The Journal of Experimental Biology 213, 445-452

doi:10.1242/jeb.036939

Temperature acclimation alters oxidative capacities and composition of membrane lipids without influencing activities of enzymatic antioxidants or susceptibility to lipid peroxidation in fish muscle

J. M. Grim*, D. R. B. Miles and E. L. Crockett

Department of Biological Science, Irvine Hall, Ohio University, Athens, OH 45701, USA and Mt Desert Island Biological Laboratory, Old Bar Harbor Road, Salisbury Cove, ME 04672, USA

*Author for correspondence (grimj@ohio.edu)

Accepted 27 October 2009

SUMMARY

Cold acclimation of ectotherms results typically in enhanced oxidative capacities and lipid remodeling, changes that should increase the risk of lipid peroxidation (LPO). It is unclear whether activities of antioxidant enzymes may respond in a manner to mitigate the increased potential for LPO. The current study addresses these questions using killifish (*Fundulus heteroclitus macrolepidotus*) and bluegill (*Lepomis macrochirus*) acclimated to 5 and 25°C for 9 days and 2 months, respectively. Because the effects of temperature acclimation on pro- and antioxidant metabolism may be confounded by variable activity levels among temperature groups, one species (killifish) was also subjected to a 9-day exercise acclimation. Oxidative capacity of glycolytic (skeletal) muscle (indicated by the activity of cytochrome *c* oxidase) was elevated by 1.5-fold in killifish, following cold acclimation, but was unchanged in cardiac muscle and also unaffected by exercise acclimation in either tissue. No changes in citrate synthase activity were detected in either tissue following temperature acclimation. Enzymatic antioxidants (catalase and superoxide dismutase) of either muscle type were unaltered by temperature or exercise acclimation. Mitochondria from glycolytic muscle of cold-acclimated killifish were enriched in highly oxidizable polyunsatured fatty acids (PUFA), including diacyl phospholipids (total carbons:total double bonds) 40:8 and 44:12. Increased oxidative capacity, coupled with elevated PUFA content in mitochondria from cold-acclimated animals did not, however, impact LPO susceptibility when measured with C11-BODIPY. The apparent mismatch between oxidative capacity and enzymatic antioxidants following temperature acclimation will be addressed in future studies.

Key words: temperature acclimation, fish, cytochrome *c* oxidase, citrate synthase, antioxidant, superoxide dismutase, catalase, lipid peroxidation, TBARS, membrane composition, membrane unsaturation.

INTRODUCTION

Aerobic organisms face challenges associated with the formation of reactive oxygen species (ROS), including superoxide ($O_2^{\bullet-}$), hydroxyl radical (–OH) and the peroxyl radical ($ROO^{\bullet}$), which can damage biological molecules, including lipids, proteins and DNA (Halliwell and Gutteridge, 1999). Some ROS can initiate lipid peroxidation (LPO), a self-propagating process in which a peroxyl radical is formed when a ROS has sufficient reactivity to abstract a hydrogen atom from an intact lipid (Halliwell and Gutteridge, 1999). The peroxidized portion of a phospholipid exists in the non-polar interior of the membrane bilayer, disrupting hydrophobic lipid–lipid interactions and placing membrane integrity at risk (Kagan, 1988; Kühn and Borchert, 2002). Cells are protected from ROS-induced damage by two classes of antioxidant defense: low-molecular-mass antioxidants (e.g. glutathione, vitamins E and C) and antioxidant enzymes [e.g. superoxide dismutase (SOD) and catalase (CAT)]. SOD catalyzes the dismutation of superoxide, and CAT eliminates H_2O_2, which is regularly formed in cells by multiple processes including SOD-catalyzed reactions. Under most physiological conditions, ROS production is closely matched by antioxidant responses, but when unmatched, organisms can experience bouts of oxidative stress including LPO (Halliwell and Gutteridge, 1999).

Temperature acclimation/adaptation typically results in restructuring of biological membranes and various metabolic changes in ectotherms, and these physiological responses should impact the susceptibility of biological membranes to LPO (Crockett, 2008). Cold-acclimated fishes possess elevated amounts of unsaturated fatty acids within phospholipids (Wodtke, 1978; Crockett and Hazel, 1995; Logue et al., 2000) and a decreased ratio of phosphatidylcholine (PC)/phosphatidylethanolamine (PE) relative to counterparts at warmer temperatures (Hazel and Landrey, 1988). Phospholipids containing polyunsaturated fatty acids (PUFA) (Holman, 1954; Cosgrove et al., 1987) and PE (Wang et al., 1994) are at particularly high risk of LPO relative to less unsaturated lipid species and lipids with a choline headgroup. The risk of LPO resulting from lipid remodeling at low temperature may be magnified because fishes acclimated to low temperatures often possess higher oxidative capacities (Guderley, 2004) and undergo significant proliferation of mitochondria (Johnston and Maitland, 1980; Egginton and Sidell, 1989) relative to warm-acclimated animals. It seems reasonable to expect that an enhancement of oxygen utilization in tissues of cold-acclimated ectotherms may result in elevated rates of ROS production, although the relationship between rates of ROS production and oxidative capacities is not simple (Brand, 2000). Finally, cold temperatures are accompanied by an elevated physical dissolution of oxygen and increased concentrations of intracellular lipids (Egginton and Sidell, 1989) in which oxygen solubility is 4-fold greater than oxygen solubility in an aqueous medium (Battino et al., 1968). These factors may enhance the availability of oxygen

to ROS-generating processes at low temperatures, yet it is unclear what risk a greater susceptibility to LPO and higher oxidative capacities pose for cold-adapted/acclimated fish.

Attempts to understand the role of temperature acclimation on oxidative capacities and enzymatic antioxidants in fishes may be confounded by variable activity patterns among temperature acclimation groups. Temperature directly influences activity levels in ectotherms. Tschantz et al. report that swimming activity of warm-acclimated centrachids (25°C), relative to 5°C counterparts, increases up to an order of magnitude, depending on the species (Tschantz et al., 2002). Similarly, O'Steen and Bennett demonstrated that warm acclimation of the tropical tinfoil barb (to 33°C) and eurythermal river barbel (to 25°C) resulted in 1.7- and 4-fold increases in voluntary swimming beyond rates in cold-acclimated equivalents (17°C, tinfoil barb; 7°C, river barbel) (O'Steen and Bennett, 2003). Variable activity patterns in ectotherms during temperature acclimation may be problematic for studies interested in the effects of temperature on oxidative capacities and antioxidants, because oxidative capacities in fishes may be enhanced by regular increases in activity (Davison, 1997), and enzymatic antioxidants appear to be elevated in more active fishes (Wilhelm-Filho, 1996).

The current study evaluates enzymatic indicators of oxidative capacities and antioxidant defense in two eurythermal fishes, killifish (*Fundulus heteroclitus macrolepidotus*) and bluegill (*Lepomis macrochirus*), which were both acclimated to 5 and 25°C. In addition, we compare phospholipid compositions and LPO susceptibility in mitochondrial and microsomal membranes prepared from killifish glycolytic muscle. Specifically, we measure the activities of citrate synthase (CS) and cytochrome *c* oxidase (CCO) and activities of two antioxidant enzymes (SOD and CAT) in cardiac muscle and glycolytic muscle (axial skeletal muscle) of cold- and warm-acclimated fishes. LPO susceptibility is quantified in biological membranes using two approaches, the frequently used thiobarbituric acid-reactive substances (TBARS) assay, as well as a method which involves one of a new generation of fluorometric probes (C11-BODIPY). This study is the first to simultaneously consider relative contributions of thermal acclimation and variable activity level to changes in oxidative capacities, antioxidant responses, LPO susceptibility and membrane composition of an ectotherm.

MATERIALS AND METHODS

Temperature acclimations

Adult killifish, *Fundulus heteroclitus macrolepidotus* (L.) (SL>55 mm), were wild-caught by Aquatic Research Organisms (Hampton, NH, USA), and adult bluegill, *Lepomis macrochirus* (Rafinesque) (SL>100 mm), were obtained from the Fish Management and Aquaculture Program at Hocking College (Nelsonville, OH, USA). Fish were held for two weeks in 15°C, 32‰ seawater (killifish) or 15°C filtered, dechlorinated tapwater (bluegill), to ensure all individuals were healthy and feeding and also to reset the thermal history of the animals prior to initiating temperature acclimations.

Both species were acclimated to 5 or 25°C. Temperatures were adjusted from ambient at a rate of ± 2.5°C day^{-1} for killifish and ±1.5–2°C day^{-1} for bluegill until the final temperature (5° or 25°C) was reached. The acclimation period commenced once final temperature had been sustained for 24 h, and animals were feeding and showing no sign of stress. Duration of acclimation was a full nine days for killifish and two months for bluegill. A short acclimation for killifish was chosen because the species occupies ecological habitats that can experience extensive tidally driven temperature fluctuations (8°C changes in water temperature in 30 min) (Bulger, 1984) and exist over a thermal gradient in which a 1° change in latitude results in a 1°C change in temperature (Powers et al., 1991). Consequently, we predicted that killifish would respond more quickly to temperature acclimation than other eurythermal species. Water quality (e.g. ammonia, nitrate, nitrite and pH) was monitored for both species, and all tanks were continuously aerated (and monitored to ensure comparable O_2 saturation levels). Animals were maintained at ambient photoperiod (16 h:8 h L:D, killifish; 12 h:12 h L:D, bluegill) throughout the acclimatory period.

Exercise acclimation

During June 2007, 60 male killifish were collected from an estuarine tidal creek on Mt Desert Island, ME, USA. Fish were returned to the Mt Desert Island Biological Laboratory (MDIBL) and held in ambient (~11°C), flow-through seawater for one week to ensure all fish were healthy and eating prior to the onset of the exercise training regime.

Killifish between 40 and 50 mm (standard length) were evenly divided between two modified Beamish-style swim tunnels and allowed to habituate to their enclosures for 24 h. Treatment fish were exercised daily for 6 h at swimming rates of 2.25 BL s^{-1}, a value significantly below the species' critical swimming speed (U_{crit}) and at approximately the midpoint of the species aerobic swimming abilities (Fangue et al., 2008) [exercise regime adapted from van der Meulen et al. (van der Meulen et al., 2006)]. The prescribed swimming speeds were comparable to sustained activity levels of warm-acclimated fish described above. Johnston and Moon (Johnston and Moon, 1980) showed that both axial glycolytic and aerobic fiber types of coalfish were active at swimming rates of 0.8–2.0 BL s^{-1}, speeds that these fish could sustain indefinitely. These data show that both muscle fiber types can be recruited during sustainable activity. Each day, the fish were allowed to adjust to the flow regime by swimming 20 min at 1.15 BL s^{-1}, followed by 20 min at 1.75 BL s^{-1}, before the final swimming rate was established. This exercise regime was maintained daily for a period of 9 days in order to mirror the duration of the temperature acclimation experiments. Exercise bouts were alternated daily between 06.00–12.00 h and 12.00–18.00 h to avoid diurnal effects. Control fish were held in an identical swim tunnel that lacked a pump. Water quality and oxygen saturation were maintained in both swim tunnels by flow-through seawater, and both photoperiod (16 h:8 h L:D) and temperature (10.5–12°C) were ambient. Both groups of fish were fed daily to satiation.

Tissue sampling and enzymatic analyses

Fish were stunned with a cranial blow and euthanized by cervical transection. Subsequently, glycolytic (skeletal) muscle and cardiac ventricle were dissected on an ice-cold glass stage and homogenized. Glycolytic muscle was homogenized 10% (w/v) in ice-cold 50 mmol l^{-1} potassium phosphate buffer (pH 7.2), while two heart ventricles were pooled and homogenized in 500 µl of the same buffer. Skeletal muscle was initially homogenized on ice with a Biospec tissuemizer (Bartlesville, OK, USA) (three 5 s bursts at low speed), and final homogenates were prepared in 7 ml Tenbroeck ground-glass homogenizers (five passes). Hearts were homogenized directly in 2 ml Tenbroeck ground-glass homogenizers (three passes). Homogenized tissues were sonicated on ice (two 2 s bursts for heart, and three 4 s bursts for skeletal muscle, with cooling intervals between bursts) to lyse all subcellular compartments.

Enzyme activities were assayed with Beckman DU640 UV/VIS (Beckman Coulter, Brea, CA, USA) and Pharmacia Ultrospec 3000 (GE Healthcare, Piscataway, NJ, USA) spectrophotometers fitted with circulating water baths and a temperature-controlled cell holder. Oxidative capacities were inferred from activities of CS and CCO [assays described in Hansen and Sidell (Hansen and Sidell, 1983)], while activities of total SOD [assay modified from Crapo et al. (Crapo et al., 1978)] and CAT [assay from Beers and Sizer (Beers and Sizer, 1952)] represented the enzymatic antioxidant responses. All assays were conducted at a temperature intermediate (15°C) to acclimation temperatures, which allows for direct comparison of enzyme activity between cold- and warm-acclimated groups. Activities were determined from assays performed in either duplicate or triplicate and were conducted using crude homogenates, except for CAT activity of glycolytic muscle in killifish. In this case, crude homogenates were centrifuged at 1000 ***g*** for 2 min, and resulting supernatants were assayed. Protein concentrations of crude homogenates and supernatants were quantified using the BCA method (Smith et al., 1985).

Membrane preparations

Biological membranes were not prepared from cardiac tissue because hearts of both species were prohibitively small, and therefore the use of these tissues was limited to enzymatic analyses only. Approximately 2 g of skeletal (glycolytic axial) muscle was used to prepare microsomal membranes as modified from Vornanen et al. (Vornanen et al., 1999). For both species, skeletal muscle pieces were homogenized in nine volumes of homogenization medium (100 mmol l^{-1} NaCl, 0.1 mmol l^{-1} EDTA, 0.1 mmol l^{-1} EGTA and 20 mmol l^{-1} MOPS at pH 7.6) using a Biospec tissuemizer. Further processing of the homogenate was made with three passes of a tight-fitting 35 ml Potter–Elvehjem homogenizer. All homogenization steps were performed at ice-cold temperatures. Homogenates were centrifuged at 3100 ***g*** for 30 min. Resulting supernatants were collected and set aside on ice while pellets were gently resuspended in 20 ml of homogenization medium using a Potter–Elvehjem homogenizer. The resuspension was centrifuged as previously. Combined supernatants were centrifuged at 100,000 ***g*** for 60 min. All centrifugation steps were performed at 4°C. Resulting pellets, containing microsomal membranes, were resuspended in <1 ml 10 mmol l^{-1} Tris-HCl (pH 7.6) using a Tenbroeck homogenizer and frozen immediately in liquid nitrogen.

Mitochondria were isolated from 2–3 g of skeletal muscle following a modification of Moyes (Moyes, 1989). Tissues were homogenized in nine volumes of ice-cold homogenization medium (140 mmol l^{-1} KCl, 20 mmol l^{-1} Hepes, 10 mmol l^{-1} EDTA, 5 mmol l^{-1} $MgCl_2$, 0.5% BSA at pH 7.1) using three successive passes of a loose, and then tight, fitting Potter–Elvehjem homogenizer. Crude homogenates were centrifuged at 800 ***g*** for 5 min. Resulting supernatants were filtered, respun, filtered as previously and further centrifuged at 9000 ***g*** for 10 min. The pellet was resuspended and centrifuged at the same higher speed. All centrifugation steps were performed at 4°C. The final pellet was collected and resuspended in 10 mmol l^{-1} Tris-HCl and frozen at –70°C.

Rates of lipid peroxidation

Rates of LPO were evaluated over time using thiobarbituric acid-reactive substances (TBARS) and/or 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY® 581/591) in membrane preparations. LPO was induced in both analyses by the production of hydroxyl radicals from the Fenton reaction between Fe^{2+} (as $FeSO_4$) and ascorbic acid (AsA) (Haberland et al., 1996).

The methods described in Uchiyama et al. (Uchiyama et al., 1978), as modified by Pamplona et al. (Pamplona et al., 1996) for TBARS, were used in the current study. Briefly, bluegill microsomes were diluted to 1 mg protein ml^{-1} in 50 mmol l^{-1} potassium phosphate buffer (pH 7.4). LPO was initiated by mixing membrane samples, Fe^{2+} (0.2 mmol l^{-1}), and AsA (1.6 mmol l^{-1}) in a volume ratio (2:1:1). LPO was allowed to proceed for 6 h at room temperature (approximately 23°C) and was sub-sampled every 60 min. Color development was initiated as previously reported. The difference between absorbance values measured at 535 nm and 520 nm represents the concentration of malondialdehyde (MDA), a secondary product of LPO. MDA concentrations were plotted for each time point and a best-fit line was plotted through the linear portion of the data. Resulting slopes ($R^2 \geq 0.89$ for all samples) as ΔMDA concentration Δmin^{-1} were used to calculate final LPO rates with an extinction coefficient of 155 A l $mmol^{-1}$.

Rates of LPO were also quantified in both species using the fluorometric reporter C-11 BODIPY. C11-BODIPY, a fatty acid analogue, indexes LPO in real-time by monitoring shifts in fluorescence upon probe oxidation. C11-BODIPY is an appropriate reporter molecule for comparing LPO in membranes of teleost fishes because it is sensitive to increasing levels of PUFA within the membrane (Drummen et al., 2002).

To incorporate the probe into membranes, killifish and bluegill membranes were diluted to final protein contents of 0.05 and 0.1 mg protein ml^{-1}, respectively, with 20 mmol l^{-1} Tris-HCl (pH 7.4). Membrane dilutions varied between study species to account for the particularly high content of readily oxidized PUFAs (e.g. 22:6) typically present in membranes of marine fish species. A working stock of BODIPY probe (1 mmol l^{-1} in 100% ethanol) was diluted to 10 μmol l^{-1} using 20 mmol l^{-1} Tris-HCl (pH 7.4). Diluted membranes (5 ml) were mixed with a small volume of the diluted probe solution to a final probe concentration of either 150 nmol l^{-1} (killifish) or 300 nmol l^{-1} (bluegill) to achieve probe-to-protein ratios that were consistent between preparations. The probe was dispersed within membranes by stirring slowly in the dark at 4°C for 60 min. Subsequently, LPO was induced in bluegill microsomes by adding 30 μl each of 0.2 mmol l^{-1} Fe^{2+} and 1.6 mmol l^{-1} AsA (final concentrations of 2.3 and 17.5 μmol l^{-1}, respectively) and in killifish membranes by adding 2.5 μl each of the inductant solutions (final concentrations of 0.19 and 1.4 μmol l^{-1}, respectively) to 2.5 ml of probe/membrane solution. Fluorescence decay was followed at 25°C (bluegill) or 15°C (killifish) with excitation/emission wavelengths of 568/590 nm. Changes in fluorescence decay were monitored until the slope was non-linear or fluorescence intensity was near zero. Linear portions of the decay slopes represented rates of LPO as Δfluorescent intensity Δmin^{-1}. An extinction coefficient of 139,444 A l mol^{-1} was used in all C11-BODIPY calculations (Drummen et al., 2004). Endogenous LPO was undetectable by either reporter method. Rates of LPO (slopes of MDA and C-11 BODIPY time course experiments) were normalized to total phospholipid content by measuring hydrolyzed phosphate according to Rouser et al. (Rouser et al., 1970).

Lipid extraction

Lipids were extracted from killifish mitochondrial and microsomal preparations in the presence of chloroform and methanol according to Bligh and Dyer (Bligh and Dyer, 1959). Total lipid extracts were sent to the Kansas State University Lipidomics Research Center for analysis of phospholipid class and molecular species. Unsaturation

index (UI) was calculated as modified from Hulbert et al. (Hulbert et al., 2007) to account for the total number of double bonds present in diacyl phospholipids rather than individual fatty acids. As a result, double bond number can range from 0 to 12, with a maximum value of 12, which would represent a phospholipid containing two fatty acid chains with six double bonds apiece: UI = (0 × mol% of fatty acids containing no double bonds) + (1 × mol% of fatty acids containing one double bonds) + (2 × mol% of fatty acids containing two double bonds) … (12 × mol% of fatty acids containing 12 double bonds).

Statistical analyses

Mean enzymatic responses (units min^{-1} g^{-1} wet tissue mass) and LPO susceptibility (normalized to phospholipid content) were compared between acclimation groups using parametric unpaired *t*-tests (JMP5; SAS, Cary, NC, USA). Data not meeting the assumption of normality were compared using Wilcoxon rank sums (JMP5). Statistical conclusions on enzyme data were based on Bonferroni-adjusted alpha values (α) of either 0.016 (three comparisons) or 0.0125 (four comparisons). All other data were evaluated relative to α=0.05. Unless otherwise noted, data are presented as means ± standard error of the mean (s.e.m.).

RESULTS

Oxidative capacity and enzymatic antioxidants

As indicated by activities of CCO, oxidative capacities were higher in glycolytic skeletal muscle, but not heart, of cold-acclimated killifish relative to warm-acclimated individuals (Fig. 1). Activities of CCO in killifish glycolytic muscle were 1.5-fold higher in 5°C-acclimated fish compared with 25°C counterparts (*t*-test; t_{18}=–4.56, *P*=0.0002; Fig. 1A). Although generally elevated, differences in CS activities between treatment groups failed to reach significance (Fig. 1A,B). Similarly, exercise training did not affect oxidative capacity of either glycolytic muscle (Fig. 2A) or cardiac muscle in killifish (Fig. 2B). Activities of enzymatic antioxidants measured in cardiac or glycolytic muscle of either species were unaffected by temperature acclimation (Fig. 3A,B). Exercise acclimation of killifish also did not change activities of CAT or SOD (Fig. 2A,B).

Phospholipid composition (killifish)

Although membrane remodeling of individual saturated and unsaturated phospholipids from both mitochondria and microsomes was relatively modest in response to temperature acclimation, there were significant differences in both the composition and the extent of remodeling in the two membrane fractions. While only the saturated phosphatidylcholine 38:0 (*t*-test; $t_{1,12}$=–2.77, *P*=0.016) varied significantly among individual phospholipids of mitochondria (Table 1), the total amount of saturated phospholipids (SFA) was 1.3-fold more abundant in 25°C-acclimated killifish mitochondria relative to those prepared from 5°C-acclimated counterparts (*t*-test; $t_{1,11}$=4.23, *P*<0.0002). Similarly, microsomal membranes prepared from 25°C-acclimated animals were enriched in the saturated phosphatidylcholine 32:0 (*t*-test; $t_{1,6}$=10.88, *P*<0.0001) and the unsaturated PC 36:2 (*t*-test; $t_{1,6}$=2.84, *P*=0.029) relative to membranes from the cold-acclimated group (Table 1). The total amount of SFA, however, was not changed in microsomes by temperature acclimation. Contents of several polyunsaturated phospholipids were altered by temperature acclimation in mitochondrial membranes, but not microsomes, from glycolytic muscle (Table 1). Mitochondria from cold-acclimated killifish were enriched in polyunsaturated phosphatidylethanolamines including 40:8 (*t*-test; $t_{1,12}$=–2.21, *P*=0.046) and 44:12 (a phospholipid

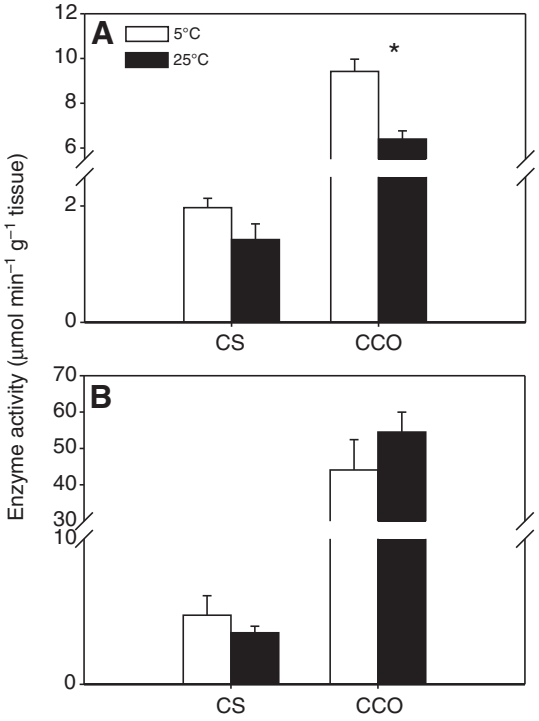

Fig. 1. Activities of oxidative enzymes from thermally acclimated killifish, *Fundulus heteroclitus*, glycolytic (A) and cardiac muscle (B). Data are presented as means ± s.e.m. Asterisk indicates significant differences (*P*<0.0125) between activities/capacities of 5 and 25°C acclimation groups *N*=9 (A), *N*=5 (B). CCO, cytochrome *c* oxidase; CS, citrate synthase.

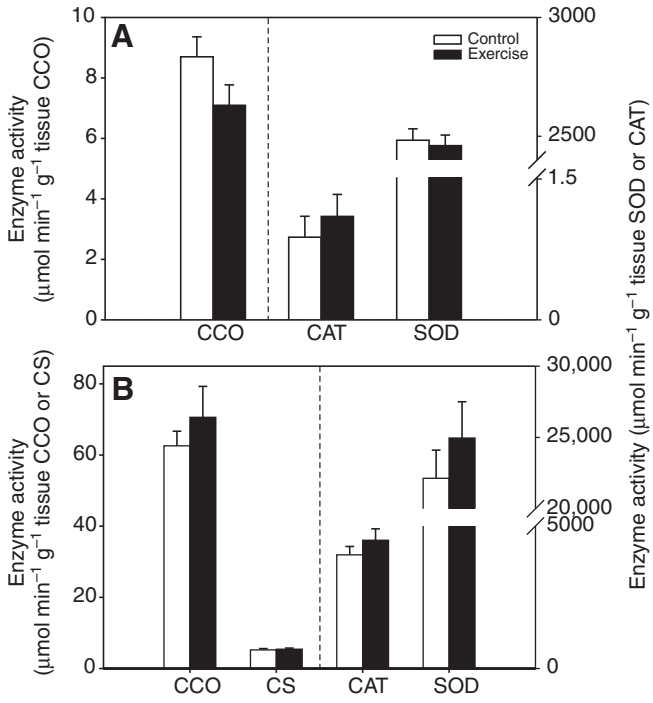

Fig. 2. Activities of oxidative and antioxidant enzymes in glycolytic (A) and cardiac muscle (B) of exercise-trained killifish, *Fundulus heteroclitus*. Data are presented as means ± s.e.m. *N*=10 (A,B). CAT, catalase; CCO, cytochrome *c* oxidase; CS, citrate synthase; SOD, superoxide dismutase.

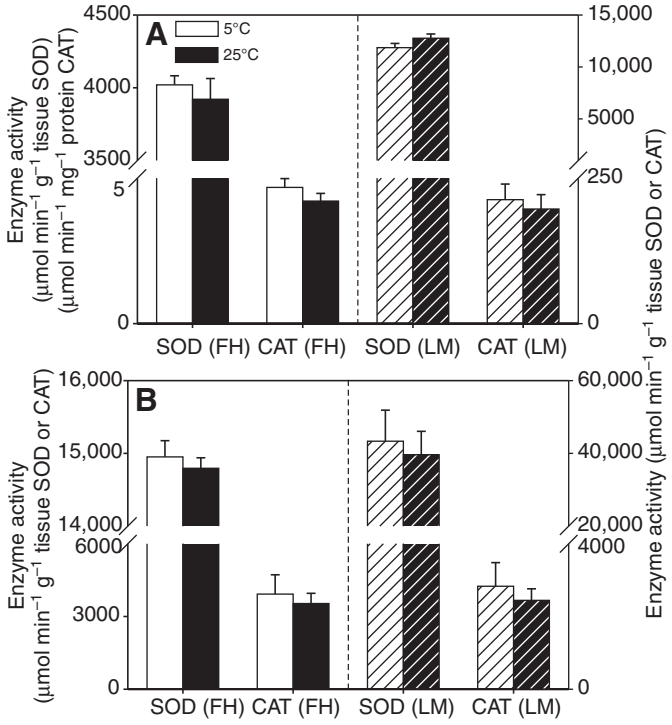

Fig. 3. Activities of antioxidant enzymes from thermally-acclimated killifish, *Fundulus heteroclitus* (FH), and bluegill, *Lepomis macrochirus* (LM), glycolytic (A) and cardiac muscle (B). Data are presented as means ± s.e.m. *N*=9 (A), *N*=5 (B). CAT, catalase; SOD, superoxide dismutase.

containing two docohexaenoyl chains) (*t*-test; $t_{1,12}$=–2.79, *P*=0.016). This remodeling, however, did not affect the total amount of mono- or polyunsaturated fatty acids in the membrane or the unsaturation index (UI) between temperature acclimation groups. Likewise, neither the amounts of mono- and polyunsaturated fatty acids nor the UI of microsomes were altered by temperature acclimation. When UIs from cold- and warm-acclimated groups are pooled, the UI of mitochondria (566) is 1.2-fold greater than the UI value of 479 observed in microsomes (*t*-test; t_{20}=–5.73, *P*<0.0001; Table 1), indicating a greater number of double bonds present in membranes of mitochondria. Finally, the ratio of the major phospholipid classes, PC/PE, was unaffected by temperature acclimation in all membranes tested (Table 1), while PC/PE ratios varied among subcellular fractions, with PC/PE being 5.2-fold higher in microsomes than in mitochondria (Wilcoxon rank sums; z_{148}=3.78, *P*=0.0002; Table 1).

Susceptibility to LPO

Analyses with both TBARS and C11-BODIPY revealed that temperature acclimation did not change the susceptibility to LPO when membranes were exposed to a hydroxyl radical generating system ($FeSO_4$/AsA; Figs 4, 5). Interestingly, subcellular fractions prepared from glycolytic muscle of killifish responded differently to oxidative challenge. Since there is no statistically significant difference between acclimation groups, pooling the results enables a comparison between mitochondrial and microsomal fractions, which revealed that LPO susceptibility was 2.6-fold greater in microsomal (*t*-test; $t_{1,9.87}$=4.17, *P*=0.002) relative to mitochondrial membranes.

DISCUSSION

Oxidative and antioxidant capacities

Activities of oxidative and antioxidant enzymes in studies involving temperature acclimations are not likely to be confounded by differences in routine locomotory behavior among temperature treatments. While acclimation to cold temperature (5°C) results in enhanced oxidative capacities (activities of CCO, but not CS, were increased) after only nine days in killifish, we found that this same duration of endurance (exercise) training altered neither oxidative capacities (activities of CCO or CS) nor activities of antioxidant enzymes in muscle tissues from killifish. The enhancement of oxidative capacities, as has so often been observed in cold-acclimated (or -adapted) ectotherms relative to warm-acclimated animals, is therefore likely to be governed by temperature alone.

Table 1. Phospholipid compositions of microsomal and mitochondrial membranes from glycolytic muscle of temperature-acclimated killifish

	Microsomes (*N*=4)		Mitochondria (*N*=7)	
	5°C	25°C	5°C	25°C
PC 32:1	1.48±0.09	1.71±0.05	1.02±0.10	1.00±0.05
PC 32:0	0.20±0.02	1.13±0.08*	–	–
PC 34:3	1.32±0.34	1.32±0.31	–	–
PC 34:2	5.91±1.48	8.12±1.05	–	–
PC 34:1	6.83±1.70	7.81±0.97	4.68±0.46	4.52±0.42
PC 36:6	1.51±0.16	0.87±0.10	–	–
PC 36:5	4.93±0.25	4.09±0.34	1.72±0.13	1.83±0.14
PC 36:4	4.94±0.63	4.61±0.19	1.68±0.14	1.75±0.19
PC 36:3	0.94±0.25	1.48±0.22	–	–
PC 36:2	0.79±0.15	1.37±0.14†	–	–
PC 38:6	18.27±0.85	17.45±0.58	14.26±1.51	15.15±1.03
PC 38:5	3.78±0.14	3.38±0.49	2.28±0.35	2.02±0.37
PC 38:0	1.05±0.04	0.92±0.05	0.81±0.09	1.08±0.05†
PC 40:8	1.40±0.18	1.59±0.12	0.96±0.07	1.01±0.05
PC 40:7	3.42±0.22	4.00±0.49	2.92±0.30	3.59±0.24
PC 40:6	1.40±0.07	1.30±0.11	0.99±0.17	1.29±0.18
PC 44:12	1.57±0.28	1.81±0.13	–	–
SM 22:1	0.78±0.12	1.02±0.17	–	–
SM 24:1	–	–	0.87±0.22	1.19±0.23
ePC 38:6	1.50±0.03	1.50±0.04	1.23±0.13	1.18±0.08
ePC 38:0	1.81±0.23	1.45±0.05	1.02±0.10	1.11±0.13
PE 38:6	1.87±0.25	2.00±0.26	8.58±1.01	7.49±0.64
PE 38:5	–	–	1.59±0.24	1.31±0.10
PE 40:8	–	–	1.87±0.19†	1.33±0.15
PE 40:7	1.22±0.19	1.11±0.12	4.44±0.48	3.93±0.30
PE 40:6	1.04±0.10	1.36±0.11	8.99±0.70	9.99±0.67
PE 40:5	–	–	1.86±0.15	1.98±0.19
PE 42:10	–	–	1.40±0.11	1.42±0.11
PE 44:12	2.58±0.59	1.98±0.31	5.31±0.63†	3.29±0.36
PE 44:11	1.76±0.23	1.30±0.12	4.74±0.36	4.51±0.19
PE 44:10	–	–	1.71±0.18	1.67±0.09
PI 38:4	1.72±0.15	1.40±0.17	–	–
PI 40:6	2.05±0.36	1.87±0.36	–	–
UI	492±20	466±15	574±16	558±9
PC/PE	5.19±0.80	6.07±0.75	1.05±0.22	1.14±0.15

Diacyl lipid composition data (headgroup total number of carbons:total number of double bonds) are presented as mean mol% ± s.e.m. Only molecular species present in amounts ≥1 mol% for at least one of the temperature treatments within a membrane preparation are shown.

*Significant difference between treatment means (*P*<0.0001); †significant difference between treatment means (*P*<0.05). See List of abbreviations in text for definitions.

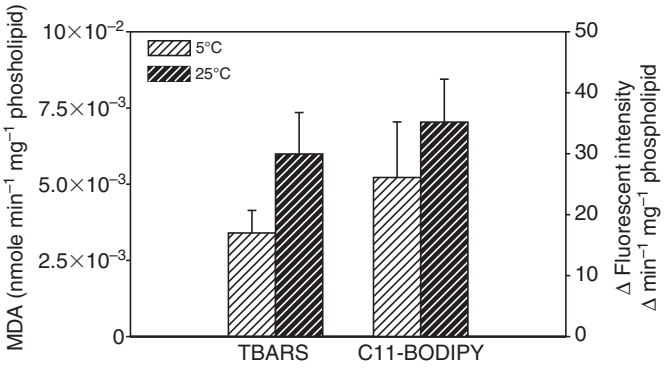

Fig. 4. Lipid peroxidation (LPO) susceptibility in microsomal membranes from glycolytic muscle of thermally acclimated bluegill, *Lepomis macrochirus*, quantified using TBARS and the fluorescent fatty acid analogue C11-BODIPY. Data are presented as means ± s.e.m. *N*=5. MDA, malondialdehyde.

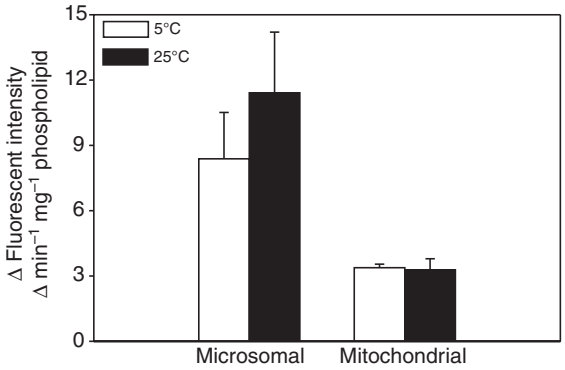

Fig. 5. Lipid peroxidation (LPO) susceptibility in microsomal and mitochondrial membranes from glycolytic muscle of thermally acclimated killifish, *Fundulus heteroclitus*, quantified using the fluorescent fatty acid analogue C11-BODIPY. Data are presented as means ± s.e.m. *N*=5.

We suggest that the level of locomotory activity need not be a concern, at least in many cases, with studies aimed at addressing questions related to the effects of acclimation temperature on oxidative and/or antioxidant activities in ecothermic animals.

The 1.5-fold rise in CCO activity from skeletal muscle of killifish following nine days of cold acclimation is the quickest change reported for this tissue, as well as for this electron transport complex, and mirrors the rapid (7–10-day) response in CS activity reported by Lucassen et al. in liver tissue from a 10°C-acclimated zoarcid, *Zoarces viviparous* (Lucassen et al., 2003). The magnitude of the observed change in CCO activity for 5°C-acclimated killifish (1.5-fold) is similar to the 1.9- [green sunfish (Shaklee et al., 1977)], 1.2- [zebrafish (McClelland et al., 2006)], and 1.7-fold [cod (Lucassen et al., 2006) and bluegill (J.M.G. and E.L.C., unpublished)] increases in CCO in skeletal muscle reported elsewhere. Similar increases in CCO activity following cold acclimation in these other studies is not unexpected given comparable acclimation lengths for species in these studies (green sunfish, 1 month; zebrafish, 1 month; cod, 1.5 months; bluegill, 2 months); however, data from killifish demonstrate that activities of CCO can be changed in glycolytic muscle much more rapidly than previously thought.

The rise in oxidative capacity at low temperature in the absence of enhanced enzymatic antioxidants indicates that changes in oxidative capacities are not necessarily matched by concomitant adjustments in enzymatic antioxidant defenses. Data from glycolytic and cardiac muscles in the current study (Figs 1, 3), as well as CAT activity measured in livers from rainbow trout (*Oncorhynchus mykiss*) acclimated to 5°C for 2 months (E.L.C., unpublished), demonstrate that temperature acclimation does not modulate antioxidant defenses. Similarly, Witas et al. (Witas et al., 1984) and Cassini et al. (Cassini et al., 1993) showed that CAT activities from liver and liver, heart and skeletal muscle, respectively, are lower in Antarctic species when compared to temperate counterparts, whereas SOD activity may (Witas et al., 1984) or may not [red-blooded Antarctic species (Cassini et al., 1993)] be elevated in cold-adapted fishes. Speers-Roesch and Ballantyne also demonstrated a mismatch between oxidative capacity and enzymatic antioxidants by reporting an inverse correlation between CCO and CAT activity in liver tissue of confamilial Arctic and temperate teleosts (Speers-Roesch and Ballantyne, 2005). Whether higher oxidative capacities at cold body temperatures, not accompanied by elevated activities of enzymatic antioxidants, leaves cold-bodied fishes with a more significant risk of oxidative damage is a question we are pursuing currently.

On the other hand, microarray analyses from zebrafish exposed for one year to a reduced temperature (18°C) suggest a positive link between oxidative capacity and antioxidant response since 2.6- and 2.0-fold increases in genes controlling various components of the CCO complex and SOD, respectively, were observed in zebrafish acclimated to 18°C (Malek et al., 2004). Additionally, Leary et al. reported a positive relationship between CCO and both CAT and SOD in heart and skeletal muscle of cold-acclimated trout (Leary et al., 2003). Conflicting reports of ROS-generating process (e.g. oxidative phosphorylation) and antioxidant defenses following temperature acclimation/adaptation demonstrate the need for further investigation into the role of temperature in the relationship between the pro- and antioxidant processes.

Closer examination of the ratios of antioxidant activity to oxidative capacity (SOD/CCO or CAT/CCO) may provide valuable insight regarding the relative levels of antioxidants required to protect against oxidative stress. While absolute activities of antioxidant enzyme activity are not affected by temperature acclimation, tissues from the warm-acclimated fishes tend to have higher levels of antioxidant activity for a given oxidative activity. SOD/CCO was 1.4- and 1.8-fold higher in glycolytic muscle from warm-acclimated killifish and bluegill, respectively, than in cold-acclimated animals. Similarly, CAT/CCO activity was 1.4-fold higher in glycolytic muscle of killifish. Because the warm-acclimated animals possess higher activities of antioxidant activities when normalized to activities of the respiratory complex, this result would suggest that warm-acclimated animals may actually require a more robust set of antioxidant defenses than cold-acclimated animals.

Temperature-induced membrane remodeling

Results from the current study and others (reviewed in Hazel and Williams, 1990) have demonstrated that not all membrane fractions respond equally to cold exposure. It has been noted that membranes from cold-acclimated/adapted ectotherms are generally enriched in unsaturated fatty acids and also that homeoviscous efficacy is typically higher in mitochondrial membranes than other membrane fractions. Although UI are similar for mitochondria from cold- and warm-acclimated killifish (Table 1), mitochondria from killifish at 5°C are significantly enriched in phospholipids containing highly unsaturated fatty acids such as PE 40:8 and 44:12. At the same time, no changes in PUFA are observed in microsomal preparations from

killifish over the acclimatory period, adding to a growing body of literature that indicates clearly that phospholipid compositions of microsomal and SR membranes are less affected by temperature change (Cossins et al., 1978; Vornanen et al., 1999).

Changes in individual phospholipid molecular species might contribute to enhanced CCO activity in cold-acclimated animals (Hazel, 1972a; Hazel, 1972b; Wodtke, 1981b; reviewed by Guderley and St. Pierre, 2002). It is well accepted that elevated levels of unsaturated FA lead to concomitant changes in membrane fluidity (homeoviscous adaptation); however, there is much debate about whether lipid remodeling, including higher PUFA content, does (Hazel, 1972a; Hazel, 1972b; Wodtke, 1981b; Wu et al., 2001; Hulbert et al., 2006; Kraffe et al., 2007; Guderley et al., 2008) or does not (reviewed by Lee, 2003) lead to enhanced catalytic rates of membrane-associated proteins such as CCO. Although increases in individual PUFA species and the enhanced CCO activities in cold-acclimated killifish mitochondria appear indeed to be correlated, we cannot say with any confidence that the changes in PUFA are responsible, even in part, for the trends in oxidative capacity.

Lipid remodeling and LPO in biological membranes

The inherent complexity of biological membranes, including interactions between pro- and antioxidants, may obscure straightforward conclusions about the impacts of lipid remodeling on susceptibility to LPO. While it is clear from studies of model lipids that rates of lipid peroxidation increase with degree of unsaturation (Holman, 1954; Cosgrove, 1987) and PE content (Wang, 1994), the modest amount of phospholipid remodeling in mitochondria and microsomes between acclimation groups in the current study is insufficient to alter LPO susceptibilities. Cardiolipin is a likely target of ROS-mediated LPO both by proximity to ROS-generating sites as well as lipid chemical properties (i.e. degree of unsaturation). As an essential phospholipid for mitochondrial respiratory complexes I and IV (Schlame et al., 2000), cardiolipin can represent a sizeable portion of phospholipids (14.9–15.3%) in mitochondria from fish glycolytic muscle (Wodtke, 1981a). In addition, this phospholipid is normally highly unsaturated (Petrosillo et al., 2009). Although our analytical method for phospholipids precluded an assessment of cardiolipin composition, our measurements of membrane LPO susceptibility in cold- and warm-acclimated fishes should include contributions from any changes in cardiolipin with temperature.

Despite the larger UI values and lower PC/PE ratios in mitochondrial membranes from killifish (compared with microsomes), the mitochondrial membranes have lower rates of LPO (Fig. 5). These results indicate that mitochondrial membranes of fishes may possess more intrinsic defenses than do microsomal membranes. The antioxidant most likely to fulfill this role is vitamin E. This low-molecular-mass, lipophilic antioxidant has been shown to be particularly abundant in mitochondrial fractions prepared from rat (Atkinson et al., 2008). The role of low-molecular-mass antioxidants that are associated with the membrane will need to be assessed in future studies that consider the consequences of temperature acclimation or adaptation. Among the potent lipophilic antioxidants, the vitamin E molecules and electron transport components (e.g. various homologues of ubiquinone) are likely to be the most important in mitigating LPO damage to biological membranes (Niki et al., 1989). It is possible that as phospholipids are remodeled, elevations in PUFA content (and consequent elevated risk of LPO) may be countered directly by fortification with one or more neutral lipid antioxidant(s) in membrane fractions. Levels of the ‘marine-derived tocopherol’ are consistently elevated in tissues of cold-adapted marine organisms, including fishes (Yamamoto et al., 2001). It would seem possible that cold acclimation may induce similar increases in vitamin E, which may then provide cold-acclimated fishes with the additional protection afforded to cold-adapted species.

Conclusions

Oxidative capacities in skeletal muscle of fishes may be altered by just nine days of temperature acclimation, while the same duration of a moderate exercise regime is not sufficient to induce a response in activities of either oxidative (CS or CCO) or antioxidant enzymes (SOD and CAT). These data demonstrate that variable activity levels among temperature treatments are not likely to confound studies aimed at addressing the effects of temperature on oxidative and/or antioxidant metabolism in ectothermic organisms. The apparent mismatch, however, between oxidative capacities (CCO) and activities of enzymatic antioxidants reported in this paper (and in previous studies) is striking. One must consider that in many instances only enzymatic antioxidants have been reported. An enhancement in the activity of enzymatic antioxidants (e.g. CAT and SOD) may be unnecessary if low-molecular-mass antioxidants fulfill this role. Despite modest changes in membrane PUFA contents, susceptibility of mitochondrial and microsomal membranes to LPO was unaffected by temperature acclimation although mitochondrial and microsomal membranes respond differently to an oxidative challenge. Despite a higher degree of membrane unsaturation and elevated levels of PEs, mitochondria were less vulnerable to LPO than were preparations of microsomes. An explanation for these counterintuitive results may reside with low-molecular-mass antioxidants (e.g. vitamins E and C, glutathione), which could reduce LPO susceptibility and/or protect animals with higher oxidative capacities. Our future studies will consider measures of both enzymatic and low-molecular-mass antioxidants in order to quantify the relative contributions of each class of antioxidants in protecting biological membranes during acclimation to varying temperatures.

LIST OF ABBREVIATIONS

BL	body length
CAT	catalase
CCO	cytochrome *c* oxidase
CS	citrate synthase
ePC	ether phosphatidylcholine
LPO	lipid peroxidation
MDA	malondialdehyde
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
SFA	saturated phospholipids
SL	standard length
SM	sphingomyelin
SOD	superoxide dismutase
TBARS	thiobarbituric acid-reactive substances
U_{crit}	critical swimming speed
UI	unsaturation index

ACKNOWLEDGEMENTS

Funding provided to J.M.G. by the Stan and Judy Fund and Stanley Bradley Fellowship at MDIBL, and to E.L.C. by Ohio University Research Challenge Award and MDIBL NIA funded by MDIBL’S NIEHS Center for Membrane Toxicity Studies (P30 ES003828-20). Experimental assistance provided by Maren Askins (MDIBL). Thanks to Dave Elliott and William Grim for assistance in constructing fish swim chambers. The Kansas Lipidomics Research Center was supported by NSF grants MCB 0455318 and DBI 0521587, and NSF EPSCoR grant EPS-0236913 with matching support from the State of Kansas through Kansas Technology Enterprise Corporation and Kansas State University. The KLRC is also supported by K-INBRE (NIH Grant P20 RR16475 from the INBRE program of the National Center for Research Resources).

REFERENCES

Atkinson, J., Epand, R. F. and Epand, R. M. (2008). Tocopherols and tocotrienols in membranes: a critical review. *Free Rad. Biol. Med.* **44**, 739-764.

Battino, R., Evans, F. D. and Danforth, W. F. (1968). The solubilities of seven gases in olive oil with reference to theories of transport through the cell membrane. *J. Amer. Oil Chem. Soc.* **45**, 830-833.

Beers, R. F. and Sizer, I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* **195**, 133-140.

Bligh, E. G. and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911-917.

Brand, M. D. (2000). Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp. Geron.* **35**, 811-820.

Bulger, A. J. (1984). A daily rhythm in heat tolerance in the salt marsh fish *Fundulus heteroclitus*. *J. Exp. Zool.* **230**, 11-16.

Cassini, A., Favero, M. and Albergoni, V. (1993). Comparative studies of antioxidant enzymes in red-blooded and white-blooded Antarctic teleost fish, *Pagothenia bernaccii* and *Chionodraco hamatus*. *Comp. Biochem. Physiol.* **106C**, 333-336.

Cosgrove, J. P., Church, D. F. and Pryor, W. A. (1987). The kinetics of the autoxidation of polyunsaturated fatty acids. *Lipids* **22**, 299-304.

Cossins, A. R., Christiansen, J. and Prosser, C. L. (1978). Adaptation of biological membranes to temperature. The lack of homeoviscous adaptation in the sarcoplasmic reticulum. *Biochim. Biophys. Acta.* **511**, 442-454.

Crapo, J. D., McCord, J. M. and Fridovich, I. (1978). Preparation and assay of superoxide dismutase. *Meth. Enzy.* **53**, 382-393.

Crockett, E. L. (2008). The cold but not hard fats in ectotherms: consequences of lipid restructuring on susceptibility of biological membranes to peroxidation, a review. *J. Comp. Physiol. B* **178**, 795-809.

Crockett, E. L. and Hazel, J. R. (1995). Cholesterol levels explain inverse compensation of membrane order in brush border but not homeoviscous adaptation in basolateral membranes from the intestinal epithelia of rainbow trout. *J. Exp. Biol.* **198**, 1105-1113.

Davison, W. (1997). The effects of exercise training on teleost fish, a review of recent literature. *Comp. Biochem. Physiol.* **117A**, 67-75.

Drummen, G. P. C., van Liebergen, L. C. M., Op den Kamp, J. A. F. and Post, J. A. (2002). C11-BODIPY$^{581/591}$, an oxidation-sensitive fluorescent lipid peroxidation probe: (Micro)spectroscopic characterization and validation of methodology. *Free Rad. Biol. Med.* **33**, 473-490.

Drummen, G. P. C., Gadella, B. M., Post, J. A. and Brouwers, J. F. (2004). Mass spectrometric characterization of the oxidation of the fluorescent lipid peroxidation reporter molecule C11-BODIPY$^{581/591}$. *Free Rad. Biol. Med.* **36**, 1635-1644.

Egginton, S. and Sidell, B. D. (1989). Thermal acclimation induces adaptive changes in subcellular structure of fish skeletal muscle. *Am. J. Physiol.* **256**, R1-R9.

Fangue, N. A., Mandic, M., Richards, J. G. and Schulte, P. M. (2008). Swimming performance and energetics as a function of temperature in killifish *Fundulus heteroclitus*. *Phys. Biochem. Zool.* **81**, 389-401.

Guderley, H. and St-Pierre, J. (2002). Going with the flow or life in the fast lane: contrasting mitochondrial responses to thermal change. *J. Exp. Biol.* **205**, 2237-2249.

Guderley, H. (2004). Metabolic responses to low temperature in fish muscle. *Biol. Rev. Camb. Philos. Soc.* **79**, 409-427.

Guderley, H., Kraffe, E., Bureau, W. and Bureau, D. P. (2008). Dietary fatty acid composition changes mitochondrial phospholipids and oxidative capacities in rainbow trout red muscle. *J. Comp. Physiol. B* **178**, 385-399.

Haberland, A., Damerau, W., Stöber, R., Schimke, I. and Baumann, G. (1996). Fe^{2+}/vitamin C – an appropriate *in vitro* model system to initiate lipid peroxidation. *J. Inorg. Biochem.* **61**, 43-53.

Halliwell, B. and Gutteridge, J. M. C. (1999). *Free Radicals in Biology and Medicine.* Oxford: Oxford University Press.

Hansen, C. A. and Sidell, B. D. (1983). Atlantic hagfish cardiac muscle: metabolic basis of tolerance to anoxia. *Am. J. Physiol.* **244**, R356-R362.

Hazel, J. R. (1972a). The effect of temperature acclimation upon succinic dehydrogenase activity from the expaxial muscle of the common goldfish (*Carassius auratus* L.). I. Properties of the enzyme and the effect of lipid extraction. *Comp. Biochem. Physiol.* **43B**, 837-861.

Hazel, J. R. (1972b). The effect of temperature acclimation upon succinic dehyrogenase activity from the expaxial muscle of the common goldfish (*Carassius auratus* L.). II. Lipid reactivation of the soluble enzyme. *Comp. Biochem. Physiol.* **43B**, 863-882.

Hazel, J. R. and Landrey, S. R. (1988). Time course of thermal adaptation in plasma membranes of trout kidney. I. Headgroup composition. *Am. J. Physiol.* **255**, R622-R627.

Hazel, J. R. and Williams, E. E. (1990). The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Prog. Lipid Res.* **29**, 167-227.

Holman, R. T. (1954). Autoxidation of fats and related substances. *Progress Chem. Fats Lipds.* **2**, 51-98.

Hulbert, A. J., Turner, N., Hinde, J., Else, P. L. and Guderley, H. (2006). How might you compare mitochondria from different tissues and different species? *J. Comp. Physiol. B* **176**, 93-105.

Hulbert, A. J., Pamplona, R., Buffenstein, R. and Buttemer, W. A. (2007). Life and death: metabolic rate, membrane composition, and life span of animals. *Physiol. Rev.* **87**, 1175-1213.

Johnston, I. and Maitland, B. (1980). Temperature acclimation in crucian carp, *Carassius carassius* L., morphometric analyses of muscle fibre ultrastructure. *J. Fish Biol.* **17**, 113-125.

Johnston, I. and Moon, T. W. (1980). Endurance exercise training in the fast and slow muscles of a teleost fish (*Pollachius virens*). *J. Comp. Physiol.* **135**, 147-156.

Kagan, V. E. (1988). *Lipid Peroxidation in Biomembranes.* Boca Raton, FL: CRC Press.

Kraffe, E., Marty, Y. and Guderley, H. (2007). Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout *Oncorhynchus mykiss*: roles of membrane proteins, phospholipids, and their fatty acids. *J. Exp. Biol.* **210**, 149-165.

Kühn, H. and Borchert, A. (2002). Regulation of enzymatic lipid peroxidation: the interplay of peroxidizing and peroxide reducing enzymes. *Free Rad. Biol. Med.* **33**, 154-172.

Leary, S. C., Lyons, C. N., Rosenberger, A. G., Ballantyne, J. S., Stillman, J. and Moyes, C. D. (2003). Fiber-type differences in muscle mitochondrial profiles. *Am. J. Physiol.* **285**, R817-R826.

Lee, A. G. (2003). Lipid–protein interactions in biological membranes: a structural perspective. *Biochim. Biophys. Acta.* **1612**, 1-40.

Logue, J. A., DeVries, A. L., Fodor, E. and Cossins, A. R. (2000). Lipid compositional correlates of temperature-adaptive interspecific differences in membrane physical structure. *J. Exp. Biol.* **203**, 2105-2115.

Lucassen, M., Schmidt, A., Eckerle, L. G. and Pörtner, H.-O. (2003). Mitochondrial proliferation in the permanent vs. temporary cold: enzyme activities and mRNA levels in Antarctic and temperate zoarcid fish. *Am. J. Phys.* **285**, R1410-R1420.

Lucassen, M., Schmidt, A., Eckerle, L. G. and Pörtner, H.-O. (2006). Mitochondrial mechanisms of cold adaptation in cod (*Gadus morhua* L.) populations from different climatic zones. *J. Exp. Biol.* **209**, 2462-2471.

Malek, R. L., Sajadi, H., Abraham, J., Grundy, M. A. and Gerhard, G. S. (2004). The effects of temperature reduction on gene expression and oxidative stress in skeletal muscle from adult zebrafish. *Comp. Biochem. Physiol.* **138C**, 363-373.

McClelland, G. B., Craig, P. M., Dhekney, K. and Dipardo, S. (2006). Temperature- and exercise-induced gene expression and metabolic enzyme changes in skeletal muscle of adult zebrafish. *J. Physiol.* **577**, 739-751.

Moyes, C. D., Buck, L. T., Hochachka, P. W. and Suarez, R. K. (1989). Oxidative properties of carp red and white muscle. *J. Exp. Biol.* **143**, 321-331.

Niki, E., Yamamoto, Y., Takahasi, M., Komuro, E. and Miyama, Y. (1989). Inhibition of oxidation of biomembranes by tocopherol. *Ann. NY Acad. Sci.* **570**, 23-31.

O'Steen, S. and Bennett, A. F. (2003). Thermal acclimation effects differ between voluntary, maximum, and critical swimming velocities in two cynprinid fishes. *Physiol. Biochem. Zool.* **76**, 484-496.

Pamplona, R., Prat, J., Cadenas, S., Rojas, C., Pérez-Campo, R., López Torres, M. and Barja, G. (1996). Low fatty acid unsaturation protects against lipid peroxidation in liver mitochondria from long-lived species: the pigeon and human case. *Mech. Ageing Develop.* **86**, 53-66.

Petrosillo, G., Matera, M., Moro, N., Ruggiero, F. M. and Paradise, G. (2009). Mitochondrial complex I disfunction in rat heart with aging: critical role of reactive oxygen species and cardiolipin. *Free Rad. Bio. Med.* **46**, 88-94.

Powers, D. A., Lauerman, T., Crawford, D. and DiMichele, L. (1991). Genetic mechanisms for adapting to a changing environment. *Annu. Rev. Genet.* **25**, 629-659.

Rouser, G., Fleisher, S. and Yamamoto, A. (1970). Two-dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* **5**, 494-496.

Schlame, M., Rua, D. and Greenberg, M. L. (2000). The biosynthesis and functional role of cardiolipin. *Prog. Lipid Res.* **39**, 257-288.

Shaklee, J. B., Christiansen, J. A., Sidell, B. D., Prosser, C. L. and Whitt, G. S. (1977). Molecular aspects of temperature acclimation in fish: contributions of changes in enzyme activities and isozyme patterns to metabolic reorganization in the green sunfish. *J. Exp. Zool.* **201**, 1-20.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85.

Speers-Roesch, B. and Ballantyne, J. S. (2005). Activities of antioxidant enzymes and cytochrome c oxidase in liver of Arctic and temperate teleosts. *Comp. Biochem. Physiol.* **140A**, 487-494.

Tschantz, D. R., Crockett, E. L., Niewiaroski, P. H. and Londraville, R. L. (2002). Cold acclimation strategy is highly variable among sunfishes (Centrarchidae).*Physiol. Biochem. Zool.* **75**, 544-556.

Uchiyama, M. and Mihara, M. (1978). Determination of malonaldehyde precursor intissues by thiobarbituric acid test. *Anal. Biochem.* **86**, 271-278.

van der Meulen, T., Schipper, H., van den Boogart, J. G. M., Huising, M. O., Kranenbarg, S. and van Leeuwen, J. L. (2006). Endurance exercise training differentially stimulates heart and axial muscle development in zebrafish (*Danio rerio*). *Am. J. Physiol.* **291**, R1040-R1048.

Vornanen, M., Tiitu, V., Kälelä, R. and Aho, E. (1999). Effects of thermal acclimation on the relaxation system of crucian carp white myotomal muscle. *J. Exp. Zool.* **284**, 241-251.

Wang, J.-Y., Wang, Z.-Y., Kouyama, T., Shibata, T. and Ueki, T. (1994). Significance of amino groups of phosphatidylethanolamine in phospholipid peroxidation of mixed liposomes. *Chem. Phys. Lipids.* **71**, 197-203.

Wilhelm-Filho, D. (1996). Fish antioxidant defenses – a comparative approach. *Braz. J. Med. Biol. Res.* **29**, 1735-1742.

Witas, H., Gabryelak, T. and Matkovics, B. (1984). Comparative studies on superoxide dismutase and catalase activities in livers of fish and other Antarctic vertebrates. *Comp. Biochem. Physiol.* **77C**, 409-411.

Wodtke, E. (1978). Lipid adaptation in liver mitochondrial membranes of carp acclimated to different environmental temperatures. Phospholipid composition, fatty acid pattern, and cholesterol content. *Biochim. Biophys. Acta.* **529**, 280-291.

Wodtke, E. (1981a). Temperature adaptation of biological membranes. The effects of acclimation temperature on the unsaturation of the main neutral and charged phospholipids in mitochondrial membranes of the carp (*Cyprinus carpio* L.). *Biochim. Biophys. Acta.* **640**, 698-709.

Wodtke, E. (1981b). Temperature adaptation of biological membranes. Compensation of the molar activity of cytochrome c oxidase in the mitochondrial energy-transducing membrane during thermal acclimation of the carp (*Cyprinus carpio* L.). *Biochim. Biophys. Acta.* **640**, 710-720.

Wu, B. J., Else, P. L., Storlein, L. H. and Hulbert, A. J. (2001). Molecular activity of Na^+/K^+ ATPase from different sources is related to the packing of membrane lipids. *J. Exp. Biol.* **204**, 4271-4280.

Yamamoto, Y., Fujisawa, A., Hara, A. and Dunlap, W. C. (2001). An unusual vitamin E constituent (α-tocomonoenol) provides enhanced antioxidant protection in marine organisms adapted to cold-water environments. *Proc. Natl. Acad. Sci. USA* **98**, 13144-13148.