OXIDATIVE STRESS IS TRANSIENT AND TISSUE-SPECIFIC DURING COLD ACCLIMATION OF THREESPINE STICKLEBACKS

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OXIDATIVE STRESS IS TRANSIENT AND TISSUE-SPECIFIC DURING COLD ACCLIMATION OF THREESPINE STICKLEBACKS

A

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Abstract

We sought to determine if oxidative stress occurs in liver, oxidative muscle or glycolytic muscle of threespine sticklebacks during cold acclimation. Fishes were held at 20°C for 12 wks and then acclimated to 8°C for 9 wks or held at 20°C for an additional 9 wks. Animals were harvested during the first four days of cold acclimation, and at wk 1, 4 and 9. Protein carbonyls were quantified as an indirect measure of the production of reactive oxygen species (ROS). The activity of superoxide dismutase (SOD), levels of SOD mRNA, and glutathione levels were quantified as indices of protection against ROS. All measurements were made in liver, glycolytic muscle and oxidative muscle. Protein carbonyl levels increased in livers of fishes after 1 wk at 8°C and decreased after wk 4. Total glutathione levels increased in livers on day 3 of cold acclimation and then decreased by wk 4. Measured at a common temperature, SOD activity increased early in all tissues and remained elevated throughout cold acclimation. Measured at the acclimation temperature, SOD activity increased only in oxidative muscle after 9 wks of cold acclimation. Together, these results indicate that oxidative stress is transient and tissue-specific during cold acclimation of fishes.

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Introduction

Changes in temperature profoundly affect the physiology of ectothermic organisms. For example, decreases in temperature decrease enzymatic activity and membrane fluidity (Guderley, 2004). Reduced enzyme activity decreases ATP production, and in order to maintain ATP levels, many fish species increase mitochondrial density in aerobic tissues through the process of mitochondrial biogenesis (Guderley, 2004; Johnston, 1982). Increases in mitochondrial density not only increase ATP production, but also increase the intracellular diffusion rate of oxygen because oxygen is more soluble within the hydrocarbon core of mitochondrial membranes than in the aqueous cytosol (Desaulniers et al., 1996; Sidell, 1998). Mitochondrial biogenesis has been well characterized in many fish species in response to cold acclimation (Johnston and Dunn, 1987; McClelland et al., 2006; Orczewska et al., 2010). However, it is not fully understood how this process is regulated and why it occurs in some tissues and not others.

In mammals, reactive oxygen species (ROS) can induce mitochondrial biogenesis (Rasbach and Schnellmann, 2007). ROS may stimulate mitochondrial biogenesis by inducing the master regulator of mitochondrial biogenesis, PGC-1α. ROS is a natural byproduct of cellular respiration, and it is estimated that between 1 and 2% of all oxygen used during oxidative phosphorylation is converted to superoxide anions (Droge, 2002). If ROS are responsible for initiating mitochondrial biogenesis in response to the cold, then ROS levels must increase in response to cold temperature. Several studies in fishes

have indicated that levels of ROS increase in response to cold temperature (Heise et al, 2006), although not all studies support these results (Grimm et al., 2010).-

A decrease in temperature may increase the production of ROS for several reasons. First, decreases in temperature decrease mitochondrial membrane fluidity, which may disrupt the transfer of electrons, increasing the production of ROS (Thieringer et al., 1998). Secondly, in order to maintain membrane fluidity at cold temperature, ectothermic organisms increase the relative proportion of polyunsaturated fatty acids (PUFAs), and PUFAs may promote ROS production (Crockett and Hazel, 1995). Thirdly, levels of ROS may increase due to an increase in cellular PO_2 as temperature decreases (Ritola et al., 1999).

I hypothesized that ROS production increases in response to cold acclimation of threespine sticklebacks (*Gasterosteus aculeatus*) and might occur within the timeframe necessary to stimulate mitochondrial biogenesis. I tested this hypothesis by measuring protein carbonyl levels, the activity of superoxide dismutase (SOD), SOD mRNA levels, and glutathione levels during cold acclimation of the threespine sticklebacks. Measurements were made in liver, oxidative muscle and glycolytic muscle. Levels of protein carbonyls were quantified as an indirect measure of ROS production. Protein carbonyl content is one of the most accurate and generally accepted measures of oxidative stress (Levine et al., 1994). Transcript levels and activity of the antioxidant superoxide dismutase (SOD) were quantified throughout cold acclimation as a measure of defense levels against ROS. The ratio of oxidized to reduced glutathione was measured

because it is considered the most important estimate of the cellular redox environment (Schafer and Buettner, 2001).

Our results indicate that oxidative stress is transient and tissue-specific during cold acclimation of threespine stickleback. Oxidative stress increased in liver tissue early during cold acclimation, while no changes in oxidative stress were observed in either oxidative or glycolytic muscle tissue. Oxidative stress occurred within the timeframe that changes in metabolism occurred in liver, suggesting that ROS might mediate metabolic remodeling in liver in response to cold acclimation in sticklebacks.

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Chapter 1: Oxidative stress is transient and tissue-specific during cold acclimation of threespine sticklebacks[1]

1.1 Summary

We sought to determine if oxidative stress occurs in oxidative muscle, glycolytic muscle or liver of threespine sticklebacks during cold acclimation. Fishes were held at 20°C for 12 wks and then acclimated to 8°C for 9 wks or held at 20°C for an additional 9 wks. Animals were harvested during the first four days of cold acclimation, and at wk 1, 4 and 9. Warm-acclimated fishes were harvested on day 0 and wk 9. Protein carbonyls were quantified as an indirect measure of the production of reactive oxygen species (ROS). The activity of superoxide dismutase (SOD), levels of SOD mRNA, and levels of glutathione were quantified as indices of protection against ROS. All measurements were made in liver, glycolytic muscle and oxidative muscle. Protein carbonyl levels increased in livers of fishes after 1 wk at 8°C and decreased after wk 4, but did not change in muscle tissue. Total glutathione levels increased in liver on day 3 of cold acclimation and then decreased by wk 4. When measured at a common temperature, the activity of SOD increased in muscles on day 2 of cold acclimation, and on day 3 in liver, and remained elevated in all tissues compared to warm-acclimated animals. When measured at the acclimation temperature, SOD activity was significantly higher in the oxidative muscle of animals at 8°C for 9 wks compared to warm-acclimated fishes at 20°C for 9 wks. Together, these results indicate that oxidative stress is transient and tissue-specific during cold acclimation of fishes.

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1.2 Introduction

Oxidative stress occurs in organisms when there is an imbalance between the production of reactive oxygen species (ROS) and the activity of antioxidants that protect against the damaging effects of ROS (Halliwell, 1999). The majority of ROS, which include superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) are produced by the electron transport chain during cellular respiration (Droge, 2002). ROS production increases when electron transfer is disrupted (Turrens, 2003), which can be brought about by a number of conditions, including food deprivation (Pascual et al., 2003), zinc deficiency (Hidalgo et al., 2002), pollutants (Torres et al., 2002), hypoxia (Dirmeier et al., 2002), hyperoxia (Ritola et al., 1999), and changes in temperature (Heise et al., 2006; Heise et al., 2007a; O'Kane et al., 1996).

Ectothermic organisms such as fish, having body temperatures that conform to the environment, may be particularly vulnerable to oxidative stress as temperature decreases for several reasons. First, decreases in temperature decrease mitochondrial membrane fluidity, which may disrupt the transfer of electrons, increasing the production of ROS (Thieringer et al., 1998). Secondly, alterations in membrane composition during homeoviscous adaptation may increase the susceptibility of fishes to oxidative stress. In order to maintain membrane fluidity at cold temperature, ectothermic organisms increase the relative proportion of polyunsaturated fatty acids (PUFAs) (Crockett and Hazel, 1995). One drawback of this process is that unsaturated fatty acids are prone to lipid peroxidation, a self-propagating process in which a peroxyl radical is formed when ROS have sufficient energy to abstract a hydrogen atom from a lipid (Halliwell, 1999). Cold

acclimation in fishes also leads to an increase in the ratio of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) (Hazel and Landrey, 1988a), which may increase the potential for oxidative stress because PE is generally more unsaturated than PC (Logue et al., 2000). Thirdly, oxidative stress may increase due to an increase in cellular PO_2 as temperature decreases (Ritola et al., 1999).

Although several studies have measured oxidative stress during cold acclimation in fishes, the results have been equivocal. In some studies, cold acclimation or acclimatization led to an increase in oxidative stress and increased levels of antioxidants. For example, mRNA levels of superoxide dismutase 1 and 2 (SOD1 and SOD2), and glutathione levels increased in skeletal muscle of zebrafish (*Danio rerio*) acclimated to 18°C compared to warm-acclimated fishes held at 28°C (Malek et al., 2004). The transcript level of SOD2 also increased in liver and muscle tissue of common carp (*Cyprinus carpio*) acclimated to 10°C compared to warm-acclimated fishes at 17°C (Gracey et al., 2004). The activity of SOD and levels of protein oxidation were elevated in the livers of winter acclimatized eelpout (*Zoarces viviparous*) compared to summer acclimatized animals (Heise et al., 2007b) and levels of protein carbonyls increased in the livers of sea bream (*Sparus aurata)* in response to cold acclimation (Ibarz et al., 2010). On the other hand, there was no change in the activity of SOD or catalase in the glycolytic or cardiac muscle of killifish (*Fundulus heteroclitus macrolepidotus*) or bluegills (*Lepomis macrochirus*) in response to cold acclimation (Grim et al., 2010). Furthermore, glutathione levels were variable with no clear trend in response to

temperature in glycolytic muscle of killifish acclimated for three weeks to temperatures between 6°C and 30°C (Leggatt et al., 2007).

The inconsistent results among these studies may be caused by differences in the cold acclimation regime and/or differences in tissues studied. Oxidative stress may be affected by the duration of cold acclimation, the magnitude of change in temperature, levels of antioxidants and rates of production of ROS. For example, oxidative stress may increase early during cold acclimation if it is due to an increase in PO_2 or a decrease in mitochondrial membrane fluidity. Alternatively, oxidative stress may increase later during cold acclimation if it is caused by changes in membrane composition and increases in PUFAs. Moreover, the susceptibility to oxidative stress may not be equivalent among different tissue types. Tissues may differ by heme, free iron, and hemoprotein content, as well as mitochondrial and lipid density, and levels of antioxidants, all of which may impact levels of oxidative stress (Lipinski et al., 2000; Perez-Campo et al., 1993; Petrat et al., 2001).

A number of studies have found that liver tissue is particularly susceptible to oxidative stress during cold acclimation (Heise et al., 2007b; Ibarz et al., 2010). There are several potential sources that may contribute to oxidative stress in liver. Liver tissue contains 2-to-20 times more iron than muscle tissue, as well as large pools of free iron necessary for synthesizing heme and cytochromes (Canli and Atli, 2003; Lipinski et al., 2000; Petrat et al., 2001). Oxidative stress may cause hemoproteins to release heme, which promotes the formation of ROS via the Fenton reaction (Jeney et al., 2002; Vercellotti et al., 1994). Free iron reacts with hydrogen peroxide, producing hydroxyl

radicals, the most damaging form of ROS (McCord, 1998). Additionally, the cytochrome P450 system catalyzes reactions involved in fatty acid metabolism and detoxifying xenobiotics, which require molecular oxygen and generate ROS as a byproduct (Lieber, 1997; Myasoedova, 2008).

We sought to determine if oxidative stress occurs during cold acclimation of threespine sticklebacks (*Gasterosteus aculeatus)* and if so, when it occurs during cold acclimation and if it differs among tissues. We chose threespine stickleback as a study species because they inhabit a vast geographic range and experience a range temperatures that made them a good candidate for our cold acclimation regime. We measured oxidative stress in liver tissue, oxidative muscle and glycolytic muscle to address whether oxidative stress is tissue specific during cold acclimation. These three tissues are metabolically very different and are also the most commonly used tissues in fish cold acclimation studies. Given results from previous studies we hypothesized oxidative stress would increase in liver tissue, and in the aerobically active oxidative muscle, but not in glycolytic muscle. We cold-acclimated our animals for 9 weeks and harvested animals throughout the cold acclimation period to determine the potential source of increases in ROS, and to determine if the variable results of different studies could be explained by the length of the acclimation period. We hypothesized that indices of oxidative stress may increase at different times during cold acclimation and that these increases may be missed by studies that only make measurements at a single endpoint.

We measured protein carbonylation, glutathione, SOD activity, and SOD mRNA expression as indices of oxidative stress during cold acclimation. This is the first study to date to examine changes in oxidative stress throughout the time course of cold acclimation.

1.3 Materials and Methods

1.3.1 Animal care and experimental design

Threespine sticklebacks were captured in Kashwitna Lake, AK (61° 50'N, 150° 00'W) in September 2007 and 2008 using minnow traps. Lakes in the area range in temperature from 4°C to 23°C. Fishes were transported to the University of Alaska Fairbanks and maintained in 114 L aquaria containing 0.35% Instant Ocean. Tanks were housed in an environmental chamber, which regulated temperature and day length. Fishes were maintained at 20°C for 12 wk on a 10-hr light, 14-hr dark cycle and fed twice daily an alternating diet of blood worms and brine shrimp. After 12 wk at 20°C, the first group of warm-acclimated fishes was harvested. The remaining animals were either acclimated to 8°C or maintained at 20°C for an additional 9 wk. Fishes were acclimated to 8°C by decreasing the temperature in the environmental chamber from 20°C to 15°C on day one, from 15°C to 10°C on day 2, and to 8°C on day 3. Twenty-six to forty animals were harvested each day, prior to decreasing the temperature and after 1, 4, and 9 wk at 8°C. The second group of warm-acclimated fishes was harvested after an additional 9 wk at 20°C. Animals were harvested prior to feeding and at the same time each day to avoid the potentially confounding effects of feeding and circadian rhythms on

measurements made. Animals were harvested and killed by immersion in liquid nitrogen and stored at -80°C until use. Fish were thawed on ice prior to dissecting pectoral adductor muscle (oxidative muscle), glycolytic muscle from the tail region, and liver tissue. Tissues were then homogenized on ice as described below.

1.3.2 Protein carbonyl levels

Levels of protein carbonyls were quantified as an indirect measure of ROS production. Protein carbonyl levels were quantified in liver, glycolytic muscle and oxidative skeletal muscles of warm- and cold-acclimated fishes harvested at all time points. Tissues were homogenized in 9 vol of ice-cold 50 mM potassium phosphate buffer, pH 7.8, using a ground-glass homogenizer. Three-hundred μl of homogenate was treated with 70 μl of 10% streptomycin sulfate and incubated for 15 min at room temperature to precipitate DNA. Samples were then centrifuged for 10 min at 9100 X *g*. Supernatant was decanted, added to 1.2 ml of ice-cold acetone, incubated for 30 min at -20°C to precipitate protein, and then centrifuged at 16000 X *g* for 15 min. The resulting pellet was washed with 80% acetone and resuspended in 100 μl of HPLC running buffer (6M guanidinium HCL, 0.5M K_2HPO_4, pH 2.5). Samples were divided into four equal aliquots. Two aliquots were derivitized with 35 μl of 10 mM 2,4-dinitrophenyl hydrazine (DNPH) in HPLC running buffer for 35 min at room temperature. An equal volume of HPLC running buffer was added to the remaining two samples and incubated for 35 min at room temperature. DNPH readily binds carbonyl groups, forming dinitrophenyl hydrazone (Levine et al., 2000). The resulting hydrazones were detected at 366 nm using

a Waters 1525 HPLC equipped with a Waters 2296 Photodiode Array Detector. Proteins were separated on Zorbax 450 and Zorbax 250 gel filtration columns (Waters, Milford, MA, USA) arranged in series and eluted with HPLC running buffer at a rate of 1 ml min^{-1}. Absorbance was monitored at 366 nm and 276 nm to detect the concentration of protein carbonyls and total protein, respectively. Background absorbance at 366 nm was determined in aliquots untreated with DNPH. Total carbonyl content was determined by subtracting the concentration of hydrazone in the untreated sample from the concentration of hydrazone in the derivatized sample as shown in equations 1-3. The extinction coefficient used for the hydrazone was 50,000 M^{-1} and 22,000 M^{-1} for protein (Levine et al., 1994).

$$(+)\text{DNPH} - (-\text{DNPH}) = \text{M carbonyl } M^{-1} \text{ protein} \quad (1)$$

$$(-)\text{DNPH} = (50{,}000\ M^{-1} \times A_{366}) / (22{,}000\ M^{-1} \times A_{276}) \quad (2)$$

$$(+)\text{DNPH} = (50{,}000\ M^{-1} \times A_{366}) / [(22{,}000\ M^{-1} \times A_{276}) - (0.43 \times A_{366})] \quad (3)$$

1.3.3 Glutathione levels

Levels of oxidized, reduced and total glutathione were quantified to determine the redox state of the cellular environment (Schafer and Buettner, 2001). Levels of oxidized and reduced glutathione were quantified in liver, glycolytic muscle and oxidative skeletal muscles of warm- and cold-acclimated fishes harvested throughout the acclimation period. Tissues were deprotonized by homogenizing in 9 vol of 5% 5-sulfosalicylic acid. Samples were then centrifuged for 5 min at 10,000 X *g* and total glutathione (GSSG +

GSH) was measured in the resulting supernatant using the DTNB-GSSG recycling assay as described by Anderson (1985). The final reaction mixture containing 115 mM $NaPO_4$, 5 mM Na_4-EDTA, 0.6 mM DTNB, and 210 mM NADPH, pH 7.2 was incubated at 20°C for 12 min. Twenty-five μL of supernatant was then added, followed by glutathione reductase at a final concentration of 1.33 U ml^{-1} to initiate the reaction. The formation of TNB was followed at 412 nm for 7 min using a Perkin Elmer Lambda 25 spectrophotometer (Downers Grove, Illinois, USA). Total glutathione was determined from a standard curve with concentrations of glutathione between 0 and 4 μM (Sigma-Aldrich, St. Louis, Missouri, USA). Each sample was measured in triplicate.

Oxidized glutathione was measured by derivatizing reduced glutathione (GSH) with 2-vinylpyridine. Two μl of 98% 2-vinylpyridine was added to 100 μl of supernatant and mixed well. Six μl of 97% triethanolamine was then added to increase the pH to 6-7 and irreversibly conjugate 2-vinylpyridine to GSH. Samples were incubated for 60 min at room temperature and glutathione concentrations measured as described above, except that glutathione reductase was added at a final concentration of 2.66 U ml^{-1} to initiate the reaction. GSSG levels were determined from a standard curve with concentrations of GSSG between 0 and 0.25 μM (Sigma-Aldrich, St. Louis, Missouri, USA). Measurements were made in triplicate in liver and glycolytic muscle, and in duplicate or triplicate in oxidative muscle.

1.3.4 Superoxide dismutase activity

The activity of superoxide dismutase was measured as an indication of levels of defense against oxidative stress. The activity of SOD was measured in liver, glycolytic muscle and oxidative skeletal muscle of cold- and warm-acclimated fishes harvested at each time point during acclimation using the xanthine oxidase / cytochrome c assay (Crapo et al., 1978). One unit of SOD was defined as the amount of SOD necessary to inhibit the rate of reduction of cytochrome c by 50% (Crapo et al., 1978). Tissues were homogenized in 9 vol of ice-cold 50 mM potassium phosphate buffer, pH 7.8, using a ground-glass homogenizer. The final reaction mixture contained 0.05 mM K_2HPO4, pH 7.8, 0.01 mM acetylated cytochrome c, 0.05 mM xanthine, and 0.01 mM KCN. Background rate was monitored for 1 min at 550 nm. The reaction was initiated by the addition of 60 µl of xanthine oxidase at a concentration sufficient to achieve an increase in the absorbance rate at 550 nm of 0.02 OD min^{-1}. The concentration of xanthine oxidase required to achieve this rate varied and therefore was determined each day prior to measuring SOD activity (Crapo et al., 1978). Fifty μl of homogenate was added to the reaction at a concentration sufficient to inhibit the reduction of cytochrome c by 50 ± 10 %. The rate of reduction of cytochrome c was monitored at 550 nm for 5 min using a Perkin Elmer Lambda 25 spectrophotometer. Samples were run in triplicate at a common temperature of 14°C and at the acclimation temperature of each fish (20°C, 15°C, 10°C, or 8°C). The pH of the assay buffer was adjusted so that all assays were conducted at a pH of 7.8. The same individuals used for measuring levels of protein carbonyls were also used for measuring the activity of SOD.

1.3.5 RNA isolation

The mRNA levels of SOD1 and SOD2 were quantified in oxidative muscle, glycolytic muscle and liver tissue of cold- and warm-acclimated fishes harvested throughout the acclimation period. RNA was isolated from 10-30 mg of tissue using the RNeasy Fibrous Tissue Mini-kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol except that liver tissues were not treated with Proteinase K and slight modifications were made to improve the removal of DNA. RNA was treated with DNase twice, once for 25 min and a second time for 20 min. RNA was eluted with 30 µl of RNase-free water, pH 8.0 and then reapplied to the column to elute remaining RNA. Samples were quantified spectrophotometrically with a Nano-Drop® (ND-1000) by measuring the absorbance at 260 nm. RNA purity was verified by measuring the ratio of the absorbance at 260 nm and 280 nm and only samples with values greater than 1.8 were used for quantifying transcript levels. RNA integrity was verified by agarose electrophoresis. Two µl of RNA was mixed with loading buffer (5 % glycerol, 0.04% bromophenol blue, 0.1 mM EDTA, pH 8.0) and separated on a 2% agarose gel. RNA was stored at -80°C.

Complementary DNA (cDNA) was synthesized using TaqMan® reverse transcription reagents (Applied Biosystems, Carlsbad, CA, USA). Each 10 μl reaction contained 5.5 mM $MgCl_2$, 0.5 μl random hexamers, 2 mM dNTPs, 4 units of RNase inhibitor, 37.5 units of reverse transcriptase and 200 ng RNA. Parallel samples were prepared, omitting the reverse transcriptase so that we could detect the presence of contaminating genomic DNA. cDNA was stored at -80°C until use.

1.3.6 Quantitative real-time PCR

Gene-specific primers were designed using sequence information obtained from Ensembl (www.ensembl.org) and the software Primer Express (Applied Biosystems, Carlsbad, CA, USA) (Table 1). At least one primer from each primer pair was designed over a splice site to ensure that no genomic DNA was amplified. Primers were synthesized commercially (Invitrogen, Carlsbad, CA, USA). Gene expression was quantified using quantitative real-time PCR (qRT-PCR) on an ABI 7900HT sequence detection system (Applied Biosystems, Carlsbad, CA, USA). Each sample was measured in triplicate in a 20 μl reaction volume containing 5 ng cDNA, 0.3 mM of each forward and reverse primer, and 10 μl Power SYBR® Green Master Mix (Applied Biosystems, Carlsbad, CA, USA). Reactions were run at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. A dissociation curve was analyzed for each reaction to verify the specificity of each primer set. Two controls were prepared for each sample, one in which the reverse transcriptase was omitted during cDNA synthesis, the second in which an equal volume of Milli Q H_2O was substituted for cDNA. Standard curves were prepared for calculating the amount of mRNA by pooling and serially diluting cDNA from four individuals harvested at each time point during acclimation. Transcript levels of each target gene were normalized to the mean transcript level of 18S in liver and EF-1α in oxidative and glycolytic muscles, which were previously identified as stable housekeeping genes (Orczewska et al., 2010).

1.4 Statistical Analysis

A one-way ANOVA comparing the eight harvest groups was run for each tissue type followed by a post-hoc Tukey's Honestly Significant Difference (HSD) test were used to identify significant differences in the activity of SOD, levels of glutathione, and physical characteristics. A one-way ANOVA for each time point comparing the three tissues followed by a post-hoc Tukey's HSD test were used to determine differences between tissues. Levels of protein carbonyls and transcript levels of SOD were not normally distributed, so significant differences were determined using a Kruskal-Wallis test and post-hoc Tukey's HSD test. Assumptions for normality were determined using residual and Q-Q plots. All tests were two sided and results were considered statistically significant at $P<0.05$. All data are presented as mean ± s.e.m.

1.5 Results

1.5.1 Physical characteristics

Body mass and length increased during the experimental period (Table 2), but condition index, hepatosomatic index, and the ratio of pectoral mass to body mass did not change. There was no effect of temperature on any of these parameters; all measurements were equivalent between cold- and warm-acclimated fishes at wk 9.

1.5.2 Protein carbonylation

We measured protein carbonyl levels in warm- and cold-acclimated animals to determine if ROS production increased during cold acclimation. Protein carbonylation

significantly changed in liver tissue during cold acclimation, $\chi^2_{(7, N = 65)} = 26.16$, ($P = 0.0005$). After 1 wk of cold acclimation, protein carbonyl levels increased 2.3-fold in liver compared to animals at 20°C, from 12.6 ± 1.7 mmol mol^{-1} protein to 29.0 ± 2.4 mmol mol^{-1} protein ($P = 0.018$) (Fig. 1.1). Protein carbonyl levels remained elevated through wk 4, ($P = 0.0042$), of cold acclimation, but by wk 9, levels were reduced and equivalent to that of warm-acclimated fishes at 20°C for 9 wk, ($P = 0.91$). Protein carbonyl levels did not significantly increase in oxidative, $\chi^2_{(7, N = 65)} = 11.89$, ($P = 0.11$), or glycolytic muscle $\chi^2_{(7, N = 40)} = 1.43$, ($P = 0.98$), in response to cold acclimation.

Differences between tissues were observed at 10°C, $\chi^2_{(2, N = 21)} = 10.81$, ($P = 0.0045$); 8°C wk 1, $\chi^2_{(2, N = 21)} = 14.72$, ($P = 0.0006$); and 8°C wk 4, $\chi^2_{(2, N = 25)} = 9.51$, ($P = 0.0086$). Protein carbonyl levels were significantly greater in liver compared to glycolytic and oxidative muscle at 10°C ($P < 0.01$), 8°C wk 1 ($P < 0.0001$), and 8°C wk 4 ($P < 0.05$) of cold acclimation. There was no significant difference in protein carbonyl levels between glycolytic and oxidative muscles ($P > 0.35$) for all time points.

1.5.3 Glutathione content

Total glutathione (2GSSG + GSH), and levels of oxidized glutathione (GSSG) were measured to determine if the cellular redox environment changed during cold acclimation. Total glutathione levels increased during cold acclimation $F_{(7,54)} = 8.65$, ($P < 0.0001$). Levels of total glutathione increased 1.6-fold in liver on day 3 of cold acclimation (8°C), from 1.84 ± 0.18 μmol g^{-1} wet mass in fishes at 20°C to 2.90 ± 0.22 μmol g^{-1} wet mass on day 3, ($P = 0.0061$) (Fig. 1.2A). By wk 4 of cold acclimation,

glutathione levels decreased and at this point, were equivalent to levels in fishes at 20°C (day 0), ($P = 0.64$), and remained at this level for the remainder of the acclimation period (Fig. 1.2A). Total glutathione levels changed during cold acclimation in glycolytic muscle $F_{(7,55)} = 2.36$, ($P = 0.034$). By wk 4 of cold acclimation total glutathione levels increased in glycolytic muscle compared to fishes at 20°C (day 0), ($P = 0.034$), but were unchanged compared to fishes held at 20°C for 9 wk, ($P = 0.74$). Levels of total glutathione remained unchanged in oxidative muscle during cold acclimation $F_{(7,52)} = 1.41$, ($P = 0.22$). Levels of total glutathione were always significantly higher in liver compared to glycolytic and oxidative muscle, ($P < 0.0001$).

Levels of oxidized glutathione changed in liver tissue during cold acclimation $F_{(7,50)} = 2.24$, ($P = 0.045$). Levels of oxidized glutathione were significantly higher in livers of fishes at 15°C (day 1) compared to animals that were cold-acclimated for 9 wk, $P = 0.0091$. No other significant changes were detected in levels of oxidized glutathione or the ratio of oxidized to reduced glutathione during cold acclimation in any tissue (Fig. 1.2B,C). However, levels of oxidized glutathione were always significantly higher in liver tissue compared to glycolytic and oxidative muscle, $P < 0.01$ at all time points. The ratio of oxidized to reduced glutathione (2GSSG/GSH) varied between tissues in fishes at 15°C (day 1), $F_{(2,19)} = 16.80$, ($P < 0.0001$), and at 20°C wk 9, $F_{(2,19)} = 4.33$, ($P = 0.30$). The ratio of 2GSSG/GSH was higher in liver tissue compared to muscle tissue in fishes at 15°C (day 1), and at 20°C wk 9, ($P < 0.05$), (Fig. 1.2C).

1.5.4 Activity of superoxide dismutase

The activity of SOD was measured at the common temperature of 14°C to determine if enzyme concentration or activity increased during cold acclimation. SOD activity increased during cold acclimation in oxidative muscle, $F_{(7,32)} = 23.04$, ($P < 0.0001$); liver, $F_{(7,32)} = 22.17$, ($P < 0.0001$), and glycolytic muscle $F_{(7,32)} = 9.96$, ($P < 0.0001$). The activity of SOD was significantly higher in oxidative, ($P = 0.021$), and glycolytic muscles, ($P = 0.0026$), of fishes on day 2 (10°C) of cold acclimation compared to fishes at 20°C (Fig. 1.3A). By day 3 (8°C) of cold acclimation, the activity of SOD had become significantly elevated in liver, ($P = 0.003$), compared to animals at 20°C (Fig. 1.3A). The activity of SOD remained elevated in all tissues from wk 1 through the remainder of the cold acclimation period. The greatest increase in the activity of SOD occurred in oxidative muscle, where by wk 9 of cold acclimation, SOD activity increased 3.3-fold, ($P < 0.0001$), from 3.5 ± 0.1 U·mg^{-1} wet mass to 11.5 ± 1.0 U·mg^{-1} wet mass compared to animals held at 20°C for 9 wk. There was a 2.3-fold increase in the activity of SOD in liver tissue, ($P < 0.0001$), from 3.5 ± 0.1 U·mg^{-1} wet mass tissue to 8.0 ± 0.6 U·mg^{-1} wet mass tissue, and a 1.7-fold increase in SOD activity in glycolytic muscle, ($P = 0.0021$), from 1.6 ± 0.1 U· mg^{-1} wet mass tissue to 2.6 ± 0.2 U·mg^{-1} wet mass tissue in fishes at 8°C for 9 wk compared to fishes held at 20°C for 9 wk (Fig. 1.3A). The activity of SOD was always significantly higher in liver and oxidative muscle compared to glycolytic muscle, ($P < 0.02$) at all time points, and higher in oxidative muscle compared to liver at wk 9 of cold acclimation, ($P < 0.016$).

We also measured the activity of SOD at the acclimation temperature of each animal to determine if the observed increase in the activity of SOD completely compensated for the Q_{10} effect. When measured at the acclimation temperature of each animal, the activity of SOD increased in oxidative muscle during cold acclimation, $F_{(7,32)} = 8.60$, ($P < 0.0001$), and was 1.4-fold greater in oxidative muscle after 9 wks of cold acclimation compared to fishes held at 20°C for 9 wks, ($P < 0.0001$), indicating that there was overcompensation for the Q_{10} effect in oxidative muscle (Fig. 1.3B). The activity of SOD remained constant in liver and glycolytic muscle during cold acclimation when measured at the acclimation temperature, indicating that there was complete compensation for the Q_{10} effect in these tissues.

1.5.5 Transcript levels of superoxide dismutase

Transcript levels of SOD1 and SOD2 were measured in fishes harvested during cold acclimation to determine if the observed increases in SOD activity were transcriptionally regulated. The mRNA levels of SOD1 did not become significantly elevated until wk 9 of cold acclimation in oxidative muscle, $\chi^2_{(7, N = 62)} = 20.75$, ($P = 0.042$), (Fig. 1.4A). At this point, SOD mRNA levels had increased by 1.8-fold, ($P = 0.024$), compared to fish held at 20°C for 9 wks. In liver tissue, mRNA levels of SOD1 also increased during cold acclimation, $\chi^2_{(7, N = 62)} = 18.58$, ($P = 0.0096$). By wk 1 of cold acclimation SOD1 mRNA levels increased1.8-fold, ($P = 0.030$), compared to fish held at 20°C (Fig.1.4B). SOD1 mRNA levels then decreased in liver and by wk 9 of cold acclimation, transcript levels were equivalent between animals held at 8°C for 9 wk and

those held at 20°C for 9 wk, (P = 0.20). The expression of SOD1 did not change during cold acclimation in glycolytic muscle, $\chi^2_{(7, N = 62)} = 11.17$, (P = 0.13), (Fig. 1.4C).

The expression of SOD2 did not change in liver or oxidative muscle in response to cold acclimation, but decreased in glycolytic muscle, $\chi^2_{(7, N = 62)} = 32.71$, (P < 0.0001). By wk 1 of cold acclimation SOD2 mRNA expression was significantly lower compared to fishes at 20°C (day 0), (P = 0.0064), and remained at this level throughout the remainder of the cold acclimation period (Fig. 1.4A,B,C).

1.6 Discussion

Results from our study indicate that oxidative stress increases in response to cold acclimation in threespine sticklebacks, but is transient and tissue-specific. Levels of oxidized proteins increased only in liver, and only within the first week of cold acclimation. These results suggest that either the production of ROS decreased and/or the activity of antioxidants increased in liver, as fishes acclimated to the cold.

1.6.1 Oxidative stress is tissue-specific

Overall, levels of antioxidants, including SOD and glutathione were higher in liver compared to muscle tissue. Previously, we quantified the activity of cytochrome c oxidase (COX) during cold acclimation in threespine sticklebacks and determined that the activity of COX was always higher in oxidative muscle compared to liver (Orczewska et al., 2010), yet the activity of SOD is similar between the two tissues. As a result, the ratio of SOD:COX is 2-3 fold higher in liver compared to oxidative muscle, suggesting

that SOD detoxifies superoxide generated by sources in addition to the mitochondrial electron transport chain in liver.

Previous studies of cold acclimation in fishes detected increases in the activity of glucose-6-phosphate dehydrogenase (G6PDH) in liver, which catalyzes the first reaction in the hexose monophosphate shunt pathway (HMP) (Campbell and Davies, 1975; Seddon and Prosser, 1997). Increases in the activity of G6PDH have been attributed to an increased demand for NADPH, produced by the HMP, for biosynthetic reactions (Campbell and Davies, 1975; Seddon and Prosser, 1997). An alternative explanation, supported by our data, is that increased levels of NADPH may be required to combat oxidative stress. NADPH is an essential co-factor for glutathione reductase, which maintains glutathione in its reduced state. This may be particularly important in liver, where oxidative stress occurs during cold acclimation.

1.6.2 SOD activity increases in response to cold acclimation

SOD is an important antioxidant because it is the only enzyme that detoxifies superoxide, dismutating it to hydrogen peroxide and oxygen (Fridovich, 1995). The two major forms of SOD encoded in eukaryotic genomes are MnSOD (SOD2), which is located in the mitochondrial matrix, and CuZn-SOD (SOD1), which is located in the cytosol and intermembrane space of mitochondria (Zelko and Folz, 2003). The activity of SOD increased by day 3 of cold acclimation (8°C) in liver, and continued to increase throughout the acclimation period. Total levels of glutathione also increased in liver by

day 3 of cold acclimation (8°C). Increases in SOD activity and glutathione may account for decreased levels of oxidized proteins in liver after 4 wk of cold acclimation.

Although we did not observe an increase in levels of oxidized proteins or total glutathione in oxidative or glycolytic muscle, the activity of SOD significantly increased in both tissues by day 2 (10°C) of cold acclimation. Measuring the activity of SOD at the acclimation temperature of each animal revealed that the increase in SOD activity in liver and glycolytic muscle compensated for the decrease in the catalytic rate of SOD at cold temperature, whereas in oxidative muscle, increases in the activity of SOD overcompensated for the decreased catalytic rate. The activity of SOD, measured at each acclimation temperature, was 1.3-fold higher in oxidative muscle of fishes held at 8°C for 9 wks compared to fishes at 20°C for 9 wks. Additionally, the ratio of SOD:COX was 1.5 fold higher in oxidative muscle of cold- acclimated fishes compared to warm-acclimated ones. Together, these data suggest that there may be a greater potential for oxidative stress in oxidative muscle of cold-acclimated fishes compared to warm-acclimated ones. Previously, we determined that mitochondrial volume density increases in oxidative muscle of threespine sticklebacks in response to cold acclimation (Orczewska et al., 2010). Corresponding elevations in mitochondrial membrane densities may warrant increases in antioxidants because mitochondrial membranes are rich in PUFAs, which promote the formation of ROS (Crockett and Hazel, 1995; Halliwell, 1999).

The increase in SOD activity occurred independently of an increase in SOD mRNA levels in all tissues. The activity of SOD increased 1 wk prior to an increase in

mRNA levels of SOD1 in liver, and over 9 wks earlier in oxidative muscle. SOD1 mRNA levels did not increase in glycolytic muscle and transcript levels of SOD2 did not increase in any tissue measured. The activity of SOD is post-translationally regulated by a copper chaperone, CCS (Casareno et al., 1998; Culotta et al., 1997). In response to an increase in superoxide levels, CCS is activated and inserts a copper atom into SOD (Brown et al., 2004). Studies in yeast have shown that pools of inactive SOD are rapidly activated by copper chaperones in response to oxidative stress, and that prolonged exposure to oxidative stress leads to an increase mRNA levels of SOD (Brown et al., 2004; Galiazzo and Labbe-Bois, 1993).

1.6.3 Potential sources of ROS during cold acclimation

The transient increase in oxidatively-modified proteins in liver suggests that the production of ROS increased early during cold acclimation. Either the disruption of the electron transport chain or an increase in intracellular PO_2 levels likely caused this increase in ROS production. Alterations in membrane composition during homeoviscous adaptation are likely not involved in promoting oxidative stress during cold acclimation. Although some changes in membrane composition, such as modifications of polar head groups, occur rapidly during cold acclimation, complete membrane remodeling resulting in significant increases in PUFAs may take several weeks (Kraffe et al., 2007; Hazel and Landrey, 1988b).

1.6.4 ROS as signaling molecules

Uncontrolled increases in ROS damage proteins, lipids and DNA, yet minor fluctuations in ROS play vital roles in intracellular signaling (Droge, 2002; Stadtman and Levine, 2002). ROS induce mitochondrial biogenesis in a variety of cell types in mammals (Lee et al., 2002; Rasbach and Schnellmann, 2007; Suliman et al., 2003). Our data suggest that ROS production increases early during cold acclimation in liver, potentially triggering a signaling cascade leading to changes in metabolism. Although mitochondrial biogenesis does not occur in the liver of threespine sticklebacks in response to cold acclimation, the expression and activity of aerobic metabolic genes does (Orczewska et al., 2010). Notably, mRNA levels of citrate synthase, and transcriptional co-regulators and regulators of aerobic metabolism, including peroxisome proliferator-activated receptor gamma coactivator-1β (PGC-1β), nuclear respiratory factor-1 (NRF-1), and mitochondrial transcription factor-A (TFAM), all increase within the first week of cold acclimation of sticklebacks, coinciding with an increase in protein carbonylation, increase in SOD mRNA levels and increase in glutathione levels (Orczewska et al., 2010). Previous studies in mammals have shown that PGC-1 family members increase in response to ROS and induce the expression of aerobic metabolic genes and antioxidants, including SOD (Irrcher et al., 2009; St-Pierre et al., 2006). Although ROS may stimulate metabolic remodeling in liver, ROS do not likely mediate mitochondrial biogenesis in oxidative muscles of sticklebacks.

1.7 Conclusions

We have demonstrated that oxidative stress is tissue-specific and occurs in a transient manner during cold acclimation of sticklebacks. Overall, our results are consistent with others that have shown increases in oxidative stress in liver tissue but not muscle during cold acclimation of fishes (Grim et al., 2010; Heise et al., 2007b). Importantly, our data also indicate that changes in mRNA levels of antioxidants do not always reflect changes in levels of proteins. Consequently, conclusions concerning changes in oxidative stress based on mRNA levels of antioxidants alone should be interpreted with caution. Additionally, SOD activity may increase during cold acclimation to offset the depressive effects of cold temperature on the catalytic rate of the enzyme, rather than to defend against elevated levels of ROS production. Only in oxidative muscle did we observe increases in SOD activity sufficient to protect against potentially elevated levels of ROS production at cold temperature, which may result from increases in mitochondrial density.

Overall, our results suggest that initial decreases in temperature during cold acclimation increase ROS production in liver, and that increases in mitochondrial content during cold acclimation may warrant elevations in antioxidant levels in oxidative muscle. It is unknown if more gradual changes in temperature, as might be experienced in natural environments, might avoid increases in oxidative stress in liver. Future studies will address this question, as well as whether or not ROS stimulate metabolic remodeling in liver during cold acclimation.

1.8 Acknowledgements

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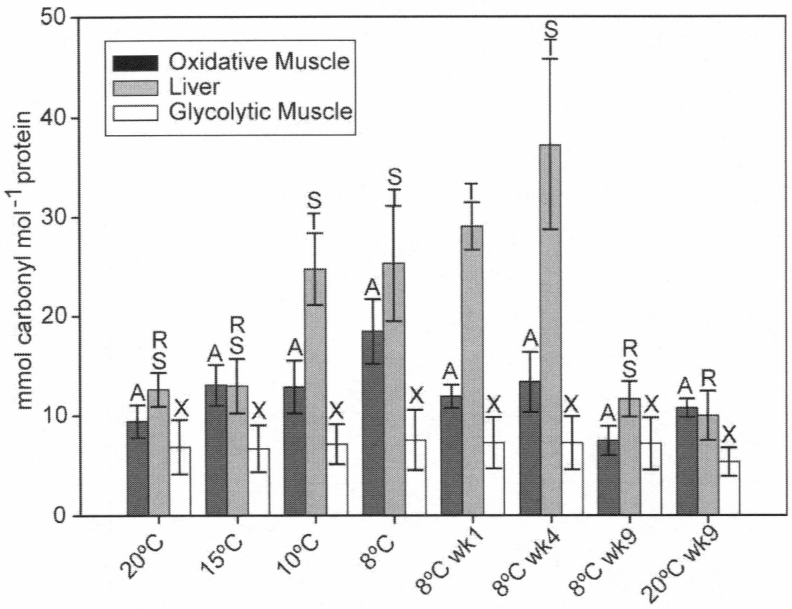

Figure 1.1. Levels of protein carbonyls in liver, oxidative muscle and glycolytic muscle. Protein carbonyl levels were quantified by derivatizing carbonyl groups with DNPH. Values are presented as means ± s.e.m. Values that do not share the same letter within a sequence are significantly different ($P < 0.05$) (N=7-10).

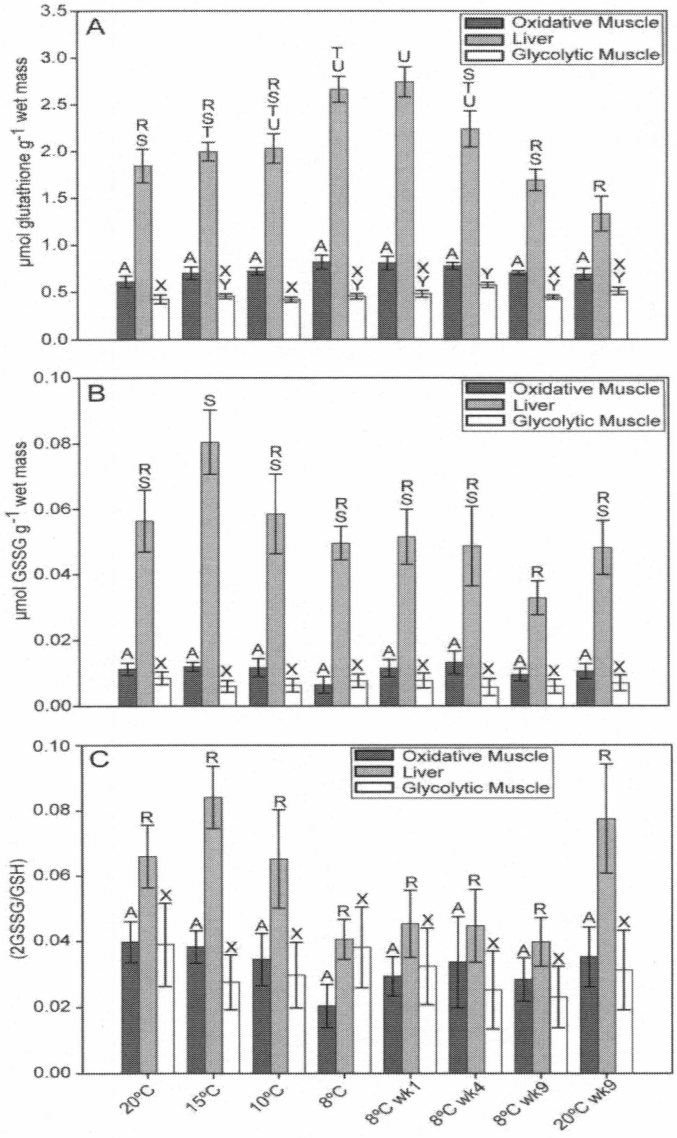

Figure 1.2. Glutathione content in liver, oxidative muscle and glycolytic muscle. Levels of total glutathione (A), oxidized glutathione (B), and the ratio of oxidized to reduced glutathione (C) were measured using the DTNB-GSSG recycling assay described by Anderson (1985). Values are presented as means ± s.e.m. Values that do not share the same letter within a sequence are significantly different ($P < 0.05$) (N=7-8).

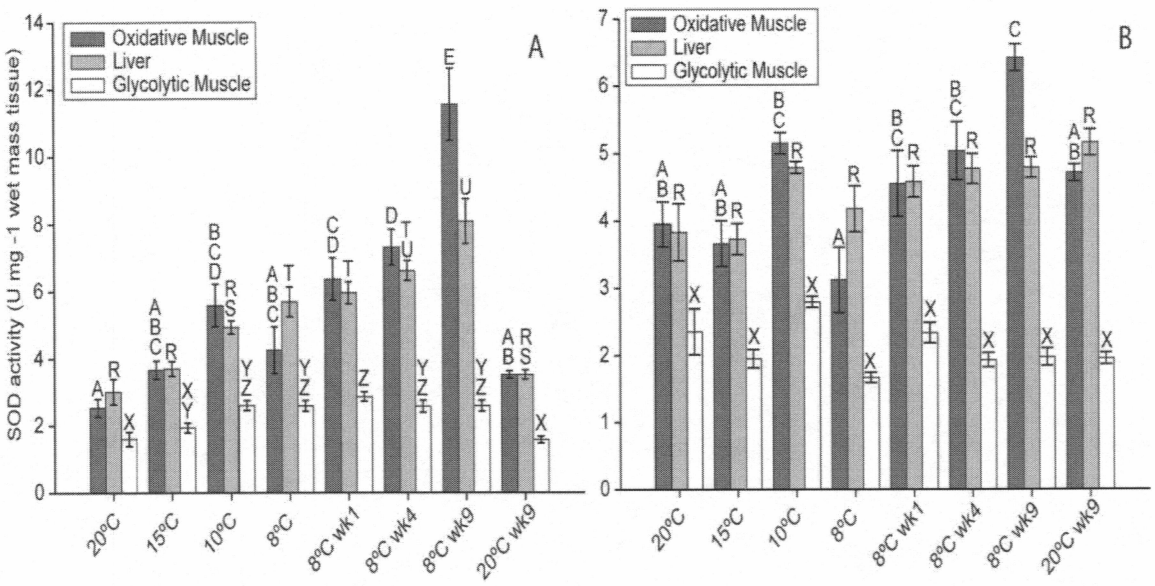

Figure 1.3. Activity of SOD in liver, oxidative muscle and glycolytic muscle. SOD activity was measured at the 14°C (A) and at the acclimation temperature of each individual (B). Values are presented as means ± s.e.m. Values that do not share the same letter within a sequence are significantly different ($P < 0.05$) (N=5).

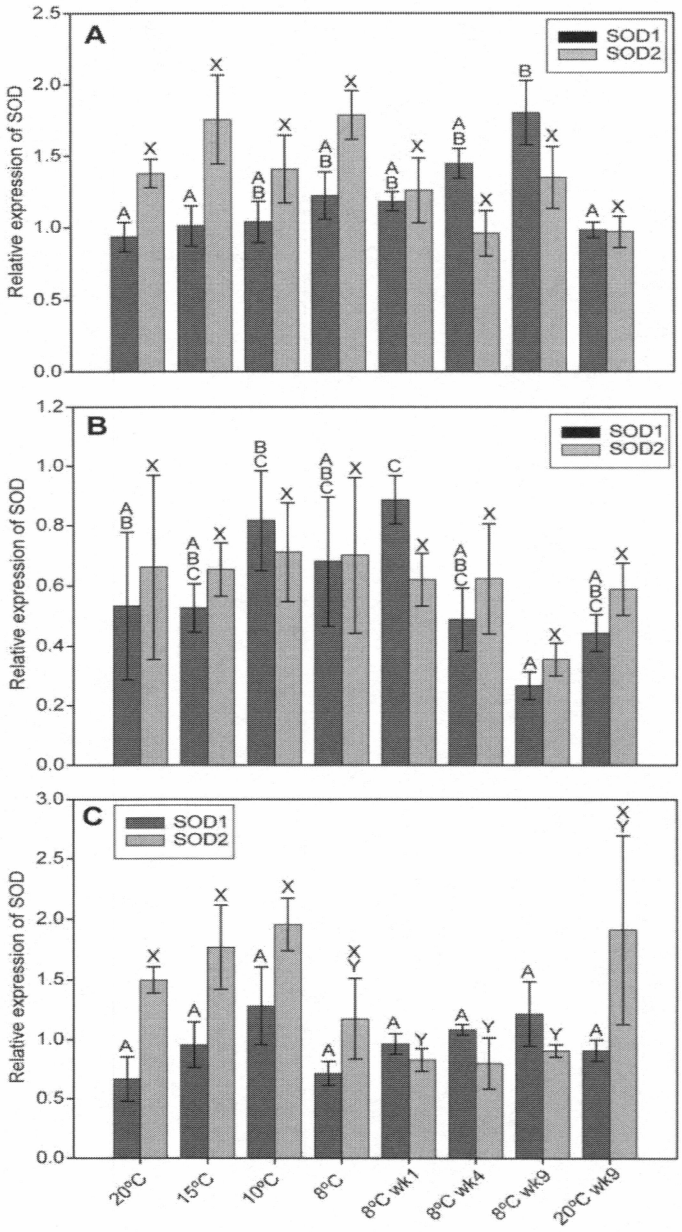

Figure 1.4. Transcript levels of SOD1 and SOD2 during cold acclimation. SOD expression was quantified using qRT-PCR and normalized to EF-1α in oxidative (A) and glycolytic muscles (C) and 18S in liver (B). Values are presented as means ± s.e.m. Values that do not share the same letter within a sequence are significantly different ($P < 0.05$) (N=7-8).

Table 1.1 *Primers used for quantitative real-time PCR*

Symbol	Amplicon size (bp)	Primer (5'-3')	
EF-1α	53	Forward	CGTCTACAAAATCGGAGGTATTGG
		Reverse	GTCTCAACACGGCCGACTG
18S	51	Forward	CCGAGTCGGGAGTGGGTAAT
		Reverse	ACCACATCCAAGGAAGGCAG
SOD1	51	Forward	TGGCCAGCATTCCATCATT
		Reverse	TCTGCTTTCTCGTGGATCACC
SOD2	51	Forward	CACTCGCAAAGGGAGATGTGA
		Reverse	TCAGAGCGGCTGGAGGG

Table 1.2 *Effect of cold acclimation on physical characteristics of G. aculeatus*

Time point (N)	Length (cm)	Mass (g)	Condition Factor	Pectoral-to-Body-Mass Ratio	Hepatosomatic Index
20°C (24)	$5.43 \pm 0.10^{\ddagger b}$	1.57 ± 0.08	0.98 ± 0.04	0.020 ± 0.001	0.057 ± 0.002
15°C (24)	$5.53 \pm 0.08^{\ddagger \alpha}$	1.66 ± 0.08	0.98 ± 0.03	0.021 ± 0.001	0.057 ± 0.002
10°C (24)	$5.39 \pm 0.08^{\ddagger b}$	1.67 ± 0.04	$1.08 \pm 0.03^{\ddagger * \alpha}$	0.019 ± 0.001	0.059 ± 0.002
8°C (24)	$5.28 \pm 0.13^{\ddagger b}$	1.53 ± 0.09	1.03 ± 0.03	0.019 ± 0.001	0.056 ± 0.002
1 week 8°C (24)	$5.57 \pm 0.07^{\ddagger \alpha}$	1.72 ± 0.08	0.99 ± 0.02	0.018 ± 0.001	0.063 ± 0.004
4 week 8°C (26)	5.69 ± 0.07	$1.83 \pm 0.05^{*b}$	1.00 ± 0.02	0.019 ± 0.001	0.063 ± 0.002
9 week 8°C (24)	$5.75 \pm 0.10^{*\alpha}$	1.66 ± 0.04	0.99 ± 0.03	0.019 ± 0.001	0.060 ± 0.001
9 week 20°C (24)	$5.88 \pm 0.12^{*\alpha}$	$1.76 \pm 0.06^{*a}$	0.98 ± 0.04	0.018 ± 0.001	0.059 ± 0.003

Values are means ± s.e.m.; N, no. of fishes. Condition factor = 100 * [body mass (g) * length $(cm)^{-3}$]. Hepatosomatic index = 100 * [liver mass (g) * body mass $(g)^{-1}$]. (*)Significantly different from animals at 20°C and (‡) significantly different from animals at 20°C wk 9; (α) $P < 0.05$, (b) $P<0.01$.

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Conclusions

The overarching goal of this research was to determine if oxidative stress increases during cold acclimation in threespine sticklebacks and if so, if it occurred within the time frame necessary to stimulate metabolic remodeling. I found that oxidative stress is tissue specific and only occurs in liver tissue during cold acclimation. Overall this result was somewhat surprising. Although we hypothesized that liver tissue would experience oxidative stress during cold acclimation, we also assumed oxidative muscle, which is aerobically very active, would be particularly vulnerable to oxidative stress. Our hypothesis that ROS may be responsible for inducing mitochondrial biogenesis in oxidative muscle in response to cold acclimation was not supported by these results.

One of the more interesting aspects of this research is the evidence we found for the potential mechanism for increases in ROS production in response to cold acclimation. We narrowed the cause down to increases in the cellular PO_2 and/or the disruption of the electron transport chain due to decreases in membrane fluidity. Two experiments could differentiate between these two possibilities. Fishes could be cold acclimated while oxygen concentrations were maintained at the equivalent levels between cold- and warm-acclimated fishes. Increases in ROS production could then be attributed to decreases in membrane fluidity. In another experiment, temperature could be maintained at a constant level at the warm-acclimation temperature, while oxygen concentration was increased to levels experienced by cold- acclimated animals. In this experiment, any increases in ROS production would be attributed to an increase in oxygen concentration.

Although I found little evidence that ROS play a role in mitochondria biogenesis in oxidative muscle, I did determine that levels of oxidized proteins and antioxidants increased in liver during cold acclimation of sticklebacks and that this increase occurred within the time frame that changes in aerobic metabolism occurred, suggesting ROS may be involved in triggering metabolic remodeling in liver during cold acclimation. Future studies would be needed to verify this link. One potential way of determining the importance of ROS for increasing aerobic metabolism would be to inject fish with antioxidants during cold acclimation. Increasing levels of antioxidants would potentially block the signaling cascade induced by ROS. If aerobic metabolic remodeling still occurred in response to cold acclimation with this treatment, ROS could be eliminated as the inducer of this pathway.

This research greatly increases our knowledge of oxidative stress in response to cold acclimation of fish. By analyzing a number of tissues throughout the acclimation period, this study explains some of the variation in results among previous studies.