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RESEARCH ARTICLE

Oxidative stress is transient and tissue specific during cold acclimation of threespine stickleback

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SUMMARY

Linkages between cold acclimation and oxidative stress in fishes are unclear and contradictory results have been published. We sought to determine whether oxidative stress occurs during cold acclimation of threespine stickleback (*Gasterosteus aculeatus*), and, if so, when it occurs and whether it varies among tissues. Fish were warm (20°C) or cold (8°C) acclimated for 9 weeks, and harvested during acclimation. Oxidative stress was assessed in oxidative and glycolytic muscles and liver by measuring levels of protein carbonyls and glutathione, and the activity and transcript levels of superoxide dismutase (SOD). Protein carbonyl levels increased in liver after 1 week at 8°C and then decreased after week 4, and remained unchanged in glycolytic and oxidative muscle. Glutathione levels increased in liver on day 3 of cold acclimation and may minimize oxidative stress later during acclimation. When measured at a common temperature, the activity of SOD increased in oxidative and glycolytic muscles on day 2 of cold acclimation, and on day 3 in liver, and remained elevated in all tissues compared with warm-acclimated animals. When measured at the acclimation temperature, the activity of SOD was significantly higher only at week 9 in oxidative muscle of cold-acclimated stickleback compared with warm-acclimated fish, and remained constant in glycolytic muscle and liver. Increased SOD activity in oxidative muscle may be required to prevent oxidative stress brought about by increased mitochondrial density. In both muscle and liver, SOD activity increased independently of an increase in transcript level, suggesting post-translational modifications regulate SOD activity.

Key words: cold temperature, fish, oxidative stress.

INTRODUCTION

Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the activity of antioxidants, which protect biological macromolecules from oxidation (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 oxidative phosphorylation (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 (Ali et al., 2010; Heise et al., 2007; Heise et al., 2006; O'Kane et al., 1996).

Ectothermic organisms, having body temperatures that conform to environmental temperatures, may be particularly vulnerable to oxidative stress as temperature decreases, for a number of reasons (Abele and Puntarulo, 2004). First, decreases in temperature decrease mitochondrial membrane fluidity (Hazel, 1995), which may disrupt the transfer of electrons, thereby increasing the production of ROS. Second, alterations in membrane composition during homeoviscous adaptation may increase susceptibility to oxidative stress (reviewed by Crockett, 2008). In order to maintain membrane fluidity at cold temperature, ectothermic organisms increase the relative proportion of polyunsaturated fatty acids (PUFAs) in the membrane (Crockett and Hazel, 1995). One drawback of this process is that unsaturated fatty acids are more prone to oxidation than saturated ones (Cosgrove et al., 1987), and the oxidation of PUFAs results in the formation of a lipid peroxyl radical, which further propagates lipid peroxidation (Halliwell, 1999). Third, oxidative stress may increase because of enhanced oxygen solubility at cold temperature, which increases 1.4-fold between 20°C and 5°C at sea level (Weiss, 1970).

Although several studies have measured oxidative stress during cold acclimation of fishes, the results have been equivocal. In some studies, cold acclimation led to an increase in oxidative stress and increased levels of antioxidants. Transcript levels of CuZn superoxide dismutase (SOD1) and MnSOD (SOD2) and levels of oxidized proteins increased in skeletal muscle of zebrafish (*Danio rerio*) acclimated to 18°C compared with warm-acclimated fish at 28°C (Malek et al., 2004). Transcript levels of several antioxidant enzymes were induced in liver and glycolytic muscle of cold-acclimated carp (*Cyprinus carpio*) (Gracey et al., 2004). Levels of oxidized lipids increased in livers of gilthead sea bream (*Sparus aurata)* in response to cold acclimation (Ibarz et al., 2010). The activity of the 20S proteasome increased in white muscle of cold-acclimated wolffish (*Anarhichas minor*), also suggesting increased protein oxidation at cold temperature (Lamarre et al., 2009). In contrast, total glutathione levels were higher in liver and muscle of warm-acclimated killifish (*Fundulus heteroclitus macrolepidotus*) compared with cold-acclimated fish, which might be due to higher metabolic activity at warmer temperatures (Leggatt et al., 2007). However, levels of oxidized glutathione were higher in liver of cold-acclimated killifish compared with warm-acclimated ones, suggesting cold temperature might increase oxidative stress in liver

but not muscle (Leggatt et al., 2007). Similarly, there was no change in the activity of SOD or catalase in glycolytic or cardiac muscle of killifish or bluegills (*Lepomis macrochirus*) in response to cold acclimation (Grim et al., 2010).

The inconsistent results among these studies may be due to differences in cold acclimation regimes, tissues analyzed and/or assays used to assess oxidative stress. Oxidative stress may increase early during cold acclimation due to an increase in oxygen solubility or a decrease in mitochondrial membrane fluidity. Alternatively, oxidative stress might increase later during cold acclimation if it is caused by membrane remodeling and an increase in PUFAs. Moreover, the susceptibility to oxidative stress may not be equivalent among different tissue types. Levels of free iron, hemoproteins and PUFAs, all of which promote ROS formation, may differ among tissues. Similarly, levels of antioxidants, which protect against ROS, may also vary and impact the extent of oxidative stress during cold acclimation (Perez-Campo et al., 1993). To accurately characterize oxidative stress, both rates of production of ROS and antioxidant defenses must be considered.

We sought to determine whether oxidative stress occurs during cold acclimation of threespine stickleback (*Gasterosteus aculeatus*) and, if so, when it occurs during acclimation and whether it differs among different tissue types. We chose threespine stickleback because they inhabit a wide geographic and temperature range, which extends from southern California to Alaska along the west coast of North America. In lakes of central Alaska, where sticklebacks were captured for this study, temperatures range between 4 and 20°C. Oxidative stress was assessed by measuring levels of oxidized proteins, glutathione, and activity and transcript levels of SOD. Measurements were made throughout cold acclimation in liver, oxidative muscle and glycolytic muscle. This is the first study we are aware of to examine changes in oxidative stress in fish throughout the time course of cold acclimation and in multiple tissues. Our results indicate that oxidative stress occurs within the first week of cold acclimation in stickleback liver but is reduced after 4 weeks of cold acclimation, and does not occur in oxidative or glycolytic muscle.

MATERIALS AND METHODS

Animal care and experimental design

All protocols were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (135490-7). Threespine stickleback (*G. aculeatus* L.) were captured in Kashwitna Lake, AK (61°50′N, 150°00′W) in September 2007 and 2008 using minnow traps. The water temperature at the time animals were captured was 16.5°C in 2007 and 12.6°C in 2008, and ranged between 4°C and 20°C throughout the year. Fish were transported to the University of Alaska Fairbanks and maintained in 114 l aquaria containing 0.35% Instant Ocean Sea Salt® (Spectrum Brands, Inc., Madison, WI, USA). Tanks were housed in an environmental chamber, which regulated temperature and day length. Fish were maintained at 20°C for 12 weeks on a 10 h light, 14 h dark cycle and fed twice daily an alternating diet of blood worms and brine shrimp. After 12 weeks at 20°C, the first group of warm-acclimated fish was harvested. Remaining animals were either cold acclimated to 8°C or maintained at 20°C for an additional 9 weeks. Fish were acclimated to 8°C by decreasing the temperature in the environmental chamber from 20°C to 15°C on day one, to 10°C on day 2, and to 8°C on day 3. Between 26 and 40 animals were harvested each day, prior to decreasing the temperature and after 1, 4 and 9 weeks at 8°C. A second group of warm-acclimated fish was harvested after an additional 9 weeks at 20°C, which was at the end of the cold-acclimation period as described previously (Orczewska et al., 2010). Animals were harvested prior to feeding and at the same time each day to avoid the potentially confounding effects of feeding and circadian rhythms. Animals were killed by immersion in liquid nitrogen and stored at –80°C. All measurements were made in liver, oxidative pectoral adductor muscle and glycolytic skeletal muscle in animals harvested throughout the acclimation period.

Protein carbonyl levels

Tissues were homogenized in 9 volumes of ice-cold 50 mmol l^{-1} K_2HPO_4/KH_2PO_4 buffer, pH 7.8, using a ground-glass homogenizer. Tissue homogenate was treated with 10% streptomycin sulfate (4.2:1) and incubated for 15 min at room temperature to precipitate DNA. Samples were then centrifuged for 10 min at 9100 ***g***. Supernatant was decanted and protein precipitated with ice-cold acetone at –20°C for 30 min. Proteins were pelleted by centrifugation at 16,000 ***g*** for 15 min, washed with 80% acetone and resuspended in HPLC running buffer (6 mol l^{-1} guanidinium HCl, 0.5 mol l^{-1} KPO_4, pH 2.5). Samples were divided into four equal aliquots. Two aliquots were derivitized with a final concentration of 7 mmol l^{-1} 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 aliquots, which were then 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 (Waters, Milford, MA, USA). 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 described previously (Levine et al., 1994). The extinction coefficients used were 22,000 l mol^{-1} cm^{-1} for the hydrazone and 50,000 l mol^{-1} cm^{-1} for protein.

Glutathione levels

Tissues were homogenized in 9 volumes of 5% 5-sulfosalicylic acid and centrifuged for 5 min at 10,000 ***g***. Total glutathione [oxidized (GSSG) + reduced (GSH)] was measured in the resulting supernatant using the DTNB–GSSG recycling assay as described by Anderson (Anderson, 1985). Briefly, the reaction mixture, containing 115 mmol l^{-1} $NaPO_4$, 5 mmol l^{-1} Na_4-EDTA, 0.6 mmol l^{-1} 5,5-dithiobis 2-nitrobenzoic acid (DTNB) and 210 mmol l^{-1} NADPH, pH 7.2, was incubated at 20°C for 12 min. Supernatant and glutathione reductase at a final concentration of 1.33 U ml^{-1} were added to initiate the reaction. The formation of reduced DTNB was followed at 412 nm for 7 min using a Perkin Elmer Lambda 25 spectrophotometer (Downers Grove, IL, USA). Total glutathione was determined from a standard curve with known concentrations of glutathione (Sigma-Aldrich, St Louis, MO, USA). Each sample was measured in triplicate.

GSSG was measured by derivatizing reduced GSH with 2-vinylpyridine: 2 µl of 98% 2-vinylpyridine was added to 100 µl of supernatant and mixed well; 6 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 concentration was 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 known concentrations of GSSG (Sigma-Aldrich). Measurements were made in triplicate in liver and glycolytic muscle, and in duplicate or triplicate in oxidative muscle.

Enzyme activity

Samples were measured in triplicate at 14°C and at the acclimation temperature of each fish (20°C, 15°C, 10°C or 8°C) using a Perkin Elmer Lambda 25 spectrophotometer equipped with a temperature-controlled circulating water bath.

Activity of superoxide dismutase (EC 1.15.1.1)

The activity of SOD was measured using the xanthine oxidase/cytochrome *c* assay (Crapo et al., 1978). Tissues were homogenized in 9 volumes of ice-cold 50 mmol l^{-1} K_2HPO_4/KH_2PO_4 buffer, pH 7.8, using a ground-glass homogenizer. The final reaction mixture contained 50 mmol l^{-1} K_2HPO_4/KH_2PO_4, pH 7.8, 0.01 mmol l^{-1} acetylated cytochrome *c*, 0.05 mmol l^{-1} xanthine and 0.01 mmol l^{-1} KCN. Background activity was monitored for 1 min at 550 nm. The reaction was initiated by adding xanthine oxidase at a concentration sufficient to achieve an increase in the absorbance rate of 0.02 OD min^{-1} at 550 nm. The concentration of xanthine oxidase required to achieve this rate varied and was determined each day prior to measuring SOD activity (Crapo et al., 1978). Homogenate was added to the reaction at a concentration sufficient to inhibit the reduction of cytochrome *c* by 50±10% and the rate of reduction of cytochrome *c* was monitored at 550 nm for 5 min. The same individuals used for measuring levels of protein carbonyls were also used for measuring the activity of SOD.

Activity of glucose 6-phosphate dehydrogenase (EC 1.1.1.49)

Tissues were homogenized by hand in a ground-glass homogenizer in 9 volumes of ice-cold phosphate buffer (100 mmol l^{-1} K_2HPO_4/KH_2PO_4, pH 7.0). Homogenates were centrifuged at 16,100 ***g*** for 30 min at 4°C and protein concentration of the supernatant was determined using the bicinchoninic acid (BCA) protein assay with bovine serum albumin as a standard (Thermo Fisher Scientific, Rockford, IL, USA). The activity of glucose 6-phosphate dehydrogenase (G6PD) was measured as described by Moreland and Sidell (Moreland and Sidell, 1981). The final reaction mixture contained 45 mmol l^{-1} imidazole, pH 7.7, 3.3 mmol l^{-1} glucose 6-phosphate and 0.2 mmol l^{-1} $NADP^+$. Preliminary experiments verified that substrate concentrations were saturating for all tissues. Background activity was measured for 5 min in the absence of the initiating substrate glucose 6-phosphate. Reaction progress was monitored by following the production of NADPH at 340 nm for 5 min, following the addition of substrate; 1 unit (U) of activity is defined as 1 μmol of product formed per min.

Gene expression

RNA was isolated from 10–30 mg of tissue using the RNeasy fibrous tissue minikit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol except that liver tissue was not treated with proteinase K and slight modifications were made to the protocol to improve 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 (Thermo Fisher Scientific) by measuring absorbance at 260 nm. RNA purity was verified by measuring the ratio of absorbance at 260 nm and 280 nm, and 230 nm and 260 nm. Only samples with values >2.0 and 1.8, respectively, were used for quantifying transcript levels. RNA integrity was verified by agarose electrophoresis: 1 μl of RNA was mixed with loading buffer (5% glycerol, 0.04% Bromophenol Blue, 0.1 mmol l^{-1} 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 mmol l^{-1} $MgCl_2$, 0.5 μl random hexamers, 2 mmol l^{-1} dNTPs, 4 U RNase inhibitor, 37.5 U reverse transcriptase and 200 ng RNA. Parallel samples were prepared omitting reverse transcriptase so that we could detect the presence of contaminating genomic DNA when measuring transcript levels. cDNA was stored at –80°C.

Gene-specific primers were designed using sequence information obtained from Ensembl (www.ensembl.org) and the software Primer Express (Applied Biosystems) (Table 1). At least one primer from each primer set was designed to anneal 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) with an ABI 7900HT sequence detection system (Applied Biosystems). Each sample was measured in triplicate in a 20 μl reaction volume containing 5 ng cDNA for target genes or 0.05 ng for 18S rRNA, 0.3 mmol l^{-1} of each forward and reverse primer, and 10 μl Power SYBR® Green Master Mix (Applied Biosystems). Reactions were run at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s 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, one in which the reverse transcriptase was omitted during cDNA synthesis, and 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 transcript levels of 18S rRNA in liver and EF-1α in oxidative and glycolytic muscles, which were

Table 1. Primers used for quantitative real-time PCR

Gene	Amplicon size (bp)	Primer	Sequence (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	TCAGAGCGGGCTGGAGGG

EF-1α, elongation factor-1α; SOD1, CuZn superoxide dismutase; SOD2, MnSOD.

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

Time point (*N*)	Length (cm)	Mass (g)	Condition factor	Pectoral:body mass ratio	Hepatosomatic index
20°C (28)	5.28±0.07†	1.45±0.06†	0.97±0.02	0.018±0.001	5.03±0.24
15°C (28)	5.41±0.07†	1.54±0.06†	0.97±0.02	0.019±0.001	5.05±0.21
10°C (28)	5.35±0.06†	1.60±0.05	1.04±0.02	0.018±0.001	5.25±0.22
8°C (27–28)	5.33±0.08†	1.63±0.07	1.07±0.03	0.019±0.001	5.19±0.26
8°C, 1 week (28)	5.38±0.06†	1.63±0.06	1.04±0.02	0.018±0.001	6.17±0.27*
8°C, 4 weeks (28)	5.71±0.06*	1.91±0.05*	1.03±0.02	0.018±0.001	6.53±0.22*,†
8°C, 9 weeks (27–28)	5.81±0.07*	1.89±0.07*	0.96±0.03	0.017±0.001	5.89±0.16
20°C, 9 weeks (28)	5.73±0.07*	1.81±0.06*	0.97±0.02	0.018±0.001	5.42±0.21

Values are means ± s.e.m.; *N*, number of fish.
Condition factor=100×[body mass×length^{-3}], where mass is in g and length is in cm.
Hepatosomatic index=100×[liver mass×body mass^{-1}], where mass is in g.
*Significantly different from animals at 20°C; †significantly different from animals at 20°C for 9 weeks (P<0.05).

previously identified as stable housekeeping genes (Orczewska et al., 2010).

Statistical analyses

Significant differences in physical characteristics, glutathione levels and enzyme activity between fish harvested at different time points were determined using a one-way ANOVA followed by a *post hoc* Tukey's honestly significant difference (HSD) test. Data were log or reciprocally transformed as necessary to maintain assumptions of normality, which was evaluated by residual and Q–Q plots. Significant differences in measurements of protein carbonyls and transcript levels of SOD between fish harvested at different time points were determined using a Kruskal–Wallis test followed by a *post hoc* Tukey's HSD test. Significant differences between tissues at each time point were determined using an ANOVA followed by a *post hoc* Tukey's HSD test. Significance was set at P<0.05. All data are presented as means ± s.e.m.

Warm-acclimated fish were harvested at only two time points during acclimation, so we compared measurements made in cold-acclimated fish harvested between day 1 (15°C) and week 4 with measurements made in warm-acclimated fish harvested at the start of the acclimation period (20°C). Measurements made in cold-acclimated fish harvested between week 4 and week 9 were compared with measurements made in warm-acclimated fish harvested at week 9, and measurements made in cold-acclimated fish harvested at week 4 were compared with both groups of warm-acclimated animals, as this time point was approximately halfway between the two time points at which we harvested each group of warm-acclimated fish.

RESULTS

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 following 9 weeks of cold acclimation. There was no effect of temperature on any of these parameters; all measurements were equivalent between cold- and warm-acclimated fish at week 9 (P>0.05).

Protein carbonylation

We measured protein carbonyl levels in warm- and cold-acclimated stickleback to determine whether ROS production increased during cold acclimation. After 1 week of cold acclimation, protein carbonyl levels increased 2.3-fold in liver compared with animals at 20°C, from 12.6±1.7 mmol mol^{-1} protein to 29.0±2.4 mmol mol^{-1} protein (P<0.05) (Fig. 1). Protein carbonyl levels remained elevated through week 4 of cold acclimation compared with fish at 20°C for 9 weeks. By week 9 of cold acclimation, levels of protein carbonyls decreased and were equivalent to those of warm-acclimated fish at 20°C for 9 weeks (P>0.05). Protein carbonyl levels did not change significantly in oxidative or glycolytic muscles in response to cold acclimation (P>0.05).

Protein carbonyl levels were significantly higher in liver than in glycolytic and oxidative muscles in stickleback at 10°C, 8°C for 1 week, and 8°C for 4 weeks (P<0.05). There were no significant differences in protein carbonyl levels between glycolytic and oxidative muscles.

Glutathione content

Glutathione was measured to determine whether the cellular redox environment changed during cold acclimation. 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} tissue wet mass in fish at 20°C to 2.90±0.22 µmol g^{-1} tissue wet mass on day 3 (P<0.05) (Fig. 2A). Glutathione levels remained elevated through week 4 of cold acclimation compared with fish at 20°C for 9 weeks, but then decreased and were equivalent for warm- and cold-acclimated fish

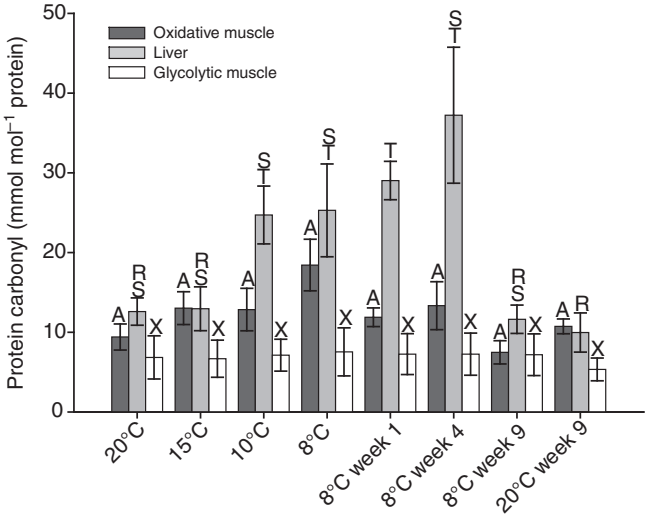

Fig. 1 Levels of protein carbonyls in oxidative muscle, liver and glycolytic muscle. Protein carbonyl levels were quantified by derivatizing carbonyl groups with dinitrophenyl hydrazine (DNPH); the resulting hydrazones were detected by HPLC. Values are presented as means ± s.e.m. Values with different letters within a sequence are significantly different (P<0.05) (N=7–10). For an explanation of sampling data points, see Materials and methods.

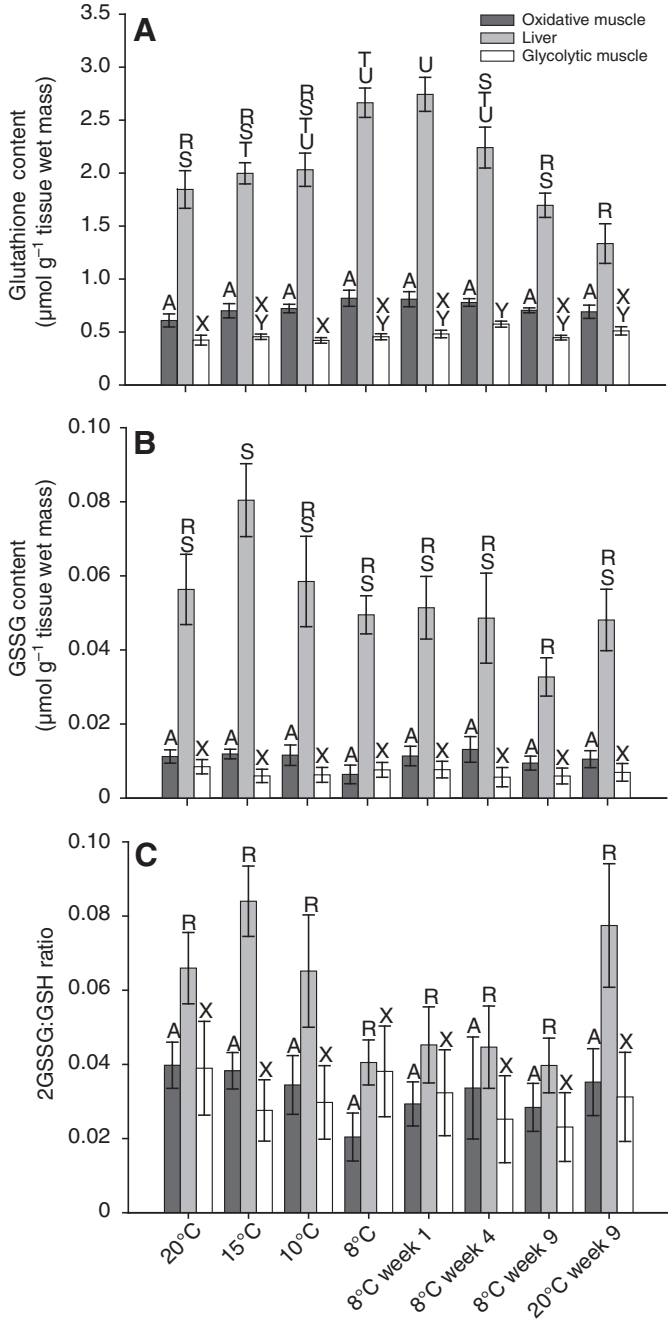

Fig. 2 Glutathione content in oxidative muscle, liver and glycolytic muscle. Levels of total glutathione (A), oxidized glutathione (GSSG) (B), and the ratio of oxidized to reduced glutathione (GSH) (C) were measured using the DTNB–GSSG recycling assay described by Anderson (Anderson, 1985). Values are presented as means ± s.e.m. Values with different letters within a sequence are significantly different (P<0.05) (N=7–8).

at week 9 (Fig. 2A). By week 4 of cold acclimation, total glutathione levels increased in glycolytic muscle compared with levels in fish at 20°C (day 0), but were unchanged compared with levels in fish held at 20°C for 9 weeks. Levels of total glutathione remained unchanged in oxidative muscle during cold acclimation and were always significantly higher in liver than in glycolytic and oxidative muscles.

Levels of oxidized glutathione and the ratio of oxidized to reduced glutathione did not change significantly in any tissues during cold acclimation (P>0.05) (Fig. 2B,C). However, levels of oxidized glutathione were always significantly higher in liver than in glycolytic and oxidative muscles, and the ratio of oxidized to reduced glutathione was higher in liver than in both muscle types in fish at 15°C (day 1) and at 20°C for 9 weeks (P<0.05) (Fig. 2B,C).

SOD activity

The activity of SOD was measured at the common temperature of 14°C to determine whether enzyme activity increased during cold acclimation. SOD activity was significantly higher in oxidative and glycolytic muscles of stickleback on day 2 (10°C) of cold acclimation compared with SOD activity in fish at 20°C (P<0.05) (Fig. 3A). By day 3 (8°C) of cold acclimation, SOD activity had become significantly elevated in liver compared with that in animals at 20°C (P<0.05) (Fig. 3A). SOD activity remained elevated in all tissues throughout the remainder of the cold acclimation period. The greatest increase in the activity of SOD occurred in oxidative muscle where, by week 9 of cold acclimation, SOD activity increased 3.3-fold from 3.5±0.1 to 11.5±1.0 U mg^{-1} tissue wet mass compared with that in animals held at 20°C for 9 weeks (P<0.05). There was a 2.3-fold increase in SOD activity in liver, from 3.5±0.1 to 8.0±0.6 U mg^{-1} tissue wet mass, and a 1.7-fold increase in SOD activity in glycolytic muscle, from 1.6±0.1 to 2.6±0.2 U mg^{-1} tissue wet mass, in fish at held 8°C for 9 weeks compared with that in fish held at 20°C for 9 weeks (P<0.05) (Fig. 3A). SOD activity was always significantly higher in liver and oxidative muscle than in glycolytic muscle at all time points, and higher in oxidative muscle than in liver at week 9 of cold acclimation (P<0.05).

We also measured the activity of SOD at the acclimation temperature of each animal to determine whether the observed increase in SOD activity completely compensated for the Q_{10} effect. When measured at the acclimation temperature of each animal, SOD activity increased in oxidative muscle during cold acclimation, and was 1.4-fold greater in oxidative muscle after 9 weeks of cold acclimation compared with that in fish held at 20°C for 9 weeks, indicating that there was overcompensation for the Q_{10} effect in oxidative muscle (P<0.05) (Fig. 3B). SOD activity remained constant in liver and glycolytic muscle during cold acclimation when measured at the acclimation temperature, indicating there was complete compensation for the Q_{10} effect in these tissues (P>0.05) (Fig. 3B).

SOD transcript levels

Transcript levels of SOD1 and SOD2 were measured in fish harvested during cold acclimation to determine whether the observed increases in SOD activity were transcriptionally regulated. The mRNA levels of SOD1 did not become significantly elevated in oxidative muscle until week 9 of cold acclimation (Fig. 4A). At this point, SOD mRNA levels had increased 1.8-fold compared with levels in fish held at 20°C for 9 weeks (P<0.05). By week 1 of cold acclimation, SOD1 mRNA levels had increased 1.8-fold in liver compared with levels in fish held at 20°C (P<0.05) (Fig. 4B). SOD1 mRNA levels then decreased in liver and by week 9 of cold acclimation transcript levels were equivalent between animals held at 8°C for 9 weeks and those held at 20°C for 9 weeks (P>0.05). The expression of SOD1 did not change during cold acclimation in glycolytic muscle (P>0.05) (Fig. 4C).

The expression of SOD2 did not change in liver or oxidative muscle in response to cold acclimation, but decreased in glycolytic muscle (Fig. 4). By week 1 of cold acclimation SOD2 mRNA expression was significantly lower in glycolytic muscle compared with that in fish at 20°C (day 0), and remained at this level

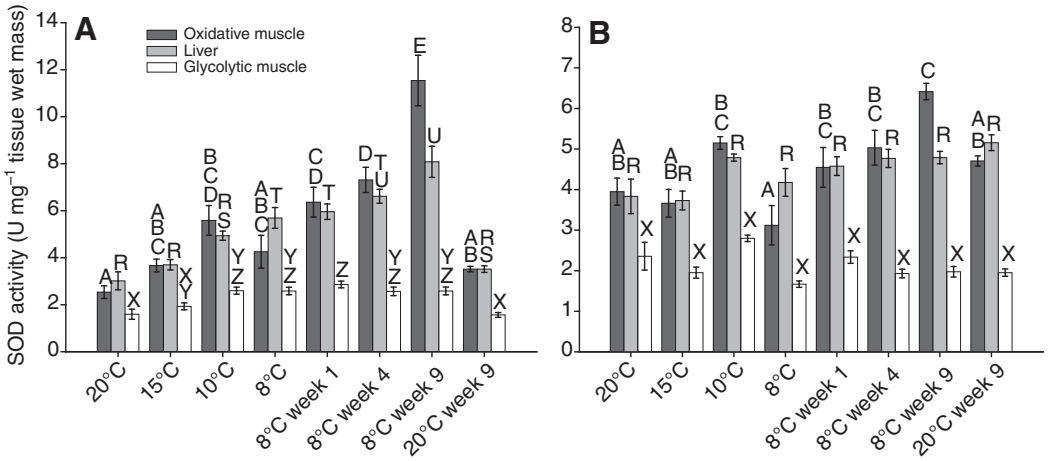

Fig. 3 Activity of SOD in oxidative muscle, liver and glycolytic muscle. SOD activity was measured at 14°C (A) and at the acclimation temperature of each fish (B). Values are presented as means ± s.e.m. Values with different letters within a sequence are significantly different (P<0.05) (N=5).

throughout the remainder of the cold acclimation period (P<0.05) (Fig. 4C).

G6PD activity

G6PD catalyzes the first rate-limiting reaction in the hexose monophosphate shunt pathway (HMP), and results in the reduction of $NADP^+$ to NADPH. Several previous studies of cold acclimation in fishes have noted an increase in the activity of G6PD and the HMP, which was attributed to an increased demand for NADPH for biosynthetic reactions (Campbell and Davies, 1978; Hochachka and Hayes, 1962; Seddon and Prosser, 1997; Stone and Sidell, 1981). Nevertheless, studies have shown that cold acclimation and acclimatization result in reduced levels of protein and lipid synthesis (Saez et al., 1982; Stone and Sidell, 1981). We hypothesized that G6PD might be up-regulated in response to cold acclimation to provide NADPH as a co-factor for glutathione reductase, which maintains glutathione in its reduced state and protects against oxidative stress. However, there was no significant increase in G6PD activity in oxidative muscle, liver or glycolytic muscle in response to cold acclimation when measured at a common temperature (P>0.05) (Fig. 5A,C). Nonetheless, G6PD activity was always significantly higher in liver than in muscle, and higher in oxidative muscle than in glycolytic muscle (P<0.05) (Fig. 5A,C).

When measured at the acclimation temperature of each animal, the activity of G6PD significantly decreased in oxidative muscle after 1 week at 8°C from 4.48±1.15 to 1.42±0.19 U g^{-1} protein (P<0.05) (Fig. 5B). After 9 weeks of acclimation G6PD activity in oxidative muscle was 2.7-fold higher in animals maintained at 20°C than in animals maintained at 8°C (P<0.05). In glycolytic muscle, G6PD activity significantly decreased on day 3 (8°C) of cold acclimation compared with that in animals at 20°C from 0.98±0.06 to 0.25±0.07 U g^{-1} protein (P<0.05) (Fig. 5B). G6PD activity remained significantly decreased in glycolytic muscle until after 9 weeks at 8°C, when activity levels were not significantly different from those in animals held at 20°C for 9 weeks (P<0.05). In liver tissue, G6PD activity significantly decreased on day 3 of cold acclimation (8°C) compared with that in animals at 20°C, from 106.53±27.18 to 38.67±4.17 U g^{-1} protein (P<0.05) (Fig. 5D). After 9 weeks of acclimation, G6PD activity was 2.2-fold higher in the liver of animals maintained at 20°C than in animals at 8°C (P<0.05) (Fig. 5D).

DISCUSSION

The results from our study indicate that cold acclimation leads to oxidative stress in threespine stickleback, but that this stress is transient and tissue specific. Levels of oxidized proteins increased only in liver, and only within the first 4 weeks of cold acclimation. This suggests that as fish acclimate to cold temperature, the production of ROS decreases and/or levels of antioxidants increase.

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. It is likely that either the disruption of electron transfer within the electron transport chain of mitochondria or an increase in oxygen concentration in the liver caused an increase ROS production, rather than it being due to alterations in membrane composition during homeoviscous adaptation. Although studies have shown that changes in the polar head groups of phospholipids occur within the first 4 days of cold acclimation in rainbow trout (*Salmo gairdneri*) kidney cells (Hazel and Landrey, 1988a), complete membrane remodeling, resulting in significant increases in long-chain PUFAs requires 10 days to 3 weeks (Hazel and Landrey, 1988b). We also cannot rule out the possibility that the rate of turnover of oxidized proteins might transiently decrease, resulting in an increase in levels of carbonylated proteins. Given that all tissues likely experience an increase in oxygen concentration and mitochondrial ROS production, and/or a decreased rate of protein turnover during cold acclimation, other properties of liver tissue must enhance its susceptibility to oxidative damage compared with muscle.

Oxidative stress is tissue specific

Overall, levels of the antioxidants SOD and glutathione, and the activity of G6PD, which supplies the co-factor NADPH for glutathione reductase, were higher in liver than in muscle. Although the majority of ROS are produced by mitochondria during oxidative phosphorylation, these differences in antioxidant defenses cannot be attributed solely to differences in aerobic metabolic capacity. The activity of cytochrome *c* oxidase (COX) is 2- to 3-fold higher in oxidative muscle than in liver during cold acclimation (Orczewska et al., 2010), yet the activity of SOD is equivalent between the two tissues, except in animals acclimated to 8°C for 9 weeks, when SOD activity is higher in oxidative muscle. Consequently, the SOD:COX ratio is approximately 2- to 3-fold higher in liver than in oxidative muscle, suggesting SOD detoxifies superoxide generated by sources in addition to the mitochondrial electron transport chain in liver. These extra-mitochondrial sources of ROS may contribute to elevations in oxidative damage during cold acclimation.

There are several potential sources of ROS in liver. Liver contains high levels of cytochromes P450, which are involved in fatty acid

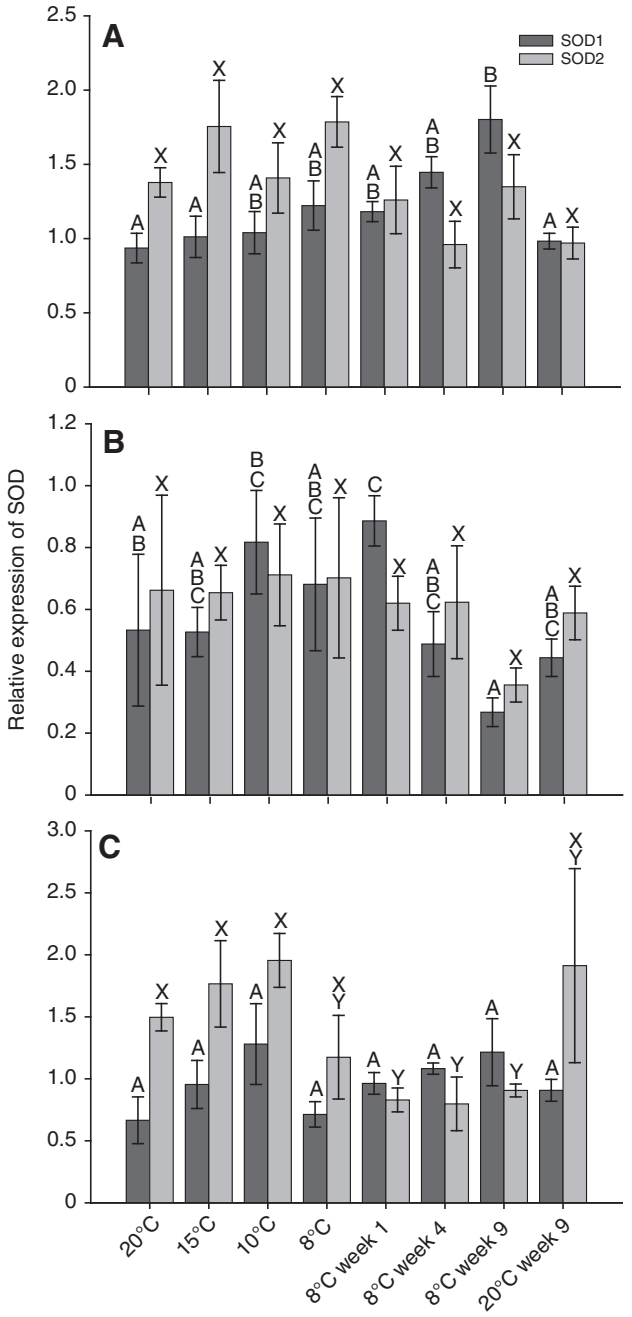

Fig. 4 Transcript levels of superoxide dismutase (SOD1) and SOD2 during cold acclimation. SOD expression was quantified using qRT-PCR and normalized to elongation factor-1α (EF-1α) in oxidative (A) and glycolytic muscles (C) and 18S in liver (B). Values are presented as means ± s.e.m. Values with different letters within a sequence are significantly different (P<0.05) (N=7–8).

metabolism and detoxify xenobiotics, generating ROS as a by-product of these reactions (Lieber, 1997; Myasoedova, 2008). Because of its high levels of cytochromes, liver contains more iron than muscle (Canli and Atli, 2003), and free iron reacts with hydrogen peroxide *via* the Fenton reaction, producing hydroxyl radicals, the most damaging form of ROS (McCord, 1998). Small amounts of ROS produced during cold acclimation may promote the release of heme from hemoproteins, and heme also catalyzes the formation of ROS *via* the Fenton reaction (Jeney et al., 2002; Vercellotti et al., 1994). High levels of cytochromes P450 and iron in liver may explain why most studies have found evidence of oxidative stress in liver in response to cold acclimation or acclimatization in fish, but not always in muscle (Gracey et al., 2004; Grim et al., 2010; Heise et al., 2007; Ibarz et al., 2010; Leggatt et al., 2007).

SOD activity increases in response to cold acclimation

SOD is a crucial antioxidant because it is the only antioxidant that detoxifies superoxide, dismutating it to hydrogen peroxide and oxygen (Fridovich, 1995). The two major isoforms of SOD are MnSOD (SOD2), located in the mitochondrial matrix, and CuZn-SOD (SOD1), located in the cytosol and intermembrane space of mitochondria (Zelko et al., 2002). We found that the activity of SOD increased by day 3 of cold acclimation (8°C) in liver, and continued to increase throughout the cold-acclimation period. Total glutathione also increased in liver by day 3 of cold acclimation (8°C). Increases in SOD activity and glutathione in liver may help reduce oxidative stress and levels of oxidized proteins after 4 weeks of cold acclimation. Mitochondrial membrane remodeling may also occur by this time, restoring membrane fluidity and reducing the formation of ROS. Studies of mitochondrial function in cold-acclimated rainbow trout have found that rates of oxidation of pyruvate and palmitoyl carnitine initially decrease, but increase between weeks 2 and 6 of cold acclimation, potentially corresponding with homeoviscous adaptation of mitochondrial membranes (Bouchard and Guderley, 2003).

Although we did not observe an increase in levels of oxidized proteins or total glutathione in oxidative or glycolytic muscle, SOD activity 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 increases 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. SOD activity, when measured at each acclimation temperature, was 1.4-fold higher in oxidative muscle of fish held at 8°C for 9 weeks compared with that in fish held at 20°C for 9 weeks. Additionally, the SOD:COX ratio was 1.5-fold higher in oxidative muscle of cold-acclimated fish than in warm-acclimated ones. Together, these data suggest there may be a greater potential for oxidative stress in oxidative muscle of cold-acclimated fish compared with warm-acclimated ones. Previously, we determined that mitochondrial volume density increases in oxidative muscle of threespine stickleback 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 (reviewed by Crockett, 2008).

An increase in SOD activity occurred independently of an increase in SOD mRNA levels in all tissues. The activity of SOD increased 1 week prior to an increase in mRNA levels of SOD1 in liver, and more than 9 weeks 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 CuZn-SOD (SOD1), the predominant form of SOD, is post-translationally regulated by the copper chaperone CCS (Casareno et al., 1998; Culotta et al., 1997). In response to an increase in ROS, CCS inserts a copper ion into the catalytic site of CuZn-SOD, in a reaction also requiring the formation of a disulfide bond between CCS and CuZn-SOD (Brown et al., 2004). Studies in yeast have shown that pools of inactive CuZn-SOD are rapidly activated by copper chaperones in response to oxidative stress, and prolonged

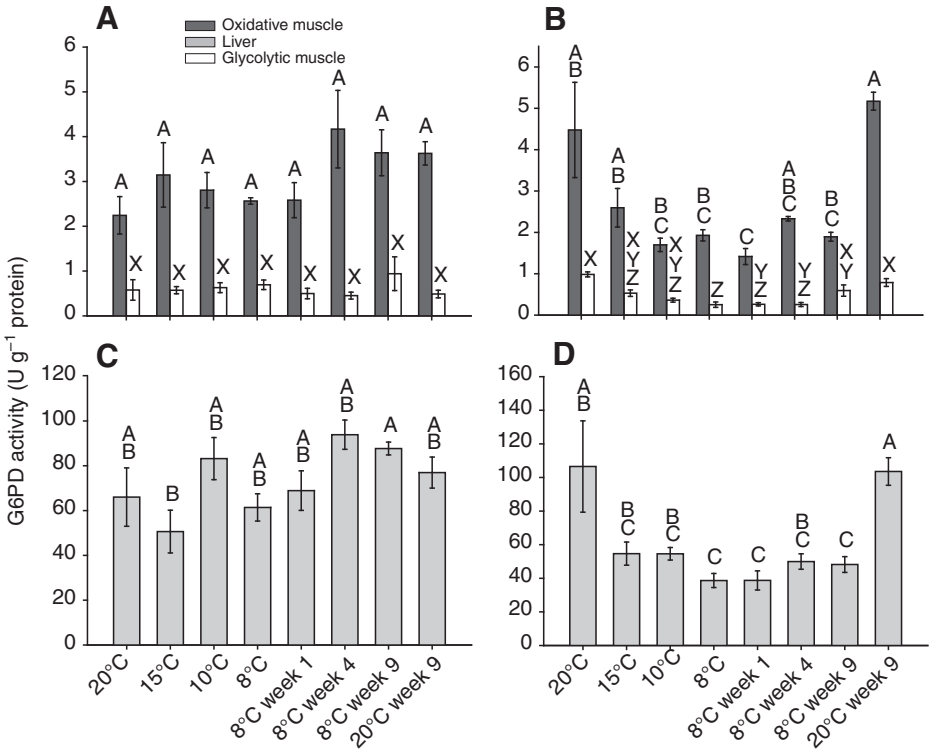

Fig. 5 Activity of glucose 6-phosphate dehydrogenase (G6PD) in oxidative and glycolytic muscles and in liver. G6PD activity was measured at 14°C (A,C) and at the acclimation temperature of each fish (B,D) in oxidative muscle, glycolytic muscle (A,B) and liver (C,D) during cold acclimation. Values are presented as means ± s.e.m. Values with different letters within a sequence are significantly different (P<0.05) (N=2–4).

exposure to oxidative stress leads to increased mRNA levels of SOD1 (Brown et al., 2004; Galiazzo and Labbe-Bois, 1993).

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 ROS production increases early during cold acclimation in liver, potentially triggering a signaling cascade leading to changes in metabolism. Although mitochondrial density does not increase in liver of threespine stickleback 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 increased within the first week of cold acclimation of stickleback (Orczewska et al., 2010), coinciding with an increase in protein carbonylation, SOD mRNA levels and glutathione levels. 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, suggesting a possible link between ROS and increases in the activity of aerobic metabolic enzymes in liver of stickleback during cold acclimation (Irrcher et al., 2009; St-Pierre et al., 2006). Additionally, NRF-1, which has been shown to be important for regulating the expression of aerobic metabolic genes in fish (LeMoine et al., 2008; McClelland et al., 2006) is redox sensitive and activated by oxidative stress (Piantadosi and Suliman, 2006; Suliman et al., 2003). Future studies will address whether or not ROS stimulate metabolic remodeling in liver during cold acclimation of stickleback.

CONCLUSIONS

Our results demonstrate that oxidative stress is tissue specific and transient during cold acclimation of stickleback. Overall, our results are consistent with others that have shown increases in oxidative stress in liver but not muscle during cold acclimation of fishes (i.e. Grim et al., 2010; Ibarz et al., 2010). Importantly, our data also indicate that changes in mRNA levels of antioxidant enzymes do not always reflect changes in the activity of proteins. The activity of SOD may be elevated by post-translational modifications rather than by an increase in gene transcription. Consequently, conclusions concerning changes in oxidative stress based on mRNA levels of antioxidant enzymes 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 an increase in SOD activity sufficient to protect against potentially elevated levels of ROS production at cold temperature, which may result from an increase in mitochondrial density.

LIST OF ABBREVIATIONS

18S	18S ribosomal RNA
COX	cytochrome *c* oxidase
DNPH	2,4-dinitrophenyl hydrazine
DTNB	dithiobis 2-nitrobenzoic acid
EF-1α	elongation factor-1α
G6PD	glucose 6-phosphate dehydrogenase
GSH	reduced glutathione
GSSG	oxidized glutathione
HMP	hexose monophosphate shunt pathway
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced form)
PUFA	polyunsaturated fatty acid
Q_{10}	temperature coefficient
qRT-PCR	quantitative real-time polymerase chain reaction
ROS	reactive oxygen species
SOD	superoxide dismutase

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