terminator v.3.1 Cycle Sequencing Kit. Products were purified and sequenced with an ABI PRISM 3100 Genetic Analyzer. Individual mRNA and protein sequence alignments were obtained using Translation Map and multiple species alignments were obtained using Color Align Conservation (<http://www.bioinformatics.org/sms2/>).

Results

```

1   F T D T E R L I G D A A K N Q V A L N P
1   cgttcactgacaccgagaggctcatcggggacgcagcaaagaaccagggtggcctttgaacc
1           10           20           30           40           50

21  S N T V F D A K R L I G R K I D D P V V
61  ctagcaacacgggtggtttgatgcaaagagactgattggtagaaagattgatgatccagtgg
61           70           80           90           100          110

41  E A D M K H W P F A V V G D G G K P K I
121 tggaggcagatatgaagcactggcccttcgcggtggttgagatggagggaaacccaaaa
121           130          140          150          160          170

61  K V E Y K G E D K T F Y P E E I S S M V
181 taaaagtggaatacaaaggggaagacaaaaccttctaccctgaggaaatttcctccatgg
181           190          200          210          220          230

81  L V K M K E I A E A Y L G Q T V S N A V
241 tcctggtaaagatgaaggaaattgctgagggcgtaccttggccaaactgtgtccaatgcag
241           250          260          270          280          290

101
301 tcgt
301

```

Fig. 1.A.1 *Chaenocephalus aceratus* HSP70 partial mRNA and amino acid sequence. Sequences were obtained in 2 individuals by using specific primers designed from *Notothenia coriiceps* partial HSP70 gene sequence (GenBank: EU588987.1). cDNA was inserted into *E. coli*, plasmids were labeled using BigDye Terminator and purified before sequencing with an ABI PRISM 3100 Genetic Analyzer


```

1   F T D T E R L I G D A A K N Q V A L N P
1  cgttcactgacaccgagaggctcatcggggacgcagcaaagaaccaggtggcctttgaacc
1      10      20      30      40      50

21  S N T V F D A K R L I G R K I D D P V V
61  ctagcaacacgggtggttgatgcaaagagactgattggtagaaagattgatgatccagtgg
61      70      80      90     100     110

41  E A D M K H W P F A V V G D G G K P K I
121 tggaggcagatatgaagcactggccctttgcggtgggttgagatggagggaaacccaaaa
121      130     140     150     160     170

61  K V E Y K G E D K T F Y P E E I S S M V
181 taaaagtggaatacaaaaggggaagacaaaaccttctaccctgaggaaatttcctccatgg
181      190     200     210     220     230

81  L V K M K E I A E A Y L G Q T V S N A V
241 tcctggtaaagatgaaggaaattgctgagggcgtaccttgccaaactgtgtccaatgcag
241      250     260     270     280     290

101
301 tcgt
301

```

Fig. 1.A.2 *Champocephalus gunnari* HSP70 partial mRNA and amino acid sequence. Sequences were obtained in 2 individuals by using specific primers designed from *N. coriiceps* partial HSP70 gene sequence (GenBank: EU588987.1). cDNA was inserted into *E. coli*, plasmids were labeled using BigDye Terminator and purified before sequencing with an ABI PRISM 3100 Genetic Analyzer

```

1   F T D T E R L I G D A A K N Q V A L N P
1   cgttcactgacaccgagaggctcatcggggacgcagcaaagaaccaggtggcctttgaacc
1           10           20           30           40           50

21  S N T V F D A K R L I G R K I D D P V V
61  ctagcaacacgggtggttgatgcaaagagactgattggtagaaagattgatgatccagtgg
61           70           80           90           100          110

41  E A D M K H W P F A V V G D G G K P K I
121 tggaggcagatatgaagcactggcccttcgcggtggttgagatggagggaaacccaaaa
121           130          140          150          160          170

61  K V E Y K G E D K T F Y P E E I S S M V
181 taaaagtggaatacaaaaggggaagacaaaaccttctaccctgaggaaatttcctccatgg
181           190          200          210          220          230

81  L V K M K E I A E A Y L G Q T V S N A V
241 tcctggtaaagatgaaggaaattgctgagggcgtaccttggccaaactgtgtccaatgcag
241           250          260          270          280          290

101
301 tcgt
301

```

Fig. 1.A.3 *Chionodraco rastrispinosus* *HSP70* partial mRNA and amino acid sequence. Sequences were obtained in 2 individuals by using specific primers designed from *N. coriiceps* partial *HSP70* gene sequence (GenBank: EU588987.1). cDNA was inserted into *E. coli*, plasmids were labeled using BigDye Terminator and purified before sequencing with an ABI PRISM 3100 Genetic Analyzer

```

1   F T D T E R L I G D A A K N Q V A L N P
1   cgttcactgacaccgagaggctcatcggggacgcagcaaagaaccaggtggcctttgaacc
1           10           20           30           40           50

21  S N T V F D A K R L X G R K I D D P V V
61  ctagcaacacgggtggttgatgcaaagagactgaYtggtagaaagattgatgatccagtgg
61           70           80           90           100          110

41  E A D M K H W P F A V V G D G G K P K I
121 tggaggcagatatgaagcactggcccttcgcggtgggttgagatggagggaaRcccaaaa
121           130          140          150          160          170

61  K V E Y K G E D K T F Y P E E I S S M V
181 taaaagtggaatacaaaaggggaagacaaaaccttctaccctgaggaaatttcctccatgg
181           190          200          210          220          230

81  L V K M K E I A E A Y L G Q T V S N A V
241 tcctggtaaagatgaaggaaattgctgaggcgtaccttgccaaactgtgtccaatgcag
241           250          260          270          280          290

101
301 tcgt
301

```

Fig. 1.A.4 *Eleginops maclovinus* *HSP70* partial mRNA and amino acid sequence. Sequences were obtained in 2 individuals by using specific primers designed from *N. coriiceps* partial *HSP70* gene sequence (GenBank: EU588987.1). cDNA was inserted into *E. coli*, plasmids were labeled using BigDye Terminator and purified before sequencing with an ABI PRISM 3100 Genetic Analyzer

```

1   F T D T E R L I G D A A K N Q V A L N P
1   cgttcactgacaccgagaggctcatcggggacgcagcaaagaaccaggtggcggttgaacc
1           10           20           30           40           50

21  S N T V F D A K R L I G R K I D D P V V
61  ctagcaacacgggtggttgatgcaaagagactgattggtagaaagattgatgatccagtgg
61           70           80           90           100          110

41  E A D M K H W P F A V V G D G G K P K I
121 tggaggcagatatgaagcactggcccttcgcggtggttgagatggaggggaagcccaaaa
121           130          140          150          160          170

61  K V E Y K G E D K T F Y P E E I S S M V
181 taaaagtggaatacaaaaggggaagacaaaaccttctaccctgaggaaatttcctccatgg
181           190          200          210          220          230

81  L V K M K E I A E A Y L G Q T V S N A V
241 tcctggtaaagatgaaggaaattgctgaggcgtaccttgccaaactgtgtccaatgcag
241           250          260          270          280          290

101
301 tcgt
301

```

Fig. 1.A.5 *Gobionotothen gibberifrons* HSP70 partial mRNA and amino acid sequence. Sequences were obtained in 3 individuals by using specific primers designed from *N. coriiceps* partial HSP70 gene sequence (GenBank: EU588987.1). cDNA was inserted into *E. coli*, plasmids were labeled using BigDye Terminator and purified before sequencing with an ABI PRISM 3100 Genetic Analyzer

```

1   F T D T E R L I G D A A K N Q V A L N P
1   cgttcactgacaccgagaggctcatcggggacgcagcaaagaaccaggtggcctttgaacc
1       10       20       30       40       50

21  S N T V F D A K R L I G R K I D D P V V
61  ctagcaacacgggtggttgatgcaaagagactgattggtagaaagattgatgatccagtgg
61       70       80       90      100      110

41  E A D M K H W P F A V V G D G G K P K I
121 tggaggcagatatgaagcactggcccttcgcggtgggttgagatggaggggaagcccaaaa
121       130      140      150      160      170

61  K V E Y K G E D K T F Y P E E I S S M V
181 taaaagtggaatacaaaaggggaagacaaaaccttctaccctgaggaaatttcctccatgg
181       190      200      210      220      230

81  L V K M K E I A E A Y L G Q T V S N A V
241 tcctggtaaagatgaaggaaattgctgaggcgtaccttgccaaactgtgtccaatgcag
241       250      260      270      280      290

101
301 tcgt
301

```

Fig. 1.A.6 *Pagothenia borchgrevinki* *HSP70* partial mRNA and amino acid sequence. Sequences were obtained in 3 individuals by using specific primers designed from *N. coriiceps* partial *HSP70* gene sequence (GenBank: EU588987.1). cDNA was inserted into *E. coli*, plasmids were labeled using BigDye Terminator and purified before sequencing with an ABI PRISM 3100 Genetic Analyzer

```

1  D I A L W K F E T A K Y Y V T I I D A P
1  cgacatcgccctgtggaagttygaractgccaaagtactacgtgaccatcattgatgcccc
1          10          20          30          40          50

21  G H R D F I K N M I T G T S Q A D C A V
61  tggacacagggatttcatcaagaacatgatcactgggtacctctcagggtgactgtgctgt
61          70          80          90          100         110

41  L I V A A G V G E F E A G I S K N G Q T
121 gctgatcggttgctgccggtgttggtgagtttgaggccggtatctccaagaacggccagac
121          130         140         150         160         170

61  R E H A L L A F T L G V K Q L I V G V N
181 ccgtgagcagcgcctgctggctttcaccctcgggtgtgaagcagctcatcgtaggagtc
181          190         200         210         220         230

81  K M D S T E P P Y S Q A R Y E E I A K E
241 caagatggactccaccgagcccccttacagccaggcccggttatgaagaaatcgccaagga
241          250         260         270         280         290

101 V S T Y I K K I G Y N P L T V P F V P I
301 agtgagcacttacatcaagaagatcgggtacaaacccttaactgtgccctttgtcccat
301          310         320         330         340         350

121 S G W H G D N M L E A S E K M T W F K G
361 ctctggatggcagcgagacaacatgctggaagccagtgaaaagatgacgtgggttcaagg
361          370         380         390         400         410

141 W K V E R K E G N A S G V T L L E S L D
421 atggaaggttgagcgtgaaggaggtaatgccagtgaggctcactctgctggagtctctcga
421          430         440         450         460         470

161 A I L P P S R P T D K P L R L P L Q D V
481 tgccatcctgcccccatcccgcccaaccgacaagcccctccgtctgcccctgcaggacgt
481          490         500         510         520         530

181  Y K I G G H
541 Ntacaagatcggcggccatc
541          550

```

Fig. 1.A.7 *C. gunnari EF-1 α* partial mRNA and amino acid sequence. Sequences were obtained in 2 individuals by using previously designed degenerate primers (Urschel and O'Brien 2008). cDNA was inserted into *E. coli*, cells were grown, and plasmids were labeled using BigDye Terminator and purified before sequencing with an ABI PRISM 3100 Genetic Analyzer

```

1  D I A L W K F E T A K Y Y G P S L M P L
1  cgacatcgccctgtggaagtttgagaccgccaagtactatggaccatcattgatgccctc
1      10      20      30      40      50

21 D T G I S S R T * S L V P L R L T A L C
61 ggacacagggatttcatcaagaacatgatcactgggtacctctcaggctgactgcgctgtg
61      70      80      90      100     110

41  * S L L P V L V S L R P V S P R T A R P
121 ctgatcgttgctgccggtgttggtgagtttgaggccggtatctccaagaacggccagacc
121      130     140     150     160     170

61  E S M P C W L T P S V * S S S L * E S T
181 cgagagcatgccctgctggcttacaccctcggtgtgaagcagctcattgtaggagtcaac
181      190     200     210     220     230

81  R W T P R A P L Q P E A L * G D H Q G S
241 aagatggactccacgagcccccttacagccagaagcgctttgaggagatcaccaaggaag
241      250     260     270     280     290

101 E H L H Q K D R L Q P R N C R V R P H L
301 tgagcacttacatcaaaaagatcggtctacaacccccgcaactgtcgggttcgtccccatct
301      310     320     330     340     350

121 W M A R R Q H A G G Q * E D E L V * G L
361 ctggatggcacggagacaacatgctggaggccagtgagaagatgagctgggtttaagggtc
361      370     380     390     400     410

141 E D R A * G G R C Q W H H P A R G S G L
421 ggaagatcgagcgttaaggaggagggtgccaatggcaccaccctgctcgaggctctggact
421      430     440     450     460     470

161 H P A P C S P H R Q A P A S A T A D V Y
481 ccatcctgccccctgctcgccccacagacaagccccctgctctgccactgcagacgtcta
481      490     500     510     520     530

181 K I G G H
541 caagatcggcggccatc
541      550

```

Fig. 1.A.8 *E. maclovinus EF-1 α* partial mRNA and amino acid sequence. Sequences were obtained in 2 individuals by using previously designed degenerate primers (Urschel and O'Brien 2008). cDNA was inserted into *E. coli*, cells were grown, and plasmids were labeled using BigDye Terminator and purified before sequencing with an ABI PRISM 3100 Genetic Analyzer

```

1  D I A L W K F E T A K Y Y V T I I D A P
1  cgacatcgccctgtggaagtttgagactgccaaagtactacgtgaccatcattgatgcccc
1          10          20          30          40          50

21 G H R D F I K N M I T G T S Q A D C A V
61  tggacacagggatttcatcaagaacatgatcactggtacctctcagggtgactgcgctgt
61          70          80          90          100         110

41  L I V A A G V G E F E A G I S K N G Q T
121 gctgatcgttgctgcccgtgttggtgagtttgaggccggtatctccaagaacggccagac
121          130         140         150         160         170

61  R E H A L L A F T L G V K Q L I V G V N
181 tcgcgagcagccctgctggctttcaccctcggtgtgaagcagctcatcgtaggagtcaa
181          190         200         210         220         230

81  K M D S T E P P Y S Q A R Y E E I A K E
241 caagatggactccaccgagcccccttacagccaggcccggttatgaagaaatcgccaagga
241          250         260         270         280         290

101 V S T Y I K K I G Y N P L T V P F V P I
301 agtgagcacttacatcaagaagatcggctacaacccttaactgtgccctttgtcccat
301          310         320         330         340         350

121 S G W H G D N M L E A S E K M T W F K G
361 ctctggatggcagcgagacaacatgctggaagccagtgaaaagatgacatgggttcaaggg
361          370         380         390         400         410

141 W K V E R K E G N A N G V T L L E S L D
421 atggaaggttgagcgtgaaggaggtaatgccaatggagtactctgctggagtctctcga
421          430         440         450         460         470

161 A I L P P S R P T D K P L R L P L Q D V
481 tgccatcctgcccccgctcccgcccaaccgacaagccccctccgtctgccccctgcaggacgt
481          490         500         510         520         530

181 Y K I G G H
541 Htacaagatcggcggccatc
541          550

```

Fig. 1.A.9 *P. borchgrevinki* *EF-1a* partial mRNA and amino acid sequence. Sequences were obtained in 2 individuals by using previously designed degenerate primers (Urschel and O'Brien 2008). cDNA was inserted into *E. coli*, cells were grown, and plasmids were labeled using BigDye Terminator and purified before sequencing with an ABI PRISM 3100 Genetic Analyzer


```

C.aceratus      cgtttcactgacaccgagaggtcatcggggacgagcaaaagaaccaggtggcttgaaccctagcaacacggtgtttgat 80
C.gunnari       cgtttcactgacaccgagaggtcatcggggacgagcaaaagaaccaggtggcttgaaccctagcaacacggtgtttgat 80
C.rastrospinosus cgtttcactgacaccgagaggtcatcggggacgagcaaaagaaccaggtggcttgaaccctagcaacacggtgtttgat 80
E.maclovinus    cgtttcactgacaccgagaggtcatcggggacgagcaaaagaaccaggtggcttgaaccctagcaacacggtgtttgat 80
G.gibberifrons  cgtttcactgacaccgagaggtcatcggggacgagcaaaagaaccaggtggcttgaaccctagcaacacggtgtttgat 80
N.coriiiceps    ctttcactgacaccgagaggtcatcggggacgagcaaaagaaccaggtggcttgaaccctagcaacacggtgtttgat 80
P.borchgrevinki cgtttcactgacaccgagaggtcatcggggacgagcaaaagaaccaggtggcttgaaccctagcaacacggtgtttgat 80

C.aceratus      gcaaagagactgattggtagaagattgatgatccagtggtggaggcagatatgaagcactggcccttcgcggtggttgg 160
C.gunnari       gcaaagagactgattggtagaagattgatgatccagtggtggaggcagatatgaagcactggcccttcgcggtggttgg 160
C.rastrospinosus gcaaagagactgattggtagaagattgatgatccagtggtggaggcagatatgaagcactggcccttcgcggtggttgg 160
E.maclovinus    gcaaagagactgattggtagaagattgatgatccagtggtggaggcagatatgaagcactggcccttcgcggtggttgg 160
G.gibberifrons  gcaaagagactgattggtagaagattgatgatccagtggtggaggcagatatgaagcactggcccttcgcggtggttgg 160
N.coriiiceps    gcaaagagactgattggtagaagattgatgatccagtggtggaggcagatatgaagcactggcccttcgcggtggttgg 160
P.borchgrevinki gcaaagagactgattggtagaagattgatgatccagtggtggaggcagatatgaagcactggcccttcgcggtggttgg 160

C.aceratus      agatggagggaacccaaaaataaaagtggaatacaaaaggggaagacaaaaccttctaccctgaggaaattctccatgg 240
C.gunnari       agatggagggaacccaaaaataaaagtggaatacaaaaggggaagacaaaaccttctaccctgaggaaattctccatgg 240
C.rastrospinosus agatggagggaacccaaaaataaaagtggaatacaaaaggggaagacaaaaccttctaccctgaggaaattctccatgg 240
E.maclovinus    agatggagggaacccaaaaataaaagtggaatacaaaaggggaagacaaaaccttctaccctgaggaaattctccatgg 240
G.gibberifrons  agatggagggaacccaaaaataaaagtggaatacaaaaggggaagacaaaaccttctaccctgaggaaattctccatgg 240
N.coriiiceps    ggatggagggaacccaaaaataaaagtggaatacaaaaggggaagacaaaaccttctaccctgaggaaattctccatgg 240
P.borchgrevinki agatggagggaacccaaaaataaaagtggaatacaaaaggggaagacaaaaccttctaccctgaggaaattctccatgg 240

C.aceratus      tcctggtaaagatgaaggaaattgctgagggcgtaccttggccaaactgtgtccaatgcagtcgt 304
C.gunnari       tcctggtaaagatgaaggaaattgctgagggcgtaccttggccaaactgtgtccaatgcagtcgt 304
C.rastrospinosus tcctggtaaagatgaaggaaattgctgagggcgtaccttggccaaactgtgtccaatgcagtcgt 304
E.maclovinus    tcctggtaaagatgaaggaaattgctgagggcgtaccttggccaaactgtgtccaatgcagtcgt 304
G.gibberifrons  tcctggtaaagatgaaggaaattgctgagggcgtaccttggccaaactgtgtccaatgcagtcgt 304
N.coriiiceps    tcctggtaaagatgaaggaaattgctgagggcgtaccttggccaaactgtgtccaatgcagtcgt 304
P.borchgrevinki tcctggtaaagatgaaggaaattgctgagggcgtaccttggccaaactgtgtccaatgcagtcgt 304

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Fig. 1.A.10 Alignment of *HSP70* in Antarctic fishes. Forward primer is highlighted in pink, reverse primer is highlighted in blue. Non-conserved nucleotides are highlighted in black. Degenerate nucleotides are indicated by R and Y (R=A or G; Y=C or T). *N. coriiiceps* partial *HSP70* sequence was previously determined (GenBank: EU588987.1)

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C.aceratus      cgacatcgccctgtggaagttgagacggccaagtactacgtgaccatcattgatgccctggacacagggatttcatca 80
C.gunnari      cgacatcgccctgtggaagttgagacggccaagtactacgtgaccatcattgatgccctggacacagggatttcatca 80
C.rastrorpinosus cgacatcgccctgtggaagttgagacggccaagtactacgtgaccatcattgatgccctggacacagggatttcatca 80
E.maclovinus   cgacatcgccctgtggaagttgagacggccaagtactacgtgaccatcattgatgccctggacacagggatttcatca 79
G.gibberifrons cgacatcgccctgtggaagttgagacggccaagtactacgtgaccatcattgatgccctggacacagagatttcatca 80
N.coriiiceps   cgacatcgccctgtggaagttgagacggccaagtactacgtgaccatcattgatgccctggacacagggatttcatca 80
P.borchgrevinki cgacatcgccctgtggaagttgagacggccaagtactacgtgaccatcattgatgccctggacacagggatttcatca 80

C.aceratus      agaacaatgatcactgggtaccttcaggctgactgcgctgtgctgatcgttgctgcgggtgttggtgagttgaggccgggt 160
C.gunnari      agaacaatgatcactgggtaccttcaggctgactgcgctgtgctgatcgttgctgcgggtgttggtgagttgaggccgggt 160
C.rastrorpinosus agaacaatgatcactgggtaccttcaggctgactgcgctgtgctgatcgttgctgcgggtgttggtgagttgaggccgggt 160
E.maclovinus   agaacaatgatcactgggtaccttcaggctgactgcgctgtgctgatcgttgctgcgggtgttggtgagttgaggccgggt 159
G.gibberifrons agaacaatgatcactgggtaccttcaggctgactgcgctgtgctgatcgttgctgcgggtgttggtgagttgaggccgggt 160
N.coriiiceps   agaacaatgatcactgggtaccttcaggctgactgcgctgtgctgatcgttgctgcgggtgttggtgagttgaggccgggt 160
P.borchgrevinki agaacaatgatcactgggtaccttcaggctgactgcgctgtgctgatcgttgctgcgggtgttggtgagttgaggccgggt 160

C.aceratus      atctcaagaacggccagaccgtagcaccgacctgctggctttcacccctcgggtgtgaagcagctcatcgttaggagtcaa 240
C.gunnari      atctcaagaacggccagaccgtagcaccgacctgctggctttcacccctcgggtgtgaagcagctcatcgttaggagtcaa 240
C.rastrorpinosus atctcaagaacggccagaccgtagcaccgacctgctggctttcacccctcgggtgtgaagcagctcatcgttaggagtcaa 240
E.maclovinus   atctcaagaacggccagaccgtagcaccgacctgctggctttcacccctcgggtgtgaagcagctcattgtaggagtcaa 239
G.gibberifrons atctcaagaacggccagaccgtagcaccgacctgctggctttcacccctcgggtgtgaagcagctcatcgttaggagtcaa 240
N.coriiiceps   atctcaagaacggccagaccgtagcaccgacctgctggctttcacccctcgggtgtgaagcagctcatcgttaggagtcaa 240
P.borchgrevinki atctcaagaacggccagaccgtagcaccgacctgctggctttcacccctcgggtgtgaagcagctcatcgttaggagtcaa 240

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Fig. 1.A.11 Alignment of *EF-1 α* in Antarctic fishes. Forward primer is highlighted in pink, reverse primer is highlighted in blue. Non-conserved nucleotides are highlighted in black. Degenerate nucleotides are indicated by H, N, R, and Y (H=A or C or T; N=A or C or G or T; R=A or G; Y=C or T). *C. aceratus*, *C. rastrorpinosus*, *G. gibberifrons*, and *N. coriiiceps* partial *EF-1 α* sequences were previously determined (GenBank: EU857824, EU857825, EU857826; Urschel and O'Brien 2008; Mueller et al. 2012)

C.aceratus caagatggactccaacgagcccccttacagccaagcccgcttatgaaagaatcccaaggaagtgagcacttacatcaaga 320
 C.gunnari caagatggactccaacgagcccccttacagccaagcccgcttatgaaagaatcccaaggaagtgagcacttacatcaaga 320
 C.rastrospinosus caagatggactccaacgagcccccttacagccaagcccgcttatgaaagaatcccaaggaagtgagcacttacatcaaga 320
 E.maclovinus caagatggactccaacgagcccccttacagccaagcccgcttatgaaagaatcccaaggaagtgagcacttacatcaaaa 318
 G.gibberifrons caagatggactccaacgagcccccttacagccaagcccgcttatgaaagaatcccaaggaagtgagcacttacatcaaga 320
 N.coriiiceps taagatggactccaacgagcccccttacagccaagcccgcttatgaaagaatcccaaggaagtgagcacttacatcaaga 320
 P.borchgrevinki caagatggactccaacgagcccccttacagccaagcccgcttatgaaagaatcccaaggaagtgagcacttacatcaaga 320

C.aceratus agatcggctacaaccccttaactgtgccctttgtcccactctctggaatggcaccggagacaacatgctggaagccagtga 400
 C.gunnari agatcggctacaaccccttaactgtgccctttgtcccactctctggaatggcaccggagacaacatgctggaagccagtga 400
 C.rastrospinosus agatcggctacaaccccttaactgtgccctttgtcccactctctggaatggcaccggagacaacatgctggaagccagtga 400
 E.maclovinus agatcggctacaaccccttaactgtgccctttgtcccactctctggaatggcaccggagacaacatgctggaagccagtga 398
 G.gibberifrons agatcggctacaaccccttaactgtgccctttgtcccactctctggaatggcaccggagacaacatgctggaagccagtga 400
 N.coriiiceps agatcggctacaaccccttaactgtgccctttgtcccactctctggaatggcaccggagacaacatgctggaagccagtga 400
 P.borchgrevinki agatcggctacaaccccttaactgtgccctttgtcccactctctggaatggcaccggagacaacatgctggaagccagtga 400

C.aceratus aagatgacatgggttaagggaatggaaggttgagcgttaaggagggtaatgccagtggagtcaactctgctggagtctctcga 480
 C.gunnari aagatgacatgggttaagggaatggaaggttgagcgttaaggagggtaatgccagtggagtcaactctgctggagtctctcga 480
 C.rastrospinosus aagatgacatgggttaagggaatggaaggttgagcgttaaggagggtaatgccagtggagtcaactctgctggagtctctcga 480
 E.maclovinus aagatgacatgggttaagggaatggaaggttgagcgttaaggagggtaatgccagtggagtcaactctgctggagtctctcga 478
 G.gibberifrons aagatgacatgggttaagggaatggaaggttgagcgttaaggagggtaatgccagtggagtcaactctgctggagtctctcga 480
 N.coriiiceps aagatgacatgggttaagggaatggaaggttgagcgttaaggagggtaatgccagtggagtcaactctgctggagtctctcga 480
 P.borchgrevinki aagatgacatgggttaagggaatggaaggttgagcgttaaggagggtaatgccagtggagtcaactctgctggagtctctcga 480

C.aceratus tgccatcctgcccccgctccgcccacagacaagcccctcgtctgccctctgacaggcgtttacaagatcggcggccatc 560
 C.gunnari tgccatcctgccccatcccgcccacagacaagcccctcgtctgccctctgacaggcgtttacaagatcggcggccatc 560
 C.rastrospinosus tgccatcctgcccccgctccgcccacagacaagcccctcgtctgccctctgacaggcgtttacaagatcggcggccatc 559
 E.maclovinus ctccatcctgccccctgctccgcccacagacaagcccctcgtctgccctctgacaggcgtttacaagatcggcggccatc 557
 G.gibberifrons tgccatcctgcccccgctccgcccacagacaagcccctcgtctgccctctgacaggcgtttacaagatcggcggccatc 560
 N.coriiiceps tgccatcctcccccgctccgcccacagacaagcccctcgtctgccctctgacaggcgtttacaagatcggcggccatc 560
 P.borchgrevinki tgccatcctgcccccgctccgcccacagacaagcccctcgtctgccctctgacaggcgtttacaagatcggcggccatc 560

Fig. 1.A.11 Continued

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**Chapter 2: Cold acclimation induces expression of heat shock proteins and sirtuins
in threespine stickleback¹**

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2.1 Abstract

Cold acclimation of fishes leads to changes in metabolism and increases in oxidative stress and levels of oxidatively modified proteins. We hypothesized that levels of molecular chaperones (heat shock proteins) might also increase during cold acclimation to assist with folding newly synthesized proteins, replacing damaged ones. We also hypothesized that increases in heat shock proteins (Hsps) would be driven by an increase in sirtuins, NAD-dependent deacetylases that regulate expression of Hsps in mammals, as well as changes in metabolism similar to those occurring during cold acclimation in fishes. Transcript levels of *SIRT1-4*, *HSP90*, *HSP70*, *HSP60*, and *HSC70*, along with protein levels of Hsp70/Hsc70 were quantified in liver and pectoral muscle of threespine stickleback (*Gasterosteus aculeatus*) cold acclimated to 8°C for 9 weeks. In liver, cold acclimation stimulated an increase in mRNA levels of *HSP60*, *HSC70*, *SIRT1*, and *SIRT2* but no changes in *HSP90* or *HSP70*. In pectoral muscle, *HSP90*, *HSP60*, *HSC70*, and *SIRT1* mRNA levels increased, *HSP70* decreased, and *SIRT2-4* did not change in response to cold acclimation. Increases in Hsps in the liver, where levels of oxidized proteins increase, may assist with folding newly synthesized proteins, whereas in the pectoral muscle, where mitochondrial biogenesis occurs during cold acclimation, Hsps may facilitate protein import into mitochondria. Our results indicate that induction of *HSPs* and *SIRTs* is tissue and isoform specific.

Key Words

cold acclimation, fish, heat shock proteins, metabolism, oxidative stress, sirtuins

2.2 Introduction

Temperate fishes may endure seasonal changes in temperature as great as 30°C, warranting metabolic remodeling to maintain ATP production (Elliott and Elliott, 2010; Hochachka and Somero, 2002). As temperature decreases, levels of metabolic enzymes often increase to offset the depressive effects of cold temperature on the catalytic rate of enzymes (Egginton and Sidell, 1989; Johnston and Maitland, 1980; Orczewska et al., 2010). Concurrent with an increase in aerobically-poised enzymes is an increase in the percent cell volume displaced by mitochondria in some tissues. For example, mitochondrial volume density is 1.6-fold higher in red muscle fibers of striped bass (*Morone saxatilis*) acclimated to 5°C compared to those at 25°C (Egginton and Sidell, 1989). Similarly, mitochondrial volume density is 1.9-fold higher in oxidative pectoral muscle of threespine stickleback (*Gasterosteus aculeatus*) at 8°C compared to stickleback at 20°C (Orczewska et al., 2010). The maximal activity of aerobic metabolic enzymes, including citrate synthase (CS) and cytochrome c oxidase (COX), also increases in many fish species in response to cold acclimation, although sometimes independently of increases in mitochondrial density (Fangue et al., 2009; Orczewska et al., 2010). The trigger for inducing alterations in metabolism in fishes in response to cold temperature is unknown. In mammals, mitochondrial biogenesis is activated by AMP-activated protein kinase (AMPK), calcium, carbon monoxide, nitric oxide, reactive oxygen species (ROS), and sirtuin-1 (Jager et al., 2007; Kang et al., 2009; Lagouge et al., 2006; Nisoli et al., 2003; Suliman et al., 2007; Wu et al., 2002).

Changes in temperature also promote oxidative stress in some fishes. For example, protein carbonyls, one form of protein oxidation and an indirect measure of ROS, are 2.3-fold higher in the liver of threespine stickleback cold acclimated to 8°C compared to those at 20°C (Kammer et al., 2011). Additionally, levels of lipid peroxidation are 1.6-fold higher in the liver of gilthead sea bream (*Sparus aurata*) cold acclimated to 8°C compared to those at 20°C (Ibarz et al., 2010). Both lipid peroxidation and protein carbonyl levels are 1.5-fold higher in eelpout (*Zoarces viviparous*) acclimated to 6°C compared to animals at 12°C (Heise et al., 2007). Levels of antioxidants also increase in response to cold acclimation in some fishes to mitigate ROS production. The maximal activity of superoxide dismutase (SOD) increases 2.3- to 3.3-fold in muscle and liver tissue of threespine stickleback in response to cold acclimation to 8°C (Kammer et al., 2011). Similarly, in the brain of zebrafish (*Danio rerio*), levels of protein carbonyls, mRNA levels of the antioxidant catalase (CAT), and activity of SOD significantly increase when temperature decreases from 28°C to 18°C (Tseng et al., 2011).

Increases in antioxidants and levels of oxidized macromolecules during cold acclimation suggest cold temperature may compromise protein integrity. Consistent with this, activity of the 20S proteasome is 1.3-fold higher in white muscle of juvenile spotted wolffish (*Anarhichas minor*) acclimated to 4°C compared to wolffish at 8°C, and 1.8-fold higher compared to animals at 12°C (Lamarre et al., 2009). Higher rates of protein degradation at cold temperature may warrant increased rates of protein synthesis and levels of heat shock proteins (Hsps) to fold newly synthesized proteins, replacing

oxidatively damaged ones. Studies in cold-adapted fishes support this conjecture. The emerald rockcod (*Trematomus bernacchii*), sharp-spined notothenia (*Trematomus pennellii*), and the bald notothen (*Pagothenia borchgrevinki*) inhabiting the Southern Ocean, where water temperatures hover near -1.9°C , have higher levels of ubiquitinated proteins and levels of Hsp70 protein than the closely-related temperate thornfish (*Bovichtus variegatus*) and Maori chief (*Notothenia angustata*) inhabiting New Zealand waters (Place et al., 2004; Todgham et al., 2007). To date, few studies have investigated whether levels of Hsps change during cold acclimation in fishes.

The molecular pathway mediating changes in metabolism and responses to oxidative stress during cold acclimation in fishes may be governed by sirtuins, a family of NAD-dependent deacetylases and ADP-ribosyltransferases that are highly conserved among prokaryotes and eukaryotes (Landry et al., 2000; Liszt et al., 2005; Smith et al., 2000). In mammals, there are seven sirtuins, Sirts1-7, localized to different compartments in the cell. Sirt1, Sirt6, and Sirt7 are found in the nucleus, whereas Sirt2 is found in the cytoplasm, and Sirts3-5 are found in mitochondria (Michishita et al., 2005). Sirtuins function as energy sensors, increasing activity as ADP or NAD^{+} increases (Gambini et al., 2011; Lin et al., 2002), and as such are central mediators of metabolism (Ahn et al., 2008; Osborne et al., 2013). Sirt1 activity is positively correlated with CS activity and COX protein levels in heart and muscle tissue of rats (Gurd et al., 2011). Sirt3 deacetylates acetyl-CoA synthetase 2, activating it and increasing the synthesis of acetyl-CoA (Schwer et al., 2006). During fasting, Sirt3 protein is upregulated, increasing the activity of long-chain acyl-CoA dehydrogenase, increasing fatty acid metabolism

(Hirschev et al., 2010). Additionally, Sirt3 deacetylates and stimulates enzymatic activity of subunits of complex I and II of the respiratory chain (Ahn et al., 2008; Finley et al., 2011).

In addition to regulating metabolic remodeling, sirtuins may also mediate responses to oxidative stress. In mouse embryonic fibroblast cells, exposure to H₂O₂ increases *SIRT2* gene expression and enhances Sirt2 deacetylation of the O class of forkhead box transcription factors (FoxO), which then transactivates the expression of *MnSOD* (Wang et al., 2007). Furthermore, in human embryonic kidney cells exposed to the superoxide-generating compound paraquat, Sirt3 deacetylates MnSOD and increases its activity 1.6-fold (Qiu et al., 2010). In mammals, Sirt1 also regulates expression of Hsps by deacetylating a key lysine residue, K80, within the DNA binding domain of the transcription factor Hsf1, which in turn promotes Hsf binding to the heat shock element of *HSP* genes, stimulating their transcription (Westerheide et al., 2009).

Few studies to date have investigated the role of sirtuins in mediating responses to changes in temperature in ectotherms. A study by Han et al. (2013) showed that sirtuins increase concurrently with Hsps in response to heat stress in the intertidal limpet *Cellana toreuma*, suggesting sirtuins may regulate levels of Hsps. Transcript levels of *HSP70*, *HSP90*, *AMPK*, and *SIRT1* all increased in response to heat shock to 40°C in *C. toreuma* (Han et al., 2013).

Previous findings in our lab have indicated that the molecular basis of aerobic metabolic remodeling differs between pectoral muscle and liver in response to cold

acclimation. In pectoral muscle mitochondrial biogenesis occurs, while in liver the concentration of aerobic metabolic enzymes increase (Orczewska et al., 2010), potentially leading to differences in the expression of Hsps and Sirts. We hypothesized that Hsp expression would increase in stickleback during early stages of cold acclimation and that sirtuins would increase concurrently, driving changes in metabolism and levels of Hsps. To test this hypothesis, stickleback were cold acclimated to 8°C for 9 weeks or maintained at 20°C. Transcript levels of *HSP90*, *HSP70*, *HSP60*, *HSC70*, and *SIRT1-4*, and protein levels of Hsp70/Hsc70 were quantified in pectoral muscle and liver tissue in stickleback harvested during cold acclimation and in those held at 20°C. Threespine stickleback are temperate fishes found along the western coast of the US from Alaska to California (Wootton, 1984). In interior Alaska, where we collected animals, stickleback experience seasonal changes in temperature between 0 and 19.1°C (nwis.waterdata.usgs.gov/ak/nwis). The stickleback genome has been sequenced, making them ideal for molecular biological studies.

2.3 Methods

2.3.1 Animal collection and experimental procedures

All protocols for the treatment and use of animals were approved by the University of Alaska Fairbanks Institutional Animal Care Committee (135490-2 and 135490-7). Threespine stickleback were captured in Kashwitna Lake, AK (61°50' N,

150°00' W) using minnow traps in August 2007 and September 2008. Water temperature at time of capture was 16.5°C in 2007 and 12.6°C in 2008. Fish were transported to the University of Alaska Fairbanks and maintained in filtered, aerated 114 L aquaria filled with water and 0.35% Instant Ocean. Fish were maintained for 12 weeks on a 10:14 h light:dark cycle at 20°C and fed bloodworms and brine shrimp twice daily. After 12 weeks, the first group of fish was harvested (20°C day 1). The fish were then either cold acclimated to 8°C or maintained at 20°C for 9 weeks. After the initial harvest on day 1, the temperature was decreased for cold-acclimated animals to 15°C on day 2, 10°C on day 3, and 8°C on day 4. Fish were kept at 8°C for an additional 9 weeks, with 6-12 fish harvested after 1 and 4 weeks. Fish were harvested each day prior to feeding and decreasing temperature. Fish were harvested and immediately frozen in liquid nitrogen and stored at -80°C.

2.3.2 RNA extraction

RNA was isolated from pectoral muscle and liver tissue using the RNeasy Fibrous Tissue Mini-kit (Qiagen), with minor modifications to increase yield, as follows. Samples were treated twice with DNase I: once for 25 min and again for 20 min. Complimentary DNA (cDNA) was synthesized using TaqMan reverse transcription reagents (Applied Biosystems). Each reaction contained 5.5 mM MgCl₂, 2.5 μM random hexamers, 2 mM deoxynucleotide triphosphates (dNTPs), 4 units of RNase inhibitor, 37.5 units of reverse transcriptase, and 200 ng RNA. For each sample, a control was also

synthesized lacking reverse transcriptase (-RT). cDNA synthesis was performed using an iCycler (Bio-Rad) programmed at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min.

2.3.3 Quantitative real-time PCR

Gene specific primers were designed using Primer Express v3.0 software (Applied Biosystems) with either a forward or reverse primer of each set annealing over a splice site when possible to ensure genomic DNA was not amplified (Table 1). Sequence information was obtained from Ensembl (www.ensembl.org). Several sequences exist for each target gene; therefore, primers were selected based on homology with other closely-related fish species. Transcript levels of *HSP90*, *HSP70*, *HSP60*, *HSC70*, *SIRT1*, *SIRT2*, *SIRT3*, *SIRT4*, *18S*, and *EF-1 α* were measured using quantitative real-time PCR (qRT-PCR) and an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) as described previously (Kammer et al., 2011; Orczewska et al., 2010). Each reaction contained 1X Power SYBR Green PCR Master Mix, 0.3 μ M of each forward and reverse primer, and 5 ng of cDNA template, except for *HSP70* and *HSC70* in pectoral muscle and *HSP90* and *HSP70* in liver where gene expression was low and 10 ng cDNA template was required to detect transcripts. In addition, only 1 ng of cDNA was used in each reaction to quantify levels of *18S*. cDNA from all temperatures and time points was pooled and serially diluted to create a standard curve for quantifying relative transcript

levels. Relative transcript levels of *HSP/HSC* and *SIRT* genes were normalized to transcript levels of *EF-1 α* in pectoral muscle and *18S* in liver tissue, which were previously determined to be suitable housekeeping genes (Orczewska et al., 2010). Only samples with critical threshold (CT) values greater than 4 CTs away from their corresponding –RT control were included. All measurements were made in triplicate in 6-11 individuals per tissue and time point.

2.3.4 Hsp70/Hsc70 protein levels

Frozen pectoral muscle and liver tissue homogenates were used from previous studies (Kammer et al., 2011; Orczewska et al., 2010). Tissues were homogenized in 10 volumes of ice-cold buffer (either 250 mM Tris·HCl, 10 mM EDTA, 10 mM EGTA, pH 7.4 or 50 mM K₂HPO₄/KH₂PO₄, 0.05% Triton X-100, pH 7.5), with both buffers being used equally between tissues and treatments. Homogenates were centrifuged for 1 min at 100 g and supernatant was collected. Protein concentration was determined by Bradford assay (Bradford, 1976). Tissue homogenates were added to SDS sample buffer (20 mM NaPO₄, 8% glycerol, 40 mM dithiothreitol, 0.025% Bromophenol Blue, 4% SDS, pH 6.8) and separated on 12% polyacrylamide gels (Bio-Rad). Homogenates from animals collected at all temperature and time points during the acclimation were run on the same gel for each tissue. Proteins were transferred to a 0.45 μ m nitrocellulose membrane using

a semi-dry transfer apparatus (GE Healthcare) set at 0.8 mA cm^{-1} of mask opening for 1 h and transfer buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol. Membranes were blocked for at least 1 h in Blotto (5% nonfat dry milk in phosphate buffered saline (PBS) with 0.1% Tween-20) at room temperature and washed in 0.1% PBS-Tween as follows: 2 quick rinses, one rinse for 15 min, and twice for 5 min each. Membranes were incubated with mouse anti-Hsp70/Hsc70 primary antibody (Pierce MA1-10891) diluted 1:2000 in Blotto overnight at 4°C . Following incubation, the membranes were washed again as described above and incubated in donkey anti-mouse horseradish peroxidase-linked secondary antibody (Pierce PA1-28748) diluted 1:2500 in Blotto for 1 h at room temperature. After a final wash as described above, membranes were developed using Amersham ECL Prime Western Blotting Detection Reagent kit (GE Healthcare) following manufacturer's instructions and visualized using an AlphaImager 3400 CCD camera and AlphaEaseFC software v4.0.1 (ProteinSimple). Protein density was quantified using ImageQuant TL v.8.1 (GE Healthcare). All measurements were made in duplicate in 4-6 individuals per tissue and treatment. Relative levels of Hsp70/Hsc70 were normalized to 75 μg of rat liver loaded on each gel with samples.

2.3.5 Statistical analyses

Significant differences in gene expression and protein levels among stickleback held at different temperatures and collected at different time points were determined using a Kruskal-Wallis test followed by a *post hoc* Tukey's honestly significant difference test for multiple comparisons using JMP v7.0.2 (SAS). Significance was set at $\alpha < 0.05$.

2.4 Results

2.4.1 Levels of heat shock proteins

In general, transcript levels of *HSP90* were low in liver and undetectable in animals on day 4 at 8°C and in animals at 20°C week 9 (Fig. 2.1a). mRNA levels of *HSP90* did not change in stickleback livers during cold acclimation ($P > 0.05$; Fig. 2.1a). In contrast, *HSP60* mRNA levels increased in response to cold acclimation in liver ($P < 0.05$; Fig. 2.1a). *HSP60* mRNA levels increased significantly by day 4 of cold acclimation (8°C). Transcript levels were highest in stickleback at 8°C on day 4 when they were 3.3-fold higher compared to fish at 20°C on day 1 and 7.9-fold higher than stickleback at 20°C for 9 weeks. Levels of *HSP60* transcripts decreased after 4 weeks of acclimation at 8°C and by that time were equivalent to those of fish at 20°C on day 1.

HSC70 mRNA levels also increased during cold acclimation in stickleback livers and were significantly greater in animals at 10°C on day 3 and in those at 8°C on day 4 compared to stickleback at 20°C on day 1 ($P < 0.05$; Fig. 2.1b). In animals at 10°C on day 3, *HSC70* mRNA levels were 7.8-fold higher than fish at 20°C on day 1, and at 8°C on day 4, levels were 10.2-fold higher than fish at 20°C on day 1. After 1 week of acclimation at 8°C, *HSC70* transcripts were equivalent to those in fish at 20°C on day 1 and 20°C for 9 weeks. *HSP70* transcript did not change in response to cold acclimation but were significantly higher in livers of stickleback at 20°C for 9 weeks compared to all other temperatures and time points (Fig. 2.1b).

In pectoral muscle, both *HSP90* and *HSP60* mRNA levels increased by day 3 of cold acclimation (10°C) ($P < 0.05$; Fig. 2.1c). *HSP90* transcripts were highest in stickleback at 8°C on day 4 where levels were 4.4- and 3.5-fold higher than fish at 20°C on day 1 and 20°C for 9 weeks, respectively. By the end of the acclimation period *HSP90* levels were equivalent between stickleback at 8°C for 9 weeks and those at 20°C for 9 weeks. *HSP60* mRNA levels were highest in stickleback at 8°C on day 4 where levels were 7.3- and 4.9-fold greater than fish at 20°C on day 1 and 20°C for 9 weeks, respectively. After 9 weeks of acclimation at 8°C, *HSP60* transcripts decreased and were comparable to stickleback at 20°C for 9 weeks.

HSC70 mRNA levels significantly increased in pectoral muscle of stickleback at 10°C on day 3 and were highest in animals at 8°C on day 4 when levels were 4.8- and 2.3-fold greater than stickleback at 20°C on day 1 and 20°C for 9 weeks, respectively ($P < 0.05$; Fig. 2.1d). By week 4 of acclimation, *HSC70* transcripts decreased and were

equivalent to those in animals at 20°C for 9 weeks. Similar to the liver, *HSP70* transcript levels did not increase in pectoral muscle during cold acclimation. However, transcripts were significantly higher in stickleback at 20°C for 9 weeks compared to those at 15°C on day 2, 10°C on day 3, 8°C on day 1, and 8°C for 1 week ($P < 0.05$).

Levels of Hsp70/Hsc70 proteins tended to increase in liver tissue during cold acclimation ($P = 0.17$; Figs. 2.2a and 2.2c) but did not change in pectoral adductor muscle ($P > 0.05$; Figs. 2.2b and 2.2d).

2.4.2 Expression of sirtuins

Transcript levels of *SIRT1* and *SIRT2* increased in liver of stickleback in response to cold acclimation ($P < 0.05$; Fig. 2.3a). *SIRT1* mRNA levels increased significantly in animals at 10°C on day 3 and remained elevated throughout the acclimation period. Transcripts were highest in stickleback at 8°C on day 4 where levels were 3.9- and 2.5-fold greater compared to fish at 20°C on day 1 and 20°C for 9 weeks, respectively. *SIRT2* transcript levels increased significantly in the liver of fish at 8°C on day 4 and remained elevated throughout the acclimation period. Transcripts were highest in stickleback at 8°C for 4 weeks where levels were 2.6- and 1.7-fold greater than fish at 20°C on day 1 and 20°C for 9 weeks, respectively. *SIRT3* mRNA levels were significantly lower in animals on 8°C day 4 compared to 20°C day 1 but not 20°C week 9 (Fig. 2.3b). Levels

of *SIRT4* mRNA did not change in the liver of stickleback at any time during acclimation ($P > 0.05$; Fig. 2.3b).

SIRT1 mRNA levels in pectoral muscle of stickleback significantly increased at 8°C on day 4 compared to 20°C on day 1 and 20°C for 9 weeks ($P > 0.05$; Fig. 2.3c). *SIRT1* transcripts were 3.2- and 3-fold higher in animals at 8°C on day 4 compared to fish at 20°C on day 1 and 20°C for 9 weeks. Levels of *SIRT2* mRNA did not change in the pectoral muscle of stickleback during acclimation ($P > 0.05$; Fig. 2.3c). Levels of *SIRT3* mRNA were 2.8-fold lower in pectoral muscle of fish at 8°C for 4 weeks compared to fishes at 20°C on day 1 but not were not significantly different from fish at 20°C for 9 weeks (Fig. 2.3d). *SIRT4* mRNA levels did not change in the pectoral muscle of stickleback during acclimation ($P > 0.05$; Fig. 2.3d).

2.5 Discussion

To our knowledge, this is the first study to show that several heat shock proteins increase during cold acclimation in fishes. Our results indicate that transcript levels of Hsps increase transiently during cold acclimation in stickleback, and that the expression of Hsp isoforms differs between liver and oxidative muscle, likely reflecting tissue-specific changes in metabolism and oxidative stress. Transcript levels of sirtuins also increased during cold acclimation, suggesting they may play a role in reorganizing metabolism and/or mediating oxidative stress responses and the expression of Hsps during cold acclimation.

2.5.1 Hsps may increase during cold acclimation in response protein denaturation

Levels of Hsps may increase in response to cold acclimation in stickleback to refold proteins denatured by cold temperature. *HSP90*, *HSP60*, and *HSC70* transcripts increased in pectoral muscle and *HSP60* and *HSC70* increased in the liver tissue within the first 3-4 days of cold acclimation in stickleback, indicating a greater demand for chaperone-mediated folding as fishes adjust to decreases in temperature. Similar to heat stress, cold stress weakens non-covalent bonds, destabilizing the tertiary structure of proteins (Adrover et al., 2012). Rates of protein folding also decline with temperature (Scalley and Baker, 1997), which may increase need for chaperone-mediated folding. Consistent with this, the Antarctic emerald rock cod expresses higher constitutive levels of *HSP70* mRNA in gill and liver compared to related New Zealand species, the Maori chief and the thornfish (Place et al., 2004). Interestingly, warm acclimation of ocellated icefish (*Chionodraco rastrospinosus*), and black rockcod (*Notothenia coriiceps*) from 0°C to 4°C for 1 week decreases *HSP70* mRNA levels in heart ventricles (Teigen and O'Brien, unpublished), and warm acclimation of the Antarctic spiny plunderfish (*Harpagifer antarcticus*) from 0°C to 6°C for 48 hours tends to decrease *HSP70* mRNA levels in liver tissue (Clark et al., 2008), suggesting that slight elevations in temperature may alleviate cold-induced protein denaturation in Antarctic fishes.

It is not entirely surprising that Hsps increase in response to cold acclimation in fishes. Increases in Hsps have been widely documented in plants and insects in response

to cold stress and have been shown to be crucial for surviving at cold temperatures. Increased expression of Hsp101, Hsp90, Hsp70, Hsp23, and Hsp17 have been linked to overwinter survival and freeze tolerance in both plants and insects (Janska et al., 2011; Lopez-Matas et al., 2004; Rinehart et al., 2006; Rinehart et al., 2007). For example, in the sweet chestnut (*Castanea sativa*), *HSP17* is quickly induced in response to cold and the protein product, Hsp17, protects the enzyme lactate dehydrogenase from cold-induced inactivation (Lopez-Matas et al., 2004). Similarly, *HSP70*, *HSP60*, *HSP25*, *HSP23*, and *HSP18* are all upregulated during winter diapause in the flesh fly (*Sarcophaga crassipalpis*), and inactivation of either *HSP70* or *HSP23* genes results in a loss of cold tolerance (Rinehart et al., 2007).

Levels of Hsps may also increase during cold acclimation to fold newly synthesized proteins replacing oxidatively damaged ones. Increases in *HSP60* and *HSC70* mRNA transcript levels in livers of stickleback coincided with increases in levels of oxidatively damaged proteins during the first week of cold acclimation (Kammer et al., 2011). Studies have also shown that the activity of the 20S proteasome increases during cold acclimation in white muscle of juvenile spotted wolffish (Lamarre et al., 2009; Perepechaeva et al., 2006). Higher rates of protein turnover might warrant higher levels of Hsps to assist with protein folding. Consistent with this, previous studies have shown that levels of Hsps increase in response to oxidative stress. Lipid peroxidation and *HSP70* and *HSP90* mRNA levels increased in hepatocytes of grass carp (*Ctenopharyngodon idellus*) exposed to 32°C heat stress for 30 min (Cui et al., 2013). Similarly, an increase in Hsp70 protein was associated with an increase of protein

carbonyls in human skin fibroblasts after exposure to hydrogen peroxide (Calabrese et al., 2001), and under expression of Hsp60 protein after application of doxycycline is associated with increased protein carbonyls in yeast (*Saccharomyces cerevisiae*) (Cabiscol et al., 2002).

2.5.2 Increases in Hsps in pectoral muscle may assist with mitochondrial biogenesis

Transcript levels of *HSP60* increased to a greater extent in pectoral muscle than liver tissue (6.9-fold versus 3.3-fold) at 8°C on day 4 compared to 20°C on day 1. Similarly, transcript levels of *HSP90* increased significantly in pectoral muscle during cold acclimation but remained unchanged in liver. Notably, Hsp90 is involved in importing nuclear-encoded proteins into mitochondria during mitochondrial biogenesis, which occurs in pectoral muscle but not liver of stickleback during cold acclimation (Orczewska et al., 2010). The mitochondrial genome encodes only 13 of the estimated 1,400 mitochondrial proteins (Baker et al., 2012; Lotz et al., 2013; Taylor et al., 2003). The majority of mitochondrial proteins are synthesized on cytosolic ribosomes and must be imported into the mitochondria. Isoforms of Hsp70 and Hsp90 are located in the cytosol where they interact with the translocase of the outer membrane (TOM) of the mitochondria to facilitate import of proteins into the mitochondrial matrix (Kang et al., 1990; Lithgow et al., 1993; Young et al., 2003). Imported proteins are then refolded by

mitochondrial isoforms of Hsp70 and Hsp60 within the mitochondrial matrix (Cheng et al., 1989; Craig et al., 1989; Mogk et al., 2001).

2.5.3 Transcript levels of *HSP70* mRNA do not change with cold acclimation

While transcript levels of the putatively constitutive form of Hsp70, *HSC70*, increased in response to cold acclimation, *HSP70* did not increase in either pectoral muscle or liver of stickleback. This suggests that Hsp70, induced by heat stress, it is not induced by cold stress, while Hsc70 may be induced by both increases and decreases in temperature. Consistent with this, levels of *HSC70* mRNA significantly increased in muscle of the common carp (*Cyprinus carpio*) in response to heat stress (26°C) and cold stress (5°C) for 1-5 h, while *HSP70* mRNA did not change in skin or spleen with cold stress (Ali et al., 2003; Ferencz et al., 2012). Similarly, *HSP70* did not change in zebrafish embryonic cells after 1-6 h of cold shock at 20°C (Airaksinen et al., 2003). Protein levels of Hsp70 and Hsc70 did not change during cold acclimation, however, the antibody we used recognizes and binds to both isoforms. Consequently, if protein levels correspond with mRNA levels, the opposing changes in Hsp70 and Hsc70 would confound our results.

2.5.4 Expression of some sirtuins change with cold acclimation

SIRT1 mRNA increased in both liver and pectoral muscle with cold acclimation, suggesting it may mediate alterations in metabolism that occur during cold acclimation of stickleback. (Imai et al., 2000; Landry et al., 2000). Increases in levels of NAD^+ , indicative of a low energy charge, increases the activity of Sirt1 and Sirt2 (Canto et al., 2009; Wang et al., 2007). Notably, activity of Sirt1 is positively correlated with CS activity, which significantly increases in response to cold acclimation in goldfish (*Carassius auratus*), killifish (*Fundulus heteroclitus*), stickleback, and zebrafish (Fangue et al., 2009; Gurd et al., 2011; LeMoine et al., 2008; McClelland et al., 2006; Orczewska et al., 2010). Additionally, Sirt1 mediates mitochondrial biogenesis by deacetylating and activating peroxisome proliferator-activated receptor- γ coactivator-1 alpha (PGC-1 α), which promotes mitochondrial biogenesis (Lehman et al., 2000).

Sirtuins also alleviate oxidative stress. In response to ROS, Sirt2 deacetylates the forkhead transcription factor FoxO, which induces expression of *SOD2* (Kops et al., 2002; Wang et al., 2007). Sirt1 also increases expression of *SOD2* through FoxO transcription factors (Tanno et al., 2010) and PGC-1 α , which induces expression of *SOD2* and *CAT* (Aquilano et al., 2013; Geng et al., 2011; Rodgers et al., 2005). Activity of SOD increases by day 3 (10°C) in the pectoral muscle and day 4 (8°C) in the liver of stickleback, which coincides with increases in *SIRT1* mRNA in our study (Kammer et al., 2011).

The hepatosomatic index increases in response to cold acclimation in channel catfish (*Ictalurus punctatus*), eel (*Anguilla anguilla*), eelpout, gilthead sea bream, and stickleback (Ibarz et al., 2005; Jankowsky et al., 1984; Kammer et al., 2011; Lannig et al., 2005; Seddon and Prosser, 1997), and may be driven by *SIRT2*, which increases in liver but not pectoral muscle during cold acclimation of stickleback. Sirt2 deacetylates and activates cyclin-dependent kinase 9, which is required for the recovery of cells from replication arrest during S phase and continuation of the cell cycle through mitosis (Zhang et al., 2013). Activation of cyclin-dependent kinase 9 results in several downstream effects, including arresting the cell cycle to allow time for DNA repair, and promoting recovery after replication arrest (Zhang et al., 2013), indicating that Sirt2 may be particularly important in stimulating the cell cycle following oxidative stress and DNA damage, which occurs in the liver of fishes during cold acclimation (Jia et al., 2011; Shao et al., 2012; Velma and Tchounwou, 2013).

2.5.5 Mitochondrial *SIRT3* and *SIRT4* do not change during cold acclimation

Both Sirt3 and Sirt4 regulate metabolism, however, neither changed in response to cold acclimation in liver or pectoral muscle. PGC-1 α drives increases in mRNA and protein expression of Sirt3 (Kong et al., 2010). Given that there is no increase in *PGC-1 α* mRNA in either liver or muscle during cold acclimation of threespine stickleback (Orczewska et al., 2010), it is not surprising that we did not find an increase in *SIRT3* mRNA. In fishes, the nuclear respiratory factor-1 binding domain of *PGC-1 α* is severely

disrupted, likely impeding its ability to induce mitochondrial biogenesis in fishes (LeMoine et al., 2010). Consistent with this, mRNA levels of *PGC-1 α* do not increase in response to cold acclimation in muscle of goldfish or stickleback (Bremer et al., 2012; LeMoine et al., 2008; Orczewska et al., 2010).

2.6 Conclusion

Our results indicate that *HSPs* are induced by cold acclimation, and may increase to re-fold cold-denatured proteins, mediate protein import into mitochondria and/or fold proteins replacing oxidatively-damaged ones. During cold acclimation of threespine stickleback, there are tissue- and isoform-specific changes in *HSP* and *SIRT* mRNA, and these changes coincide with metabolic changes. In pectoral muscle, mRNA levels of three *HSPs* involved in protein import and folding in the mitochondria increased (*HSP90*, *HSP60*, and *HSC70*), while in the liver we found increases in *HSP60* and *HSC70* likely responding to increases in oxidative stress. Increases in *HSP* mRNAs in liver correlated with increases in *SIRT1* and *SIRT2* in liver, suggesting sirtuins may mediate responses to oxidative stress in this tissue. Conversely, there was only a modest increase in *SIRT1* in pectoral muscle during cold acclimation, suggesting metabolic remodeling may be driven by other factors in muscle in addition to Sirt 1.

2.7 Figures

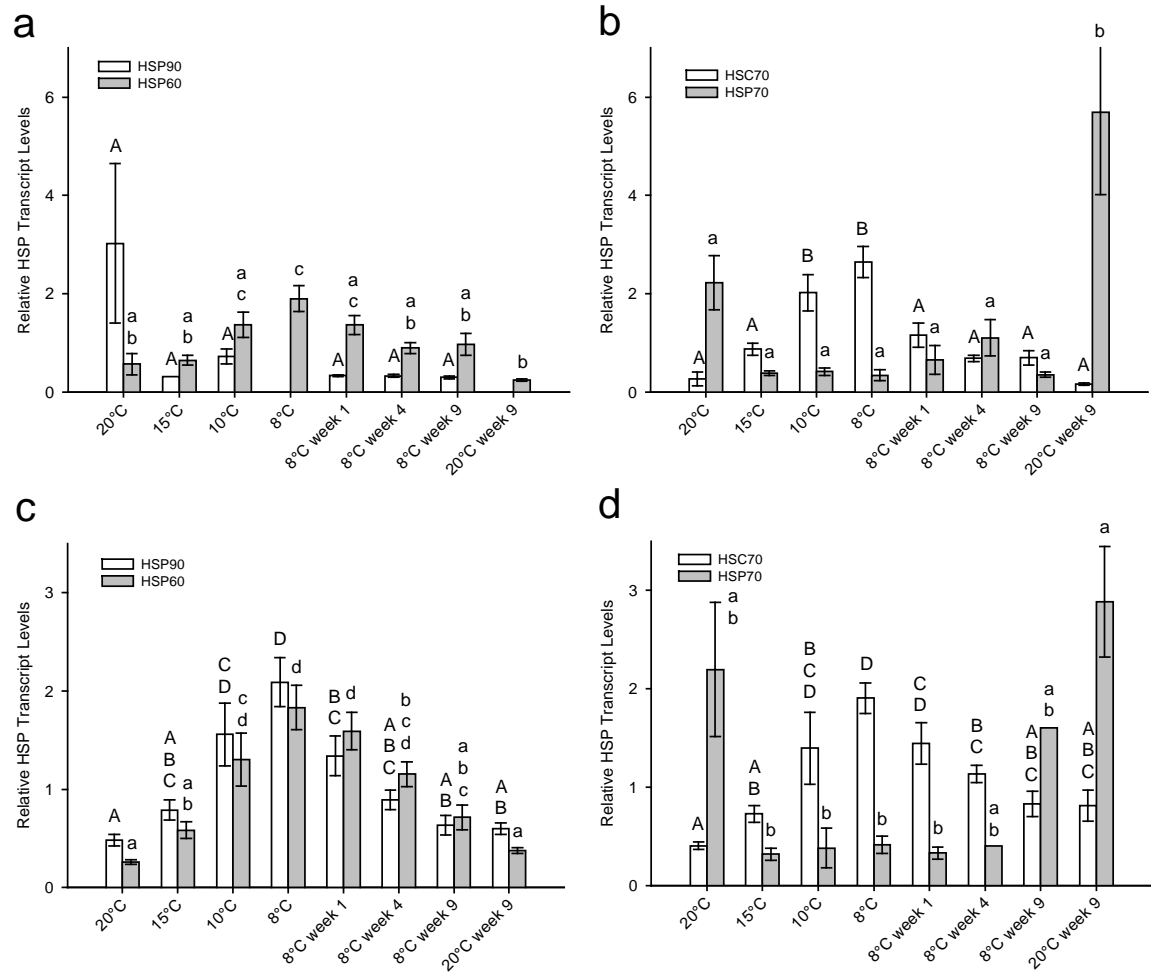


Fig. 2.1 *HSP* mRNA transcript levels in liver (a & b) and pectoral muscle (c & d) of threespine stickleback during cold acclimation. Transcript levels of *HSPs* were normalized to transcript levels of the housekeeping gene *EF-1 α* in pectoral muscle and *18S* in liver. Significant differences are indicated by different letters ($P < 0.05$). Values are mean \pm SEM, $N = 1-9$.

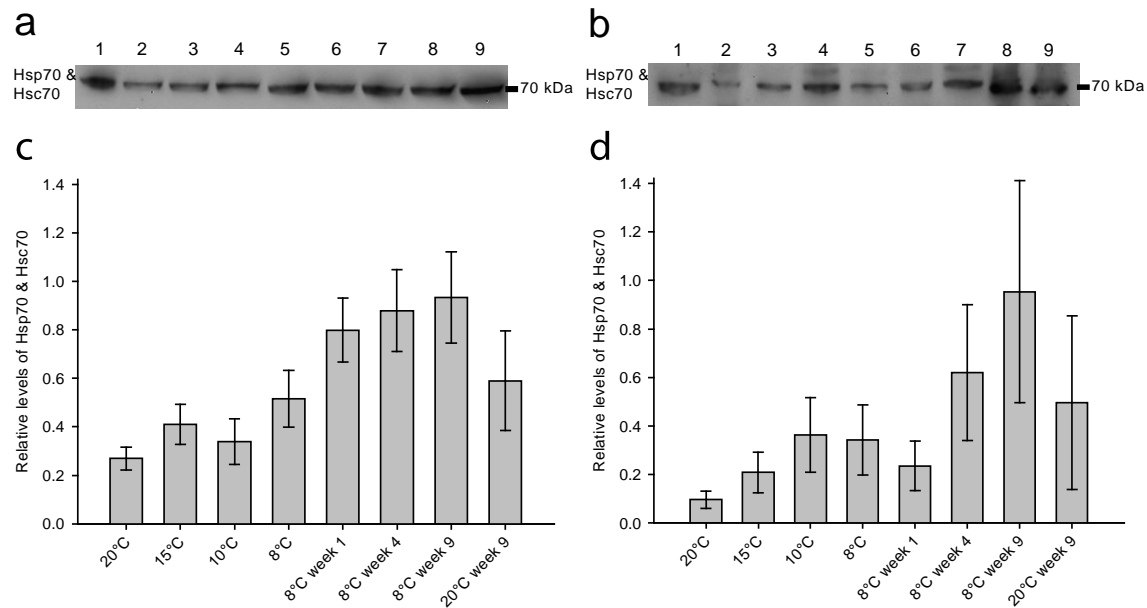


Fig. 2.2 Relative levels of Hsp70 and Hsc70 in liver (a & c) and pectoral muscle (b & d) of threespine stickleback during cold acclimation. Representative Western blot of Hsp70 and Hsc70 levels (a & b). Lane 1, rat liver; Lane 2, 20°C; Lane 3, 15°C; Lane 4, 10°C; Lane 5, 8°C; Lane 6, 8°C week 1; Lane 7, 8°C week 4; Lane 8, 8°C week 9; Lane 9, 20°C week 9. Analysis of Western blot, normalized to rat liver (c & d). 100 μ g of protein was loaded for each sample, except rat liver (75 μ g). Hsp70/Hsc70 levels tended to increase in liver, however there was no significant difference ($P = 0.17$). Values are mean \pm SEM, N = 4-6.

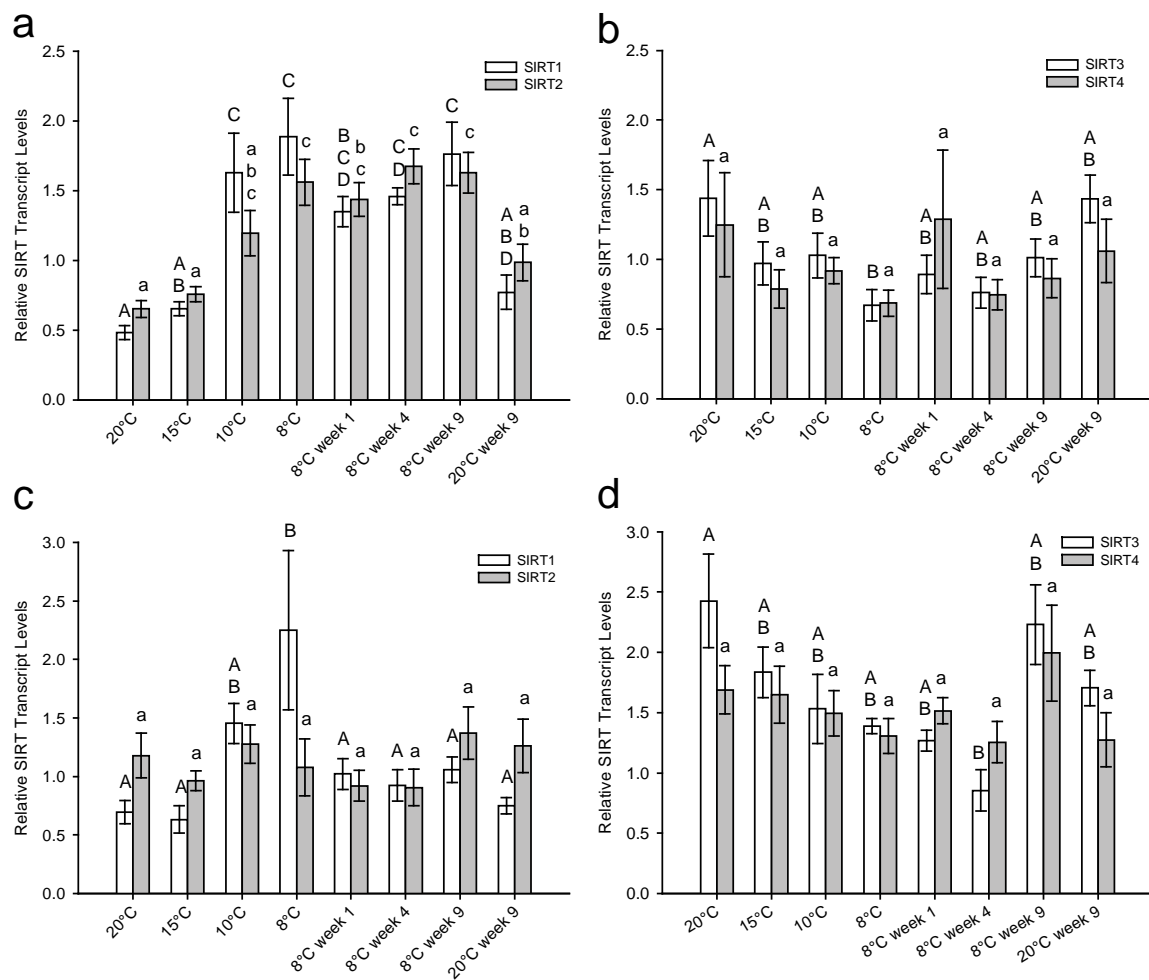


Fig. 2.3 *SIRT* mRNA transcript levels in liver (a & b) and pectoral muscle (c & d) of threespine stickleback during cold acclimation. Transcript levels of *SIRT*s were normalized to transcript levels of the housekeeping gene *EF-1 α* in pectoral muscle and *18S* in liver. Significant differences are indicated by different letters ($P < 0.05$). Values are mean \pm SEM, $N = 3-11$.

2.8 Tables

Table 2.1 Primers used for quantitative real-time PCR.

Gene (Ensembl ID ENSGACT000000)	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Amplicon (bp)
18S (21793)	ACCACATCCAAGGAAGGCAG	CCGAGTCGGGAGTGGGTAAT	51
EF-1 α (02143)	CGTCTACAAAATCGGAGGTATTGG	GTCTCAACACGGCCGACTG	53
HSP90 (17054)	TCAGCTGGAGTTCCGTGCTT	TCGAAAAGACGCCCTTCTGG	51
HSP60 (11881)	AAGTTGGCCGCAAGGGAG	CGTGCAGAGTCTTCCCATCC	51
HSC70 (13955)	CAGTGGAGGACGAGAAGCTG	TCTGTCTGTCCTCTTCGCTGAT	51
HSP70 (17285)	CTTCAGAGAGACAGGGTCTCCG	GAAGGCGTAGGACTCCAAGGA	51
SIRT1 (03411)	GCAAACGCCTTGAGACAGG	GCTCGACGTCTCGTCCTCAG	101
SIRT2 (05747)	ACCCAGGACAGTTTAAGCCGA	TGTAGCAGCGCCTCAAGTACC	81
SIRT3 (15722)	CGAGGACAAGCAGGACGC	GAGAGACGGTTTGTCTGCACAG	102
SIRT4 (01497)	CGCCATTGTGAACATTGGG	TTCAGCTCAGCCAGGTGGTC	51

18s, 18s rRNA; EF-1 α , elongation factor-1 α , HSP, heat shock protein; HSC, heat shock cognate; SIRT, sirtuin.

2.9 Acknowledgements

This research was supported by a National Science Foundation grant to K.M.O. (IOS-0643857).

2.10 Appendix A: Alignment of *HSP* Genes in Several Fishes

Methods

Hsp amino acid and gene sequences in *Gasterosteus aculeatus* were obtained from Ensembl (ensembl.org). For comparison, sequences from several fish species were obtained from the NCBI Protein and Nucleotide Databases (<http://www.ncbi.nlm.nih.gov/>). Sequence alignments were obtained using Color Align Conservation (<http://www.bioinformatics.org/sms2/>). Sequence specific primers (See Table 2.1) were designed from *G. aculeatus* sequences that showed the greatest homology with other fishes. To ensure Hsp70 and Hsc70 did not amplify the same product, *G. aculeatus* amino acid sequences were aligned based on homology with Hsp70 and Hsc70 in other fishes. Furthermore, cDNA sequences were compared between *HSP70* and *HSC70* in *G. aculeatus* to ensure primers were not specific for both isoforms.

Results

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ENSGACT017054 EMLQSKILKVIRKNLVKKCDLFELAEDKNYKYYEQFSKNIKLGIHEDSQNRRKLSELLRYTSNSGDETVSLKDY 471
D.rerio EMLQSKILKVIRKNLVKKCDLFELAEDKNYKYYEQFSKNIKLGIHEDSQNRRKLSDLLRYTSNSGDEMVSLKDY 474
E.coioides EMLQSKILKVIRKNIVKKCELFAELAEDKENYKFYEGFSKNIKLGIHEDSQNRRKLSELLRYHSSQSGDETTSLTEY 472
S.maximus EMLQSKILKVIRKNIVKKCELFAELAEDKENYKFYEGFSKNIKLGIHEDSQNRRKLSELLRYQSSQSGDETTSLTEY 474
S.salar EMLQSKILKVIRKNIVKKCELFAELAEDRENYKFYDGFSKNLKLGIHEDSQNRRKLSELLRYHSSQSGDELTSLTEY 469
T.albonubes EMLQSKILKVIRKNIVKKCELFAELAEDKENYKFYDAFSKNLKLGIHEDSQNRRKLSELLRYQSSQSGDEMTSLTEY 472

ENSGACT017054 VSRMKDNQKIYYITGETKDQVASAFVERLRKAGEVIYIMEPIDEYCVQQLKEYDGKNLVSVTKEGLELPEDEENLK 551
D.rerio VSRMKDTQKIYYITGETKDQVASAFVERLRKAGEVIYIMEPIDEYCVQQLKEYDGKNLVSVTKEGLELPEDEEKK 554
E.coioides LTRMKENQKIYYITGESKDQVASAFVERVRKRGEVLYMEPIDEYCVQQLKEFDGKSLVSVTKEGLELPEDEEKK 552
S.maximus LSRMKENQKIYYITGESKDQVASAFVERVRKRGEVLYMEPIDEYCVQQLKEFDGKSLVSVTKEGLELPEDEEKK 554
S.salar LTRMKDNQKIYYITGESKDQVASAFVERVRKRGEVLYMEPIDEYCVQQLKEFDGKTLVSVTKEGLELPEDEEKK 549
T.albonubes VSRMKENQKIYYITGESKDQVASAFVERVCKRGEVLYMEPIDEYCVQQLKDFDGKSLVSVTKEGLELPEDEDEKK 552

ENSGACT017054 QEEIKNKFENICKIMKDILDKKIEKVTSNRLVSSPCCIVTSTYGWTANERIMKSQALRDNSTMGYMAKKHLEINPLH 631
D.rerio QDEIKAKYENLCKIMKDILDKKIEKVTSNRLVSSPCCIVTSTYGWTANERIMKSQALRDNSTMGYMAKKHLEINPAH 634
E.coioides MEEIKAKFENLCKLMKEILDKKVEKVTSNRLVSSPCCIVTSTYGWTANERIMKSQALRDNSTMGYMAKKHLEINPDH 632
S.maximus MEEIKAKFENLCKLMKEILDKKVEKVTSNRLSSPCCIVTSTYGWTANERIMKSQALRDNSTMGYMAKKHLEINPDH 634
S.salar MDEIKTKFENLCKLMKEILDKKVEKVTSNRLVSSPCCIVTSTYGWTANERIMKSQALRDNSTMGYMAKKHLEINPDH 629
T.albonubes MEEIKAKFENLCKLMKEILDKKVEKVTSNRLVSSPCCIVTSTYGWTANERIMKSQALRDNSTMGYMAKKHLEINPDH 632

ENSGACT017054 PIVETLRVKAEADKNDKAVKDLVILLFETALLSSGFTLDDPQTHNRIYRMIKLGLGID-DDSAVDDLIH-PAEDMPV 709
D.rerio PIVETLRVKAEAEKNDKAVKDLVILLFETALLSSGFTLDDPQTHNRIYRMIKLGLGID-DDSVVEEISQ-PAEDMPV 712
E.coioides PIVETLRVKADADKNDKAVKDLVILLFETALLSSGFSLDDPQTHSNRIYRMIKLGLGID-DDVPAEEATSTSVPDEIPP 711
S.maximus PIVETLRVKADADKNDKAVKDLVILLFETALLSSGFSLDDPQTHSNRIYRMIKLGLGID-DDVPVEETTSAAVPDEIPP 713
S.salar PIVETLRVKADLDKNDKAVKDLVILLFETALLSSGFSLDDPQTHSNRIYRMIKLGLGID-DDEVIPEEPTSAPAPDEIPP 708
T.albonubes PIVETLRVKADADKNDKAVKDLVILLFETALLSSGFSLDDPQTHSDRIYRMIKLGLGID-DEDVPVEEPTSAPAPEEIPP 712

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Fig. 2.A.1 Alignment of *Gasterosteus aculeatus* Hsp90 amino acid sequence with other fishes. *G. aculeatus* sequence was obtained from Ensembl (ENSGACT00000017054) and Hsp90 sequences from the other fishes were obtained from the NCBI Protein Database (Accession ABU50778, ACV04938, ADK27678, NP_571403, and NP_001117004). Underlined text indicates conserved amino acid, black text without underline indicates amino acid of similar grouping (GAVLI, FYW, CM, ST, KRH, DENQ, or P), and gray text indicates amino acids of different groups.

ENSGACT017054	<u>L</u> -- <u>EGDDD</u> <u>SRMEEVD</u>	723
D.rerio	<u>L</u> -- <u>EGDDD</u> <u>SRMEEVD</u>	726
E.coioides	<u>LEG</u> <u>EGEDD</u> <u>SRMEEVD</u>	727
S.maximus	<u>LEG</u> <u>EGEDD</u> <u>SRMEEVD</u>	729
S.salar	<u>L</u> -- <u>EGDDD</u> <u>SRMEEVD</u>	722
T.albonubes	<u>L</u> -- <u>EGEDD</u> <u>SRMEEVD</u>	726

Fig. 2.A.1 Continued

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ENSGACT011881 MRLPTIMQRPVCRALAPHLTRAYAKDVKFGAEARAMLQGVDLLADAVAVTMGPKGRNVIEQSWGSPKVTKDGVTV 80
C.auratus MRLPSVMQRPVCRALAPHLTRAYAKEVKFGADARAMLQGVDLLADAVAVTMGPKGRTVIEQSWGSPKVTKDGVTV 80
D.rerio MRLPSVMQRPVCRALAPHLTRAYAKDVKFGADARAMLQGVDLLADAVAVTMGPKGRTVIEQSWGSPKVTKDGVTV 80
C.idella MRLPSVMQRPVCRALAPHLTRAYAKDVKFGADARAMLQGVDLLADAVAVTMGPKGRTVIEQSWGSPKVTKDGVTV 80
T.albonubes MRLPSVMQRPVCRALAPHLTRAYAKDVKFGADARAMLQGVDLLADAVAVTMGPKGRTVIIDQSWGSPKVTKDGVTV 80

ENSGACT011881 AKSIDLKDRYNIGAKLVQDVANNTNEEAGDGTTTATVLARAIAKEGFDTISKGANPVEIRRGVMAVEIVINELKNQSK 160
C.auratus AKSIDLKDRYNIGAKLVQDVANNTNEEAGDGTTTATVLARAVAKEGFDTISKGANPVEIRRGVMAVEIVISELKKISK 160
D.rerio AKSIDLKDRYNIGAKLVQDVANNTNEEAGDGTTTATVLARAVAKEGFDTISKGANPVEIRRGVMAVEIVISELKKNSK 160
C.idella AKSIELKDRYNIGARLVQDVANNTNEEAGDGTTTATVLARAVAKEGFDTISKGANPVEIRRGVMAVEIINELKKISK 160
T.albonubes AKSIELKDRYNIGARLVQDVANNTNEEAGDGTTTATVLARAIAKEGFDTISKGANPVEIRRGVMAVEIISELEKISK 160

ENSGACT011881 PVTTPEEIAQVATISANGDVEIGIISNAMKKVGRKGVITVKDGKTLHDELEIIEGMKFDRGYISPYFINTAKGQKEFQ 240
C.auratus PVTTPEEIAQVATISANGDVEVGIISNAMKKVGRKGVITVKDGKTLHDELEVIEGMKFDRGYISPYFINTAKGQKEFQ 240
D.rerio PVTTPEEIAQVATISANGDVEVGIISNAMKKVGRKGVITVKDGKTLHDELEIIEGMKFDRGYISPYFINTAKGQKEFQ 240
C.idella PVTTPEEIAQVATISANGDVEVGIISNAMKKVGRKGVITVKDGKTLHDELEIIEGMKFDRGYISPYFINTAKGQKEFQ 240
T.albonubes PVTTPEEIAQVATISANGDVEVGIISNAMKKAGRKGVITVKDGKTLHDELEIIEGMKFDRGYISPYFINTAKGQKEFQ 240

ENSGACT011881 DAYVLLSEKKISSVQSIVPALEIANQHRKPLVIVAEDVDGEALSTLVLNRLKVGLQVVAVKAPGFGDNRKNQLDMAVAT 320
C.auratus DAYVLLSEKKISSVQSIVPALEIANQHRKPLVIVAEDVDGEALSTLVLNRLKVGLQVVAVKAPGFGDNRKNQLDMAIST 320
D.rerio DAYVLLSEKKISSVQSIVPALEIANQHRKPLVIVAEDVDGEALSTLVLNRLKVGLQVVAVKAPGFGDNRKNQLDMAVST 320
C.idella DAYVLLSEKKISSVQSIVPALEIANQHRKPLVIVAEDVDGEALSTLVLNRLKVGLQVVAVKAPGFGDNRKNQLDMAVST 320
T.albonubes DIYVLLSEKKISSVQSIVPALEIANQHRKPLVIVAEDVDGEALSTLVLNRLKVGLQVVAVKAPGFGDNRKNQLDMAVST 320

ENSGACT011881 GGTVFGDEAGLAIEDIQAHDFGKVGEVIVTKDDTLLKGRGDPAAIEKRANEIAEQLESTNSDYEKEKLNERLAKLSDG 400
C.auratus GGTVFGDEAGLAIEDIQAHDFGRVGEVIVTKDDTLLKGRGDPAAIEKRANEIAEQLESTNSDYEKEKLNERLAKLSDG 400
D.rerio GGTVFGDEAGLAIEDIQAHDFGKVGEVIVTKDDTLLKGRGDASAIEKRVNEIAEQLESTNSDYEKEKLNERLAKLSDG 400
C.idella GGTVFGDEAGLAIEDIQAHDFGRVGEVIVTKDDTLLKGRGDPSAIEKRVNEIAEQLESTNSDYEKEKLNERLAKLSDG 400
T.albonubes GGTVFGDEAGLAIEDIQAHDFGKVGEVIVTKDDTLLKGRGDASAIEKRANEIAEQLESTNSDYEKEKLNERLAKLSDG 400

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Fig. 2.A.2 Alignment of *G. aculeatus* Hsp60 amino acid sequence with other fishes. *G. aculeatus* sequence was obtained from Ensembl (ENSGACT00000011881) and Hsp60 sequences from the other fishes were obtained from the NCBI Protein Database (Accession ABI26641, ADK27679, ADU34083, and NP_851847). Underlined text indicates conserved amino acid, black text without underline indicates amino acid of similar grouping (GAVLI, FYW, CM, ST, KRH, DENQ, or P), and gray text indicates amino acids of different groups.

ENSGACT011881	<u>VAVLKIGGTS</u> <u>SDVEVNEKKDRVTDALNATRAAVE</u> <u>EGIV</u> <u>GGGCALLRCIP</u> <u>SLDTIK</u> <u>TANADQKIGVEIIR</u> <u>RALRIPAMTIA</u>	480
C.auratus	<u>VAVIKVGGT</u> <u>SDVEVNEKKDRVTDALNATRAAVE</u> <u>GGIV</u> <u>GGGCALLRCIP</u> <u>LDNIK</u> <u>TANNDQKIGIEIIR</u> <u>SALRIPAMTIA</u>	480
D.rerio	<u>VAVIKVGGT</u> <u>SDVEVNEKKDRVTDALNATRAAVE</u> <u>EGIV</u> <u>GGGCALLRCIP</u> <u>LDNIK</u> <u>TANADQKIGIDIIR</u> <u>RSALRIPAMTIA</u>	480
C.idella	<u>VAVIKVGGT</u> <u>SDVEVNEKKDRVTDALNATRAAVE</u> <u>EGIV</u> <u>GGGCALLRCIP</u> <u>LENIK</u> <u>TANSQKIGIDIIR</u> <u>RALRIPAMTIA</u>	480
T.albonubes	<u>VAVIRVGGT</u> <u>SDVEVNEKKDRVTDALNATRAAVE</u> <u>EGIV</u> <u>GGGCALLRCIP</u> <u>LENIK</u> <u>TANSQKIGIDIIR</u> <u>RALRIPAMTIA</u>	480
ENSGACT011881	<u>KNAGVEGSLVVEKILQ</u> <u>GPVDVGYDAM</u> <u>GEYVNMVEKGI</u> <u>IDPTKVVRTALLDAAGVASLL</u> <u>STAEAVVTE</u> <u>IPKEEKE</u> <u>MPGGG</u>	560
C.auratus	<u>KNAGVDGSLVVEKILQ</u> <u>SAPEIGYDAM</u> <u>NGEYVNMVERGI</u> <u>IDPTKVVRTALLDAAGVASLL</u> <u>ATAEAVVTE</u> <u>IPKEEKD</u> <u>MPAGG</u>	560
D.rerio	<u>KNAGVEGSLVVEKILQ</u> <u>SSTEIGYDAM</u> <u>NGEYVNMVERGI</u> <u>IDPTKVVRTALLDAAGVASLL</u> <u>STAEAVVTE</u> <u>IPKEEKE</u> <u>MPAGG</u>	560
C.idella	<u>KNAGVEGSLVVEKILQ</u> <u>SAPEIGYDAM</u> <u>GEYVNMVEKGI</u> <u>IDPTKVVRTALLDAAGVASLL</u> <u>STAEAVVTE</u> <u>LPKEEKD</u> <u>MPAGG</u>	560
T.albonubes	<u>KNAGVEGSLVVEKILQ</u> <u>STQDIGYDAM</u> <u>GEYVNMVERGI</u> <u>IDPTKVVRTALLDAAGVASLL</u> <u>STAEAVVTE</u> <u>LPKEEKE</u> <u>MPAGG</u>	560
ENSGACT011881	<u>MGMGMGMGMGMGMGF</u>	575
C.auratus	<u>MGMGMGMGMGMGMGF</u>	575
D.rerio	<u>MGMGMGMGMGMGMGF</u>	575
C.idella	<u>MGMGMGMGMGMGMGF</u>	575
T.albonubes	<u>MGMGMGMGMGMGMGF</u>	575

Fig. 2.A.2 Continued


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ENSGACP013930  M--SKGPAVGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNOVA  NPNTVFDAKRLIGRRF  78
O.mykiss_HSC70  M--SKGPAVGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNOVA  NPNTVFDAKRLIGRRF  78
D.erio_HSC70    M--SKGPAVGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNOVA  NPNTVFDAKRLIGRRF  78
ENSGACP017251  MSAAKGTATIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNOVA  NPSNTVFDAKRLIGRKV  80
D.erio_HSP70    MSSPKGIAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNOVA  NPNTVFDAKRLIGRRF  80
O.mykiss_HSP70  MSSAKGPSIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNOVA  NPNTVFDAKRLIGRKV  80

ENSGACP013930  DDNVVQSDMKHWPFIVIDDSTRPKVQVE  KGETK  FY  PEEISSMVL  KMKEIAEAYLGKTV  NAVVTVPAYFNDSQRQAT  158
O.mykiss_HSC70  DDVVQSDMKHWPFIVIDDSTRPKLQVE  KGETK  FY  PEEISSMVL  KMKEIAEAYLGKTV  NAVVTVPAYFNDSQRQAT  158
D.erio_HSC70    DDVVQSDMKHWPFIVIDNSRPKVQVE  KGESK  FY  PEEISSMVL  KMKEIAEAYLGKTV  NAVITVPAYFNDSQRQAT  158
ENSGACP017251  EDFVVQSDMKHWPFI  VVGDGCKPKIQVE  KGEK  FY  PEEISSMVL  KMKEIAEAYLGKTV  NAVVTVPAYFNDSQRQAT  160
D.erio_HSP70    DDVVQSDMKHWPFI  VVSDGCKPKVAVE  KGENK  FY  PEEISSMVL  KMKEIAEAYLGKTV  NAVITVPAYFNDSQRQAT  160
O.mykiss_HSP70  NDVVQSDMKHWPFI  VVSDGCKPKVQVD  KGENK  FY  PEEISSMVL  KMREIAEAYLGKTV  NAVITVPAYFNDSQRQAT  160

ENSGACP013930  KDAGTISGLNVLRIINEPTAAA  IAYG  DKKVGAE  ERNVLI  FDLGGGTFDVSILTIEDGIFEVK  TAGDTHLGGEDFDNRMV  238
O.mykiss_HSC70  KDAGTISGLNVLRIINEPTAAA  IAYG  DKKVGAE  ERNVLI  FDLGGGTFDVSILTIEDGIFEVK  TAGDTHLGGEDFDNRMV  238
D.erio_HSC70    KDAGTISGLNVLRIINEPTAAA  IAYG  DKKVGAE  ERNVLI  FDLGGGTFDVSILTIEDGIFEVK  TAGDTHLGGEDFDNRMV  238
ENSGACP017251  KDAGVIA  GLNVLRIINEPTAAA  IAYG  DRGKTG  ERNVLI  FDLGGGTFDVSILTIEDGIFEVK  TAGDTHLGGEDFDNRMV  240
D.erio_HSP70    KDAGVIA  GLNVLRIINEPTAAA  IAYG  DKGKSS  ERNVLI  FDLGGGTFDVSILTIEDGIFEVK  TAGDTHLGGEDFDNRMV  240
O.mykiss_HSP70  KDAGVIA  GLNVLRIINEPTAAA  IAYG  DKGKSR  ERNVLI  FDLGGGTFDVSILTIEDGIFEVK  TAGDTHLGGEDFDNRLV  240

ENSGACP013930  NHFISEFKRKY  KKDI  DNKRAVRLRLTACERAKRTLSSSTQASIEIDSLYEGVDFYTSITRARFEELNA  DLFRGTLDPVE  318
O.mykiss_HSC70  NHFIAEFKRKY  KKDI  DNKRAVRLRLTACERAKRTLSSSTQASIEIDSLYEGIDFYTSITRARFEELNA  DLFRGTLDPVE  318
D.erio_HSC70    NHFITEFKRKH  KKDI  DNKRAVRLRLTACERAKRTLSSSTQASIEIDSLYEGIDFYTSITRARFEELNA  DLFRGTLDPVE  318
ENSGACP017251  NHFVEEFRRKH  KKDI  QNKRALRRLRLTACERAKRTLSSSSQASIEIDSLYEGVDFYTSVTRARFEELCS  DLFRGTLDPVE  320
D.erio_HSP70    NHFVEEFKRKH  KKDI  QNKRALRRLRLTACERAKRTLSSSSQASIEIDSLYEGIDFYTSITRARFEELCS  DLFRGTLDPVE  320
O.mykiss_HSP70  SHFVEEFKRKH  KKDI  QNKRALRRLRLTACERAKRTLSSSSQASIEIDSLYEGIDFYTSITRARFEEMCS  DLFRGTLDPVE  320

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Fig. 2.A.3 Alignment of Hsp70 and Hsc70 amino acid sequences in several fishes. *G. aculeatus* sequences were obtained from Ensembl (ENSGACP00000013930 and ENSGACP00000017251). *Oncorhynchus mykiss* and *Danio rerio* sequences were obtained from the NCBI Protein Database (Accession AAF70445, BAB72233, NP_001117704 and NP_001103873). Sequences were aligned according to homology using MAFFT Alignment (<http://mafft.cbrc.jp/alignment/server/>). Underlined text indicates conserved amino acid, black text without underline indicates amino acid of similar grouping (GAVLI, FYW, CM, ST, KRH, DENQ, or P), and gray text indicates amino acids of different groups.

ENSGACP013930 KSLRDAKMDKAQIHDIVLVGGSTRIPKIQKMLQD FNGKELNKSINPDEAVAYGAAVQAAILSGDKS NVQDLLLLDVT 398
 O.mykiss_HSC70 KSLRDAKMDKAQVHDIVLVGGSTRIPKIQKLQD FNGKELNKSINPDEAVAYGAAVQAAILSGDKS NVQDLLLLDVT 398
 D.terio_HSC70 KALRDAKMDKAQIHDIVLVGGSTRIPKIQKLQD FNGKELNKSINPDEAVAYGAAVQAAILSGDKS NVQDLLLLDVT 398
 ENSGACP017251 KALNDAKMDKGQIHDIVLVGGSTRIPKIQKLQD FNGRELNKSINPDEAVAYGAAVQAAILSGDTS NVQDLLLLDVA 400
 D.terio_HSP70 KALRDAKMDKAQIHDIVLVGGSTRIPKIQKLQD FNGRELNKSINPDEAVAYGAAVQAAILSGDTS NVQDLLLLDVA 400
 O.mykiss_HSP70 KALGDAKMDKAQIHDIVLVGGSTRIPKIQKLQD FNGRELNKSINPDEAVAYGAAVQAAILSGDKS NVQDLLLLDVA 400

 ENSGACP013930 LSLGIETAGGVMTVLIKRNTTIPTKQTO FTTYS DNQPGV IQVFEGERAMTKDNN LGKFELTGIPPA PRGVPQIEVTF 478
 O.mykiss_HSC70 LSLGIETAGGVMTVLIKRNTTIPTKQTO FTTYS DNQPGV IQVFEGERAMTKDNN LGKFELTGIPPA PRGVPQIEVTF 478
 D.terio_HSC70 LSLGIETAGGVMTVLIKRNTTIPTKQTO FTTYS DNQPGV IQVFEGERAMTKDNN LGKFELTGIPPA PRGVPQIEVTF 478
 ENSGACP017251 LSLGLETAGGVMTALIKRNTTIPTKQTO FSTY DNQPGV IQVFEGERAMTKDNN LGKFDLTGIPPA PRGVPQIEVTF 480
 D.terio_HSP70 LSLGIETAGGVMTALIKRNTTIPTKQTO FTTYS DNQPGV IQVFEGERAMTKDNN LGKFELTGIPPA PRGVPQIEVTF 480
 O.mykiss_HSP70 LSLGIETAGGVMTALIKRNTTIPSKQTO FTTYS DDQPGV IQVFEGERAMTKDNN LGKFELSGIPPA PRGVPQIEVTF 480

 ENSGACP013930 DIDANGI NVSAADKSTGKENKIIITNDKGRLSKEDIERMVQEA KYK EDDVQREK VSAKN LESYAF NMKSTV DEKL 558
 O.mykiss_HSC70 DIDANGI NVSAADKSTGKENKIIITNDKGRLSKEDIERMVQEA KYK EDDVQRDK VSSKN LESYAF NMKSTV DEKL 558
 D.terio_HSC70 DIDANGI NVSAVDKSTGKENKIIITNDKGRLSKEDIERMVQEA KYK EDDVQRDK VSAKN LESYAF NMKSTV DEKL 558
 ENSGACP017251 DVDANGI NVSAVDKSTGKENKIIITNDKGRLSKEEIERMVQAD KYK EDDLQRDR VSAKN LESYAF SVSSSL DENL 560
 D.terio_HSP70 DIDANGI NVSAADKSTGKQNKIIITNDKGRLSKEEIERMVQEA MYK EDDLQREK ISAKN LESYAF NMKSSV DDNL 560
 O.mykiss_HSP70 DIDANGI NVSAVDKSTGKENKIIITNDKGRLSKEDIERMVQAD KYK EDDAQREK MAAKN LESYAF NMKSSV DDNM 560

 ENSGACP013930 KDKISEEDRQKIVEKCS EVI WLDGNO AEKEEFEH QKQK ELEKLCNPI TKLYQ NAGGMP GGM PGMPGGMPGGF GGAGG 638
 O.mykiss_HSC70 QGKISDEDKTKILEKCS EVI WLDKNO AEKEEYEH QKQK ELEKVCNPI TKLYQ GAGGMP GGM PGMAGGF ---GAGGA 635
 D.terio_HSC70 KGKISDEDKQKILDKCS EVI WLDKNO AEKEEFEH QKQK ELEKVCNPI TKLYQ SAGGMP GGM PGMPGGFP ---GAGAA 635
 ENSGACP017251 RGQVSAEDLKVTEKCE ETV WLENNQ AEKEEYEH QKQK ELEKVCNPI SKLHQ ---- GGP PAASC -----GQAR 627
 D.terio_HSP70 KGKISEEDKKRVIEKCS EAV WLENNQ ADKEEYEH QKQK ELEKVCNPI SKLYQ ---- GGM PAGGC -----GAQAR 627
 O.mykiss_HSP70 KGKIRQEDKKVMDRCN QTI SWLENNQ GDKEEYEH QKQK ELEKVCPI TKLYQ ---- GGM PTGCC -----GDQAR 628

 ENSGACP013930 APGGGASS GP TIEE -----VD 654
 O.mykiss_HSC70 APGGGGSS GP TIEE -----VD 651
 D.terio_HSC70 P---GGGSS GP TIEE -----VD 649
 ENSGACP017251 ----AGSQ GP TIEE -----VD 639
 D.terio_HSP70 GASGPALF GP LKRWIKTPHELNKLDKKT IL 658
 O.mykiss_HSP70 TSSGVSSQ GP TIEE -----ID 644

Fig. 2.A.3 Continued

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HSP70 atgtctgcagccaagggcagcagcgtcggcattgacctggggcaccacctactcctgctgtggcgctttccaaacaggcaa 80
HSC70 atgtct-----aagggcagcagcgtcggcattgacctggggcaccacctactcctgctgtaggcgctttccagcatggcaa 74

HSP70 agtggaaatcatcgccaaagcagggcaacaggaccacgcccagctatgtggcgttcaccgataccgagaggctcatcgg 160
HSC70 agtggagatcatcgccaatgatcagggaaacggcaccacgcccagctacgtggccttcacggatactggagaggctcatcgg 154

HSP70 ggcagcgggccaagaaccaggtggcctgaaccccagcaacacggtgtttgacggcaaaaggctgatcgggaggaaggctg 240
HSC70 gagaagcagccaagaaccaggtggcctgaaccccacaacacggtatattgagcacaaggcgtctcattggcggctcggttt 234

HSP70 gaggatccggctggtgcagcggacatgaagcactggcccttcaagggtgggtgggagatggaggggaagcccaagatccaggt 320
HSC70 gatgataaatgttgtccagtcagacatgaaacactggccatttacagtcattggatgatccgacacgcccacaaggtacaggt 314

HSP70 ggagcacaaggaggagagaagagcttctaccaggagagatctctccatgggtcctggtgaaatgaaggagattgcag 400
HSC70 agagcacaaggaggagacaaggccttctaccagagagatctctccatgggtgctcataaatgaaggagattgcag 394

HSP70 aggcatacctgggcacaaaggtgtccaacggcgtggttacggctccggcgtacttcaacgactccagagccaggccacc 480
HSC70 aggcatacctgggaaagactgtaaccaatgagtggtgtactgtacctgctacttcaacgactccagcggcaggccacc 474

HSP70 aaagagcgcggcgtcatcggggactcaacgtcctgaggatcatcaacgagccacagcggccgccatcgcctacggctct 560
HSC70 aaagatgcagggaccattctggactaaagtctctcgtatcatcaacgagccaacggctgctgccatcgcctacggcct 554

HSP70 ggaccgaggcaagcgggagaaagcaacgtcctgatcttgacctggggcggggcaccttcgaaggtgtccatctgacaa 640
HSC70 agacaaaaaggtgggagctgagaggaatgtcctcatcttgacctggggcggggcaccttcgaaggtgtccatctgacaa 634

HSP70 tcgaggacggcatcttcgaggtcaaggccacggcgggagacacgcacttgggaggagagacttgacaaccgcatggtc 720
HSC70 ttgaaagacggcatcttcgaggtcaagtcaacggcaggagacacgcacttgggtggggaagacttgacaaccgcatggtc 714

HSP70 aaccactttgtggaggatttcaggaggaaaacaagaaggacatcaggcagaacaagagggccttgaggaggctgctgcac 800
HSC70 aaccacttcatctcagaattcaaaaggcaagtacaaaaggacatcaggcagaacaagagggccttcggcggctgctgcac 794

HSP70 ggcgtagagagggccaaggagactttgtctccagcctcaggccagcatggagatcgactcctgttcgagggcgtg 880
HSC70 agcgtagagagggccaaggagactttgtctccagcctcaggccagcatggaaatcgactcctgttcgagggcgtg 874

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Fig. 2.A.4 Alignment of *G. aculeatus* HSP70 and HSC70 genes. Sequences were obtained from Ensembl (ENSGACT00000013955 and ENSGACT00000017285). Black shading indicates non-conserved nucleotides. HSP70 primers are highlighted in pink and HSC70 primers are highlighted in blue.

HSP70 acttctacacgtcctaccagggcctcgatttcgaggagctgtgctctgacctcttccggggaactctggaaccctgtggag 960
HSC70 acttctacacctcaataccggggcctgggttcgaggagctgaaacgcagacctgttccggggaactctggaaccctgtggag 954

HSP70 aaagctctgaacgacgccaagatggacaaggggcagatccacgatgttggtcttggtcgggggtccacccgcatcccaa 1040
HSC70 aagctctctcgtgacgccaagatggacaaggctcagatccacgatattggtcttggtcggagggtcaactcgtattcccaa 1034

HSP70 gatccaaaagctctctgacaggactcttcaacggcaggagctgaaacaagagcatcaaccgacgaggcgtggctacg 1120
HSC70 gatccagaagatgctgacaggactcttcaacgggaaggagctgaaacaagagcattaaaccagacgaaggcgtggctacg 1114

HSP70 ggcctgctcaggctgcatcctcaccggggacacctcggggaacgtcaggactgtctctgctggaagtggctccc 1200
HSC70 gtgcaggcgtcaggctgcatcctgtctggggacaaactcgggaacgtcaggactgtctctgctggaagtggctccc 1194

HSP70 ctgtctctgggcttgagacggcggagggtcatgacggcgtgatcaaacggcaacacgacgatcccaacaagcagac 1280
HSC70 ttgtctctgggcatgagacggcggagggtcatgacgtgctgatcaaacgggaacacacacatcccaacaagcagac 1274

HSP70 ccaggtcttcagcacctacgacgacaaccaaccgggggtctctatccaggctctacgaaggggagcggcgcctgactaagg 1360
HSC70 ccagactcttcagcacctactcagacaaccaaccctgggtgtgctcatccaggctcttgaggggagaaggggcctgactaagg 1354

HSP70 acaacaaccctgtgggcaatttgatctgacaggaaatcccaccggctccgcggggtcccgagatcgaaggtcaccttt 1440
HSC70 acaacaaccatttgggcaatttgagctgacaggaaatcccaccggcaccgagggagtccctcagattgaagtgactctt 1434

HSP70 gacgtggagccaac----- 1455
HSC70 gacatcgtatgccaacggcatcatgaatgtgtccgcggtgacaagagcaccgggaaggaaaacaagatcacgatcaccaa 1514

HSP70 -----ggcatctgaaagtgtcggcggtgg 1480
HSC70 cgacaaaggtcgcttgagcaaggaggatctcgagcgtatggtccaggaagccgatggcatctgaaagtgtcggcggtgg 1594

HSP70 acaagagcaccggcaaggagaacaagatcaccaatcaccaacgacaaaggcctgagcaaggaggagatcgagaggatg 1560
HSC70 acaagagcaccgggaaggagaacaagatcaccaatcaccaacgacaaaggcctgagcaaggaggagatcgagaggatg 1674

HSP70 gtcaggacgccgacaaatacaaggccgaggatgaccttcagag-gacaaggctctccccaagaactccttcgagtccta 1640
HSC70 gtcaggagccgataaatacaaggccgaggatgatcttcagagggagaaaggctctccccaagaatggctcttcgagtccta 1754

HSP70 cgccttcagctgagcagcagcctgctgggacgagaaactgaggggctcaggtgagcgcggaggactgaagaagggtgacgg 1720
HSC70 cgccttcacatgaagtccaagtcgagggacgagaaactgaggggcaagaatcagcgaaggaggacagacagaagattgtgg 1834

Fig. 2.A.4 Continued

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HSP70 agaagtgcgaagagaccgtcggcttggtggagaacaaacagctggccgagaaggaggagtaccaacaagcaggaggag 1800
HSC70 agaagtgtagcagggtcatcactggcttgatgggaaacagctcgcagagaaggaggagttagagcatcagcagaaggag 1914

HSP70 ctggagaaggtgtgtaaccgggcatcagcaagctgcaccag-----ggagggaaggcctg-----cggccagct 1864
HSC70 ttggagaaggtgtgtaaccgatcatgaccaagctctaccaaaatgctgggggatgcctggtgggatgcccggggat 1994

HSP70 gt-----ggagagcaggca-----cgagcggc-----tccagggccaccattgagg 1909
HSC70 gcctggtgggatgccagggtggcttcggcggagctgggtggtgctcccggcggtggcgctcccgccaccattgagg 2074

HSP70 aggtggactaa 1920
HSC70 aggtggactaa 2085

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Fig. 2.A.4 Continued

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Conclusion

Results from my studies suggest that evolution at cold temperature in Antarctic fishes and cold acclimation in temperate threespine sticklebacks (*G. aculeatus*) leads to an increase in heat shock proteins (Hsps), suggesting cold temperature denatures proteins and/or slows rates of protein folding, warranting higher levels of Hsps.

My studies revealed that levels of Hsps decline with moderate warming in Antarctic notothenioid fishes. Previous studies have shown that evolution of notothenioids for 12 million years in the constantly cold Southern Ocean at temperatures less than 5°C (Clark 1980), has led to an increase in levels of Hsps compared to temperate notothenioids (Place et al. 2004; Place and Hofmann 2005). Constitutive levels of *HSP70* are higher in the Antarctic emerald rockcod (*T. bernacchii*) compared to the New Zealand Maori chief (*N. angustata*) and thornfish (*B. variegatus*) (Place et al. 2004). Additionally, Antarctic species (emerald rockcod, sharp-spined notothenia (*T. pennellii*), bald notothen (*P. borchgrevinki*), and eelpout (*Lycodichthys dearborni*)) have higher levels of ubiquitin-conjugated proteins compared to temperate species (Maori chief, thornfish, and twister (*Bellapiscis medius*)), suggesting that cold temperature denatures proteins. I found lower levels of *HSP70* mRNA in *N. coriiceps* and *C. rastrospinosus* warm acclimated to 4°C compared to control animals, indicating that moderate elevations in temperature may reduce protein denaturation and the need for folding newly synthesized and misfolded proteins. While Antarctic notothenioids are believed to be extremely stenothermic fishes, my study reveals that they have some capacity to adjust to

warming temperatures and interestingly, slight elevations in temperature may reduce stress. Consistent with this, warm acclimation of *N. coriiceps* and *C. rastrospinosus* results in a decrease in transcript levels of antioxidants (Mueller et al. 2014). Decreases in levels of antioxidants suggest oxidative stress also declines, which may reduce levels of denatured proteins needing to be replaced by newly synthesized proteins and folded by Hsps. These data, along with my data showing lower levels of *HSP70* with warm acclimation, suggest that moderate warming reduces levels of stress and protein denaturation in Antarctic fishes.

Mine is the first study to show that cold acclimation results in an increase in Hsps in a temperate fish species, threespine stickleback. I found a transient increase in the expression of *HSP90*, *HSC70*, and *HSP60*, beginning on day 3 (10°C) in pectoral muscle, and *HSP60* and *HSC70* beginning on day 4 (8°C) and day 3 (10°C), respectively, in liver of cold-acclimated stickleback. In both tissues, mRNA levels began to decline by week 1 (8°C), suggesting elevated levels of nascent or misfolded proteins during the first week of cold acclimation. Another important finding of these studies is that different isoforms of Hsps change in different tissues of stickleback during cold acclimation, suggesting that Hsps have different functions in facilitating cold acclimation. I found increases in *HSP60* and *HSC70* mRNA transcripts levels in liver of stickleback during cold acclimation that coincided with increases in levels of oxidatively damaged proteins (Kammer et al. 2011). This is consistent with previous studies showing increases in oxidative stress coinciding with increases in Hsps in ectotherms. Lipid peroxidation, *HSP70*, and *HSP90* levels increased in hepatocytes of grass carp (*C. idellus*) exposed to 32°C heat stress for 30 min

(Cui et al. 2013). Other studies have shown that oxidative stress increases with cold exposure in fishes. For example, the eelpout *Z. viviparus* has higher lipid peroxidation and carbonyl levels in the liver when acclimated to 6°C compared to 12°C (Heise et al. 2007). Similarly, lipid peroxidation levels are higher in the liver of gilthead sea bream (*S. aurata*) when acclimated to 8°C compared to 20°C (Ibarz et al. 2010). This suggests that Hsps may increase in cold-acclimated stickleback in response to increases in oxidative stress.

Increases in levels of Hsps in stickleback during cold acclimation may also facilitate protein import into mitochondria. Hsp70 is involved in protein import and folding in the mitochondria (Craig et al. 1989; Scherer et al. 1990). Additionally, Hsp90 is also involved in mitochondrial protein import, and Hsp60 assists with protein folding within the mitochondria (Young et al. 2003; Mogk et al. 2001; Cheng et al. 1989). I found increases in levels of *HSP90*, *HSP60*, and *HSC70* in the pectoral muscle of cold-acclimated stickleback, corresponding with an increase in mitochondrial volume density (Orczewska et al. 2010). Increases in mitochondrial volume density during cold acclimation might warrant increased levels of these Hsps to facilitate protein import and folding. High mitochondrial densities in hearts of Antarctic icefishes (O'Brien and Mueller 2010) may also explain why transcript levels of *HSP70* are higher compared to red-blooded species, despite their lower levels of oxidative stress. When pooled, I found 3.3-fold higher levels of *HSP70* mRNA in white-blooded notothenioids compared to red-

blooded notothenioids, which corresponds to their 2-fold higher mitochondrial densities (O'Brien and Mueller 2010; O'Brien and Sidell 2000).

Higher levels of *HSP70* mRNA in hearts of white-blooded notothenioids compared to red-blooded notothenioids was unexpected. I hypothesized that Hsps would be higher in hearts of red-blooded fishes and correlated with levels of oxidized proteins. A possible explanation of these unexpected results is that other Hsps may increase in response to increases in oxidative stress in Antarctic notothenioids. For example, increases in *HSP90* mRNA levels in grass carp hepatocytes is correlated with increases in lipid peroxidation when exposed to a heat stress of 32°C for 30 min (Cui et al. 2013). In addition to high expression of *HSP70* mRNA in Antarctic fishes (Place et al. 2004), *HSP90* expression is also elevated in the Antarctic notothenioid *Dissostichus mawsoni* compared to Atlantic salmon (*Salmo salar*), killifish (*F. heteroclitus*), medaka (*Oryzias latipes*), stickleback, and zebrafish (*D. rerio*), which inhabit temperate or tropical waters (Chen et al. 2008). My data from cold-acclimated stickleback showed that cold acclimation induces expression of *HSP60* and *HSC70* but not *HSP70* in the liver, where oxidative stress increases. It is possible that these Hsps are responding to the high levels of oxidative stress in red-blooded notothenioids instead of Hsp70, by restoring levels of properly folded proteins.

Results from my research have revealed several unanswered questions about the function of Hsps at cold temperature. For example, although red-blooded notothenioids have higher rates of protein synthesis and levels of oxidatively modified proteins (Haschemeyer 1983; Motta et al. 2004; Mueller et al. 2012), I found that they have lower

levels of *HSP70* than white-blooded notothenioids. This may be due to higher mitochondrial volume density associated with a lack of oxygen binding proteins (O'Brien and Mueller 2010) and, thus, higher levels of mitochondria-associated Hsps, such as Hsp70. To determine whether or not this holds true, a study could be designed to quantify levels of Hsps associated with mitochondrial protein import and folding in both red- and white-blooded notothenioids to determine if greater mitochondrial volume density is correlated with higher levels of Hsps. Additionally, we do not know how levels of other Hsps change with heat stress in Antarctic notothenioids. Future studies should expose both red- and white-blooded notothenioids to acute and long-term heat stresses and quantify levels of Hsp90, Hsp60, and Hsc70.

Finally, the molecular pathway regulating changes in metabolism during cold acclimation of fishes has yet to be determined. In mammals, mitochondrial biogenesis is activated by AMP-activated protein kinase, calcium, carbon monoxide, nitric oxide, ROS, and Sirt1 (Jager et al. 2007; Kang et al. 2009; Lagouge et al. 2006; Nisoli et al. 2003; Suliman et al. 2007; Wu et al. 2002). Our results suggest that Sirt1 is likely not involved in metabolic remodeling in muscle during cold acclimation of fishes since we found only a modest increase in *SIRT1* mRNA in the pectoral muscle, where mitochondrial biogenesis occurs. Research in cold-acclimated stickleback suggests that nitric oxide is also not responsible for metabolic remodeling (Mueller and O'Brien 2011). Future studies should investigate the possible roles AMP-activated kinase, calcium,

carbon monoxide, and ROS in the regulation of metabolic remodeling during cold acclimation of fishes.

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