**Cold acclimation increases levels of some heat shock protein and sirtuin isoforms in
threespine stickleback**

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Abstract

Molecular chaperones [heat shock proteins (HSPs)] are known to increase in response to rapid changes in temperatures, but long-term acclimation to cold temperature may also warrant elevations in HSPs. In fishes, cold acclimation increases mitochondrial density and oxidative stress in some tissues, which may increase demand for HSPs. We hypothesized that levels of HSPs, as well as sirtuins (SIRTs), NAD-dependent deacetylases that mediate changes in metabolism and responses to oxidative stress (including increases in HSPs), would increase during cold acclimation of threespine stickleback (*Gasterosteus aculeatus*). Transcript levels of *hsp70*, *hsc70*, *hsp60* and *hsp90- α* , *sirts1-4*, as well as protein levels of HSP60, HSP90 and HSC70 were quantified in liver and pectoral adductor muscle of threespine stickleback during cold acclimation from 20 °C to 8 °C. In liver, cold acclimation stimulated a transient increase in mRNA levels of *hsp60* and *hsc70*. Transcript levels of *sirt1* and *sirt2* also increased in response to cold acclimation and remained elevated. In pectoral muscle, mRNA levels of *hsp60*, *hsp90- α* , *hsc70* and *sirt1* all transiently increased in response to cold acclimation, while levels of *sirts2-4* remained constant or declined. Similar to transcript levels, protein levels of HSC70 increased in both liver and pectoral muscle. Levels of HSP90 also increased in liver after 4 weeks at 8 °C. HSP60 remained unchanged in both tissues, as did HSP90 in pectoral muscle. Our results indicate that while both HSPs and SIRTs increase in response to cold acclimation in stickleback, the response is tissue and isoform specific, likely reflecting differences in metabolism and oxidative stress.

Key Words

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

1. Introduction

Molecular chaperones assist with the folding and assembly of proteins (Hartl, 1996). Chaperones were first identified as proteins induced by heat stress, and so aptly named heat shock proteins (HSPs) (reviewed by Lindquist, 1986). We now know that many HSPs are induced by a variety of stressors. Virtually any perturbation of the cellular milieu (ie; oxidative, osmotic, temperature, chemical stress) will lead to an increase in levels of HSPs (Lindquist, 1986). Moreover, chaperones not only assist with folding proteins, both those produced *de novo* and denatured by stress, but also assemble multimeric proteins, participate in signaling pathways, and assist with the degradation of proteins by the ubiquitin- proteasome system (Kim et al., 2013). The different families of HSPs are categorized according to molecular mass and include HSP100, HSP90, HSP70, HSP60, and small HSPs (Mogk et al., 2001).

Changes in levels of HSPs have been well characterized in fishes and other ectotherms in response to acute temperature stress (ie; Dietz and Somero, 1992; Donaldson et al., 2008; Feder and Hofmann, 1999; Todgham et al., 2005; Tomanek and Somero, 1999). Yet, to date, the role of HSPs in mediating acclimatory responses to decreases in temperature in fishes has not been fully explored. Nevertheless, many aspects of acclimation to cold temperature may warrant elevations in HSPs, including the proliferation of mitochondria, defenses against oxidative stress, and preventing thermal denaturation of proteins.

Temperate fishes may endure seasonal changes in temperature as great as 30 °C, stimulating 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 in some tissues, is an increase in the percent cell volume displaced by mitochondria (Egginton and Sidell, 1989; Orczewska et al., 2010).

Mitochondrial biogenesis and increases in levels of aerobic metabolic enzymes in response to cold acclimation may increase demand for HSPs. 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 imported into mitochondria. Isoforms of HSP/HSC70 and HSP90 are located in the cytosol where they interact with the translocase of the outer mitochondrial membrane (TOM) to facilitate protein import (Kang et al., 1990; Lithgow et al., 1993; Young et al., 2003). Imported proteins are then refolded by mitochondrial isoforms of HSP/HSC70 and HSP60 within the mitochondrial matrix (Cheng et al., 1989; Craig et al., 1989; Mogk et al., 2001).

Cold acclimation also promotes oxidative stress in some fish tissues. Protein carbonyls, one form of protein oxidation and an indirect measure of ROS, transiently increase during cold acclimation and are 2.3-fold higher in the liver of threespine stickleback cold acclimated to 8 °C for 4 weeks compared to those at 20 °C (Kammer et al., 2011). Similarly, both lipid peroxidation and protein carbonyl levels are 1.5-fold higher in livers of eelpout (*Zoarces viviparus*) acclimated to 6 °C compared to animals at 12 °C (Heise et al., 2007). Increases in 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 HSPs to assist with targeting damaged proteins to the proteasome and with folding newly synthesized proteins. HSPs are also essential for preventing thermal denaturation and aggregation of proteins that may occur as temperature decreases. Studies in cold-adapted fishes support this conjecture. The Antarctic fishes *Trematomus bernacchii*, *Trematomus pennellii* and *Pagothenia borchgrevinki* have higher levels of ubiquitinated proteins and levels of HSP70 compared to the New Zealand species *Bovichtus variegatus* and *Notothenia angustata* (Place et al., 2004; Todgham et al., 2007).

The molecular pathways mediating metabolic remodeling and responses to oxidative stress during cold acclimation of fishes are largely unknown. Sirtuins (SIRT), a family of highly conserved NAD-dependent deacetylases and ADP-ribosyltransferases mediate both pathways in mammals, and also induce expression of HSPs, potentially providing a unifying explanation for the molecular drivers of cold acclimation in fishes (Brenmoehl and Hoeflich, 2013; Hori et al., 2013; Westerheide et al., 2009).

In mammals, there are seven sirtuins, SIRT1-7, localized to different compartments in the cell. SIRT1, SIRT6, and SIRT7 are found in the nucleus, whereas SIRT2 is found in the cytoplasm, and SIRT3-5 in mitochondria (Michishita et al., 2005). SIRTs function as energy sensors, increasing activity as NAD⁺ levels increase, and consequently, are central mediators of metabolism (Ahn et al., 2008; Gambini et al., 2011; Osborne et al., 2013). SIRTs also enhance defenses against oxidative stress. SIRT2 deacetylates the O class of forkhead box transcription factors (FoxO), which drives increases in the key antioxidants, superoxide dismutase and catalase (Wang et al., 2007). Additionally, SIRT1 deacetylates the transcriptional regulator of HSP70, HSF1, prolonging its binding to the HSP70 promoter (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 acute 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).

We hypothesized that levels of HSPs and SIRTs would change in response to cold acclimation of threespine stickleback. Previous studies in our lab have shown that the molecular basis of aerobic metabolic remodeling differs between pectoral muscle and liver in threespine stickleback in response to cold acclimation. In pectoral muscle, mitochondrial biogenesis occurs, whereas in liver, concentrations of aerobic metabolic enzymes increase in the absence of mitochondrial biogenesis (Orczewska et al., 2010). Additionally, in liver but not pectoral muscle, levels of oxidized proteins increase during cold acclimation (Kammer et al., 2011). These differences may be reflected in differences in the expression and levels of HSP and sirtuin isoforms. To characterize changes in patterns of expression of HSPs and SIRTs, stickleback were cold acclimated from 20 °C to 8 °C for 9 weeks or maintained at 20 °C for 9 weeks. Transcript levels of *hsp90-α*, *hsp60*, *hsp70*, *hsc70*, and *sirts1-4*, and protein levels of HSP60, HSP90, and HSC70 were quantified in pectoral muscle and liver tissue in stickleback harvested during cold acclimation and in animals held at 20 °C.

2. Materials and methods

2.1 Animals

All protocols for the treatment and use of animals were approved by the University of Alaska Fairbanks Institutional Animal Care and Use 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 for transcript measurements and in August 2013 for measuring levels of HSP proteins. Water temperature at time of capture was 16.5 °C in 2007 and 13.1 °C in 2013. 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. In 2007, 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 start). Remaining fish were then either cold acclimated to 8 °C or maintained at 20 °C for 9 weeks. After the initial harvest on day 0 (start), the temperature was decreased for cold-acclimated animals to 15 °C on day 1, 10 °C on day 2, and 8 °C on day 3. Fish were held at 8 °C for an additional 9 weeks. Between 20 and 40 animals were harvested each day before the temperature was decreased and after 1, 4, and 9 weeks at 8 °C. The acclimation regime was similar in 2013 except that animals were held for 20 weeks at 20 °C before beginning cold acclimation and harvested at the start (20 °C), after 4 weeks and 9 weeks at 8 °C, and at 20 °C for an additional 9 weeks. All animals were harvested at the same time each morning before feeding to avoid the potentially confounding effects of circadian rhythms on gene expression (Podrabsky and Somero, 2004). Fish were netted, immediately frozen in liquid nitrogen and stored at -80 °C. Body mass, length, and masses of liver and oxidative muscles were recorded for each individual.

2.2 Quantitative real-time PCR

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.

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 (supplementary material Fig. S1-S4). Transcript levels of *hsp90-α*, *hsp70*, *hsp 60*, *hsc70*, *sirt1*, *sirt2*, *sirt3*, *sirt4*, *18S*, and *EF-1α* 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 µM of each forward and reverse primer, and 5 ng of cDNA template, except for *hsp70* and *hsc70* in pectoral muscle and *hsp90-α* 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*. Reaction efficiency and relative gene expression levels were determined using a standard curve. Optimum primer concentration was determined by testing efficiency using 200 nM, 300 nM and 400 nM of primers. Complementary DNA from animals harvested at all temperatures and time points was

pooled and serially diluted to create a standard curve for quantifying relative transcript levels. Separate standard curves were prepared for each gene and tissue type. Primer efficiency was determined from the slope of the standard curve. A dissociation curve was run for each reaction at 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s (slow ramp) to verify the specificity of each primer set. Two types of controls were used: one for each primer set in which the cDNA template was replaced with an equal volume of Milli Q H₂O. The second control, which was run in parallel with each sample, contained template from the cDNA synthesis reactions in which the reverse transcriptase (RT) was omitted. Both of these controls allowed us to identify and omit samples containing contaminated genomic DNA. 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 controls were included in the final analyses. All measurements were made in triplicate in 6-11 individuals per tissue and time point (20 °C start, 15 °C, 10 °C, 8 °C, 8 °C week 1, 8 °C week 4, 8 °C week 9 and 20 °C week 9).

2.3 Western blotting

Livers were homogenized in 4 volumes and pectoral muscles in 8 volumes of ice-cold buffer (250 mM Tris·HCl, 10 mM EDTA, 10 mM EGTA, pH 7.4). Homogenates were centrifuged for 5 min at 500 g and supernatant collected. Protein concentration was determined by a Bradford assay (Bradford, 1976). Tissue homogenates were resuspended in SDS sample buffer (20 mM NaPO₄, 8% glycerol, 40 mM dithiothreitol, 0.025% Bromophenol Blue, 4% SDS,

pH 6.8) at a concentration of 8 ug uL^{-1} for liver and 4 ug uL^{-1} for pectoral adductor muscle. Liver and pectoral muscle samples from animals harvested at 20 °C start, 8 °C week 4, 8 °C week 9 and 20 °C week 9 (100 μg each) were separated on 10% polyacrylamide gels. Each gel contained samples from each temperature and time point. HSP protein levels prior to week 4 were not measured because we anticipated that changes in protein levels would occur more slowly than changes in transcript levels. Proteins were transferred to 0.20 μm nitrocellulose membrane using a semi-dry transfer apparatus (GE Healthcare) set at 0.8 mA cm^{-1} of mask opening for 1.5 h and using 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 overnight at 4 °C in either 1:2500 anti-HSC70 salmon (StressMarq SPC-303D) or both 1:5000 anti-HSP60 salmon (StressMarq SMC-111A/B) and 1:2500 anti-HSP90 salmon (StressMarq SMC-107A/B). Following incubation, the membranes were washed again as described above and incubated in either 1:5000 anti-rabbit IgG (whole molecule) peroxidase antibody produced in goat (Sigma A6154) or 1:2500 anti-mouse IgG (whole molecule) peroxidase antibody produced in rabbit (Sigma A9044), respectively, for 2 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 4-6 individuals per tissue and temperature treatment. Relative levels of each HSP were normalized to the average HSP level in

individuals at 20 °C (start) for each tissue. HSP70, using the antibody anti-HSP70 (salmon) (StressMarq SPC-313D) could not be detected in either tissue.

2.4 Statistical analyses

Significant differences in gene expression 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). Significant differences in HSP protein levels were determined using an ANOVA followed by a *post hoc* Tukey's test except for levels of HSC70 in pectoral adductor muscle. These data had unequal variance as determined by a Levene's test and so means were compared using a Welch's ANOVA. Data were log transformed as necessary to maintain assumptions of normality as determined by a Shapiro-Wilk's test. Significance was set at $\alpha < 0.05$ except for analysis of HSC70 levels in pectoral muscle in which $P < 0.10$ because of the small sample size ($N = 4$).

3. Results

3.1. Physical characteristics

Body mass and length increased during the time period of the experiment but there was no effect of temperature on physical characteristics (Table 2). There was no difference in length, body mass or condition index between animals held at 20 °C for 9 weeks and animals held at 8 °C for 9 weeks, nor were there any differences in the condition index among animals collected at different time points and temperatures during the experiment ($P > 0.05$; Table 2).

3.2. Transcript levels of heat shock proteins

Hsp60 mRNA levels increased significantly by day 3 of cold acclimation (8°C) in liver and at this time were 3.3-fold higher compared to fish at 20 °C (start) and 7.9-fold higher than stickleback at 20 °C for 9 weeks ($P < 0.05$; Fig. 1A). Levels of *hsp60* transcripts decreased by week 4 of cold acclimation and by then, levels were equivalent to stickleback at 20 °C at the start and end of the acclimation period ($P > 0.05$; Fig. 1A). Transcript levels of *hsp90-α* were not consistently detected in liver (ie; most samples had a CT value ≤ 4 CT values higher than the – RT control). The efficiency of the primers was high (Table 1) and therefore we believe the inability to detect transcripts was due to the low abundance of the transcript.

In pectoral muscle, both *hsp90-α* and *hsp60* mRNA levels increased by day 2 of cold acclimation (10°C) ($P < 0.05$; Fig. 1B). *Hsp90-α* transcripts were highest in stickleback at 8 °C (day 3) at which point levels were 4.4- and 3.5-fold higher than fish at 20 °C (start) and 20 °C for 9 weeks, respectively. *Hsp60* mRNA levels were also highest in stickleback at 8°C (day 3) when levels were 7.3- and 4.9-fold greater than fish at 20°C (start) and 20°C for 9 weeks, respectively. By the end of the acclimation period, *hsp60* and *hsp90-α* levels were equivalent between stickleback at 8 °C for 9 weeks and those at 20 °C for 9 weeks.

Hsc70 mRNA levels increased during cold acclimation in stickleback livers and were significantly greater in stickleback at 10 °C (day 2) and at 8 °C (day 3) compared to stickleback at 20 °C at the start of the acclimation period ($P < 0.05$; Fig. 1C). *Hsc70* mRNA levels were 7.8-fold higher in livers of stickleback at 10 °C than in fish at 20 °C (start), and levels were 10.2-fold higher in livers of stickleback at 8 °C compared to stickleback at 20 °C (start). After 1 week of

acclimation at 8 °C, *Hsc70* transcripts were equivalent to those in fish at 20 °C (start) and in stickleback held at 20 °C for 9 weeks.

Similar to liver, *Hsc70* mRNA levels increased in response to cold acclimation in pectoral muscle and were highest in animals at 8 °C (day 3) when levels were 4.8- and 2.3-fold greater than stickleback at 20 °C (start) and 20 °C for 9 weeks, respectively ($P < 0.05$; Fig. 1D). By week 4 of acclimation, *Hsc70* transcripts decreased and were equivalent to those in animals at 20 °C for 9 weeks.

Hsp70 was not consistently detected in either tissue (ie; most samples had a CT value ≤ 4 CT values higher than the –RT control). Primer efficiency was high (Table 1) so we believe the inability to detect the mRNA was due to low abundance of the transcript.

3.3 Protein levels of heat shock proteins

Although *Hsp90- α* transcript levels were not robustly expressed in the liver, HSP90 protein levels were high. Levels of HSP90 tended to increase in stickleback livers during cold acclimation and reached a maximum after 9 weeks at 8 °C, at which time they were 5.7-fold higher than animals at 20 °C (start) ($P = 0.001$) and 1.7- fold higher than animals at 20 °C after 9 weeks ($P = 0.11$; Fig. 2a, b). Levels of HSP60 did not change in response to cold acclimation in liver or pectoral adductor muscle (Fig. 2A,B and C), nor did levels of HSP90 change in pectoral muscle in response to cold acclimation (Fig. 2A,C).

Levels of HSC70 increased in both liver and pectoral adductor muscle during cold acclimation. In liver, levels of HSC70 were significantly higher after 4 weeks at 8 °C compared to stickleback at 20 °C (start) and higher in stickleback after 9 weeks of cold acclimation compared to stickleback at 20 °C (start and week 9; $P < 0.05$; Fig 2D, E). In pectoral adductor

muscle, levels of HSC70 were significantly greater in animals after 4 weeks of cold acclimation compared to those at 20 °C (start and week 9; $P = 0.074$; Fig 2D, F) but then decreased and by week 9 of cold acclimation were not significantly different than stickleback held at 20 °C for 9 weeks ($P > 0.10$; Figs. 2 D,F).

3.4 Transcript levels of sirtuins

Transcript levels of *sirt1* and *sirt2* increased in liver of stickleback in response to cold acclimation ($P < 0.05$; Fig. 3A). *Sirt1* mRNA levels were significantly higher in animals at 10 °C (day 2) and remained elevated throughout the cold acclimation period. Transcripts were highest in stickleback at 8 °C on day 3 when levels were 3.9- and 2.5-fold greater compared to fish at 20 °C (start) and 20 °C for 9 weeks, respectively. *Sirt2* transcript levels increased significantly in stickleback livers by day 3 of cold acclimation (8 °C) and remained elevated throughout the acclimation period. Transcript levels were highest in stickleback at 8 °C for 4 weeks when levels were 2.6- and 1.7-fold greater than fish at 20 °C (start) and 20 °C for 9 weeks, respectively.

In pectoral muscle, *sirt1* mRNA levels were significantly higher in stickleback at 8°C (day 3) compared to animals at 20 °C (start) and animals at 20 °C for an additional 9 weeks ($P < 0.05$; Fig. 3B). *Sirt1* transcripts were 3.2- and 3-fold higher in animals at 8 °C (day 3) compared to fish at 20 °C (start) and at 20 °C for an additional 9 weeks, respectively. Transcript levels of *sirt2* did not change significantly during cold acclimation in pectoral muscle ($P > 0.05$; Fig. 3B).

In liver, *sirt3* mRNA levels were significantly lower in animals on 8 °C (day 3) compared to 20 °C (start) (Fig. 3C). Similarly, levels of *sirt3* mRNA declined during cold acclimation in pectoral muscle and were 2.8-fold lower at 8 °C for 4 weeks compared to fish at

20 °C (start) (Fig. 3D). Levels of *sirt4* mRNA did not change in the liver or pectoral muscle of stickleback in response to cold acclimation ($P > 0.05$; Fig. 3C,D).

4. Discussion

Results from this study show that transcript levels of some HSPs and SIRTs increase transiently during cold acclimation in stickleback, and that the pattern of expression of HSP and SIRT isoforms differs between liver and oxidative muscle. Importantly, HSP protein levels do not always change concurrently with transcripts, highlighting the importance of measuring both to fully understand the role of HSPs in organismal responses to temperature stress.

Levels of HSPs may increase in response to cold acclimation in stickleback to refold proteins denatured by cold 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 demand for chaperone-mediated protein folding. *Hsp90- α* , *Hsp60*, and *Hsc70* transcripts increased in pectoral muscle and *Hsp60* and *Hsc70* increased in the liver tissue within the first 2-3 days of cold acclimation in stickleback. HSC70 proteins increased in both liver and pectoral muscle in response to cold acclimation, and HSP90 protein levels tended to increase in liver. Together, these data suggest a greater demand for chaperone-mediated folding as fish adjust to decreases in temperature. Another possibility is that activity of HSPs declines with cold acclimation due to the Q_{10} effect, and stickleback may simply increase HSP levels to maintain chaperone activity.

Levels of HSPs may also increase during cold acclimation to ameliorate oxidative stress. Levels of oxidatively damaged proteins increased in stickleback livers by week 1 of cold acclimation from 20 °C to 8 °C and remained elevated after 4 weeks at 8 °C, but then declined

and were equivalent to levels in animals at 20 °C by week 9 (Kammer et al., 2011). Similarly, transcript levels of *Hsp60* and *Hsc70* transiently increased during cold acclimation and were highest in animals at 8 °C (day 3) and by week 9 at 8 °C were equivalent to levels in animals at 20 °C (start and week 9). HSP90 and HSC70 protein levels were also higher in stickleback livers after 4 weeks at 8 °C but unlike mRNA levels, HSC70 protein levels remained high. Similar to results from our study, oxidative stress was correlated with *Hsp* expression in hepatocytes of grass carp (*Ctenopharyngodon idellus*), where lipid peroxidation and *hsp70* and *hsp90* mRNA levels increased following exposure to 32°C heat stress for 30 min (Cui et al., 2013).

Increases in oxidative stress in stickleback liver might warrant higher levels of HSPs to assist with folding *de novo* proteins, preventing protein denaturation, and/or targeting oxidized proteins to the proteasome. Overexpression of HSP60 protects proteins from oxidation in yeast (*Saccharomyces cerevisiae*) (Cabisco et al., 2002). It has been proposed that HSP60 binds denatured, iron-centered proteins, preventing the release of free iron, which promotes the production of ROS through the Fenton reaction (Cabisco et al., 2002). While HSP60 did not increase in stickleback livers in our study, other chaperones may function in a similar fashion. Both HSP90 and HSP70 participate in the ubiquitin-proteasome system for degrading damaged proteins. HSP90 and HSP/HSC70, along with the co-chaperone CHIP, present proteins to the E3 ubiquitin ligase for ubiquitination (Esser et al., 2004). HSP90 also interacts with the proteasome and promotes degradation of oxidatively damaged proteins and prevents inactivation of the proteasome by oxidative stress (Conconi et al., 1998; Whittier et al., 2004). These functions of HSP90 are consistent with its increase in liver where levels of oxidized proteins increase in response to cold acclimation, and its higher level in liver compared to pectoral muscle; levels of

oxidized proteins and oxidized glutathione are also higher in liver compared to pectoral muscle (Kammer et al., 2011).

The mismatch between transcript levels of *hsp90-α* and protein levels of HSP90 in both liver and pectoral muscle may be due to the presence of two isoforms of the protein, HSP90- α and HSP90- β . We designed primers to quantify transcript levels of HSP90- α because in mammals, HSP90- α is considered to be the inducible isoform, whereas HSP90- β is considered the constitutive isoform (Csermely et al., 1998). The antibody we used recognizes both isoforms of the protein. Our data suggests that HSP90- β , detectable at the protein level, is more highly expressed in liver compared to HSP90- α , which we were unable to detect using qRT-PCR. The increase in *hsp90-α* mRNA observed in pectoral muscle in response to cold acclimation may have gone undetected at the protein level because of low abundance of this isoform.

Increases in HSC70 in the pectoral adductor muscle and liver may facilitate mitochondrial biogenesis and import of nuclear-encoded proteins into mitochondria. While mitochondrial biogenesis occurs in pectoral muscle but not liver in response to cold acclimation, the activity of enzymes located within the mitochondrion increase in both pectoral muscle and liver (Orczewska et al., 2010). Activity of citrate synthase (CS), reflecting protein levels, increased in liver by week 1 of cold acclimation to 8 °C and in pectoral muscle by week 9 (Orczewska et al., 2010). Cytochrome c oxidase (COX) activity increased by day 2 of cold acclimation in pectoral muscle but not until week 9 in liver (Orczewska et al., 2010). HSC70 protein levels increased by week 4 of acclimation to 8 °C in both liver and pectoral adductor muscle but may have increased prior to this; we did not measure protein levels prior to week 4. Transcript levels of *hsc70* significantly increased by day 2 of cold acclimation (10 °C) in both

tissues, suggesting protein levels may have increased earlier than week 4 to assist with protein import to the mitochondrion.

HSP60 was abundant in both liver and pectoral muscle and while mRNA levels of *hsp60* increased in both tissues in response to cold acclimation, protein levels remained unchanged. HSP60 is a chaperonin that provides an enclosed barrel-like structure for protein folding (Saibil, 2013). It is localized to both the mitochondrion and cytosol where it folds *de novo* proteins and ones denatured by stress (Saibil, 2013). The discordance between HSP60 transcript abundance and protein levels suggests post-transcriptional regulation of protein levels. Additionally, the increase in *hsp60* mRNA levels may be due to a decrease in decay rate, rather than gene induction, as shown for COX and housekeeping genes in cold-acclimated goldfish (Bremer and Moyes, 2014).

While transcript levels of the putative constitutive isoform of HSP70, *hsc70*, increased in response to cold acclimation, *hsp70* was not consistently detected in either pectoral muscle or livers of stickleback. Protein levels of HSP70 may have been undetectable because we used an antibody raised against the C-terminus of the protein in salmon, a region that is not well conserved (supplemental material Fig. S3). The lack of increase in *hsp70* suggests that HSP70, well-known to be induced by heat stress, may not be as strongly 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).

SIRT6 has been shown to regulate the expression of HSPs (Westerheide et al., 2009). In mammals, acetylation of K80 within HSF1 attenuates the heat-shock response by reducing DNA

binding of HSF1 to the heat-shock element (HSE) (Westerheide et al., 2009). Deacetylation by SIRT1 enhances HSF1 binding to HSE and maintains high levels of expression of HSP70 (Westerheide et al., 2009). The kinetics of induction of *sirt1* and *sirt2* in liver were similar to the induction of *hsp60* and *hsc70* in stickleback (this study, Orczewska et al., 2010). Thus, the increase in *sirt1* in liver, where oxidative stress occurs during cold acclimation, may drive increases in HSPs to refold oxidatively damaged proteins.

SIRT1 may also mediate adjustments in metabolism that occur during cold acclimation of stickleback. (Imai et al., 2000; Landry et al., 2000). Increases in NAD^+ , indicative of a low energy charge, increase activity of SIRT1 and SIRT2 (Canto et al., 2009; Wang et al., 2007) and activity of SIRT1 is correlated with CS activity (Gurd et al., 2011). In our study, we did not measure SIRT protein levels but both *sirt1* and *sirt2* mRNA levels increased in liver and *sirt1* increased in pectoral muscle during cold acclimation. In liver, mRNA levels of *sirt1* increased on day 2 of cold acclimation (10 °C), coinciding with an increase in CS mRNA and preceding an increase in CS activity that occurs at week 1 of cold acclimation (this study and Orczewska et al., 2010). Similarly, increases in *sirt1* mRNA in pectoral muscle on day 3 (8 °C) precede increases in CS mRNA levels at 1 week of cold acclimation and CS activity at week 9 of cold acclimation, suggesting SIRT1 may enhance aerobic metabolic capacity during cold acclimation of stickleback (this study and Orczewska et al., 2010).

In pectoral adductor muscle, SIRT1 may also activate mitochondrial biogenesis. In mammals, SIRT1 stimulates mitochondrial biogenesis by deacetylating and activating peroxisome proliferator-activated receptor- γ coactivator-1 alpha (PGC-1 α), considered the master regulator of mitochondrial biogenesis in mammals (Lehman et al., 2000). In fish, the NRF-1 binding domain of *PGC-1 α* is not conserved, likely impeding its ability to induce

mitochondrial biogenesis (LeMoine et al., 2010). This may explain why mRNA levels of *PGC-1 α* do not increase in response to cold acclimation or exercise in muscle of goldfish or stickleback despite increases in aerobic metabolic capacity (Bremer et al., 2012; LeMoine et al., 2008; Orczewska et al., 2010). Although NRF-1, rather than PGC-1 α , appears to mediate mitochondrial biogenesis in fish, SIRT1 may play a similar role, deacetylating and activating NRF-1.

Sirtuins also activate pathways that alleviate oxidative stress. In response to ROS in mammals, 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 2 (10 °C) in the pectoral muscle and day 3 (8 °C) in the liver of stickleback, which coincides with increases in *sirt1* mRNA, although SOD transcripts did not increase in response to cold acclimation in either tissue (Kammer et al., 2011).

Both SIRT3 and SIRT4 regulate metabolism, however, neither increased 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.

Conclusions

During cold acclimation of threespine stickleback, there are tissue- and isoform-specific changes in *hsp* and *sirt* mRNA, which may reflect differences in metabolic remodeling and/or

oxidative stress between the two tissues. Thus, HSPs are not only essential for enhancing survival during periods of acute changes in temperature, but also for adjusting to moderate decreases in temperature during cold acclimation. Sirtuins may play a role in regulating levels of HSPs and/or changes in metabolism during cold acclimation; further studies are warranted to verify this. The lack of correlation between changes in HSP transcript and protein levels highlights the importance of cautiously interpreting the physiological implications of changes in HSP transcript levels.

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Figure legends

Fig. 1. *HSP* mRNA transcript levels in liver (A & C) and pectoral adductor muscle (B & 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 = 5-9.

Fig. 2. Levels of HSP protein in liver (B & E) and pectoral adductor muscle (C & F) of threespine stickleback during cold acclimation. Representative western blots of HSP 60 (A), HSP 90 (A) and HSC 70 (D) in liver (lanes 1-4) and pectoral adductor muscle (lanes 5-8). Lanes 1 and 5, 20 °C; lanes 2 and 6, 8 °C week 4; lanes 3 and 7, 8 °C week 9; lanes 4 and 8, 20 °C week 9. Protein levels were standardized to levels in animals at 20 °C for each HSP in each tissue. Significant differences are indicated by different letters ($P < 0.05$, except for HSC70 in pectoral muscle in which $P < 0.10$). Values are mean $\pm$ SEM, N = 4-6.

Fig. 3 *SIRT* mRNA transcript levels in liver (A & C) and pectoral adductor muscle (B & D) of threespine stickleback during cold acclimation. Transcript levels of *SIRTs* 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.

Figure 1

Figure 2

Figure 3

Table 1. Primers used for quantitative real-time PCR.

Gene (Ensembl ID ENSGACT000000)	Forward Primer	Reverse Primer	Amplicon (bp)	Efficiency in pectoral muscle	Efficiency in liver
18S (21793)	5'ACCACATCCAAG GAAGGCAG3'	5'CCGAGTCGGGAG TGGGTAAT3'	51	N/A	1.62
EF-1 α (02143)	5'CGTCTACAAAAT CGGAGGTATTGG3'	5'GTCTCAACACGG CCGACTG3'	53	0.94	N/A
HSP90- α (17054)	5'TCAGCTGGAGTT CCGTGCTT3'	5'TCGAAAGACGCC CTTCTGG3'	51	0.98	0.81
HSP60 (11881)	5'AAGTTGGCCGCA AGGGAG3'	5'CGTGCAGAGTCT TCCCATCC3'	51	0.96	1.00
HSC70 (13955)	5'CAGTGGAGGACG AGAAGCTG3'	5'TCTGTCTGTCCT CTTCGCTGAT3'	51	0.93	1.02
HSP70 (17285)	5'CTTCAGAGAGAC AGGGTCTCCG3'	5'GAAGGCGTAGG ACTCCAAGGA3'	51	0.95	0.85
SIRT1 (03411)	5'GCAAACGCCTTG AGACAGG3'	5'GCTCGACGTCTC GTCCTCAG3'	101	1.06	0.83
SIRT2 (05747)	5'ACCCAGGACAGT TTAAGCCGA3'	5'TGTAGCAGCGCC TCAAGTACC3'	81	0.98	0.93
SIRT3 (15722)	5'CGAGGACAAGC AGGACGC3'	5'GAGAGACGGTTT GTCTGCACAG3'	102	0.95	0.99
SIRT4 (01497)	5'CGCCATTGTGAA CATTGGG3'	5'TTCAGCTCAGCC AGGTGGTC3'	51	0.95	1.04
18s, 18s rRNA; EF-1 α , elongation factor-1 α ; HSP, heat shock protein; HSC, heat shock cognate; SIRT, sirtuin.					

Table 2. Effect of cold acclimation on physical characteristics of *G. aculeatus*

Temperature and time point of harvest	Length (cm)	Body mass (g)	Condition factor
20 °C (start)	4.78 ± 0.31 ^A	1.01 ± 0.23 ^A	0.92 ± 0.16
8 °C week 4	4.93 ± 0.43 ^{AB}	1.13 ± 0.21 ^A	0.94 ± 0.09
8 °C week 9	5.43 ± 0.25 ^B	1.63 ± 0.19 ^B	1.03 ± 0.10
20 °C week 9	5.32 ± 0.49 ^{AB}	1.55 ± 0.38 ^B	1.02 ± 0.09

Table includes physical characteristics of animals used for measuring protein levels of HSPs.
Physical characteristics

of animals used for measuring transcript levels have been reported previously (Orczewska et al., 2010). Condition

factor = 100 * [body mass (g) * length (cm)⁻³]; N = 6; Different letters represent significant differences among animals

harvested at different time points and temperatures (P < 0.05).