The Effects of Acclimation Temperature on the Susceptibility of Biological Membranes in Fish Muscle to Lipid Peroxidation and the Role of Phospholipid Composition on Antioxidant Defenses in Vertebrates

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This dissertation titled

The Effects of Acclimation Temperature on the Susceptibility of Biological Membranes in Fish Muscle to Lipid Peroxidation and the Role of Phospholipid Composition on Antioxidant Defenses in Vertebrates

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ABSTRACT

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The Effect of Acclimation Temperature on the Susceptibility of Biological Membranes in Fish Muscle to Lipid Peroxidation and the Role of Phospholipid Composition on Antioxidant Defenses in Vertebrates (149 pp.)

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The rate of lipid peroxidation (LPO) of biological membranes depends on both phospholipid composition and the balance between pro- and antioxidant metabolism. At cold temperatures, fishes are enriched in phospholipids containing polyunsaturated fatty acids and ethanolamine headgroups, which should increase LPO susceptibility. LPO at cold temperatures may be exacerbated by increases in the production of reactive oxygen species accompanying enhanced oxidative capacities. The central hypothesis of Chapters 1 and 2 of the current work is that animals at cold and warm body temperatures may experience similar rates of LPO because the physiological changes at cold temperature (phospholipid remodeling and oxidative metabolism) may offset the shift in kinetics of LPO and ROS generation as a consequence of temperature. This work addresses the effects of acclimation temperature on pro- and antioxidant metabolism, phospholipid composition, and susceptibility of LPO using eurythermal fishes including killifish (Fundulus heteroclitus macrolepidotus), bluegill (Lepomis macrochirus), and striped bass (Morone saxatilis). In Chapter 1, killifish and bluegill were acclimated for 9 days and 2 months, respectively, to 5°C and 25°C. Following the acclimation period, activities of oxidative and antioxidant enzymes were quantified in glycolytic and cardiac muscle, and LPO susceptibility was measured at an intermediate temperature (15°C) in mitochondrial
and microsomal membranes. In Chapter 2, striped bass were acclimated to 7° and 25°C for six weeks and mitochondrial and sarcoplasmic reticular membranes were prepared from aerobic muscle fibers. Subsequently, LPO susceptibility was quantified in membranes at both intermediate (16°C) and physiological (acclimation) temperatures (7° and 25°C), and phospholipid composition was determined.

Phospholipid compositions of vertebrate taxa are also influenced by diet, yet the link between naturally-occurring dietary PUFA and antioxidant defenses has not been well-studied. Marine fishes are generally enriched in omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), relative to other animals. Most vertebrates (including marine fishes) are dependent upon dietary sources to obtain adequate levels of these compounds. Diets of marine vertebrates are enriched in omega-3 fatty acids, while those of terrestrial vertebrates are not. It is unclear whether the highly oxidizable content of omega-3 fatty acids in marine fishes requires a fortification of antioxidants that can both protect and repair (i.e., GPx4) these lipids from LPO. Chapter 3 addresses this question by quantifying activities of oxidative (citrate synthase) and antioxidant (glutathione peroxidase 1 & 4 and catalase) enzymes, GPx4 protein, and phospholipid composition of livers from seven vertebrate species including, hagfish (*Myxine glutinosa*), sea lamprey (*Petromyzon marinus*), dogfish (*Squalus acantbias*), killifish, longhorn sculpin (*Myxocephalus octodecemspinosus*), red-spotted newt (*Notopthalmus viridescens*), and mouse (*Mus musculus*).

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Overview

Temperature

The vulnerability of biological membranes to lipid peroxidation (LPO) is a function of both phospholipid composition and the balance between pro- and antioxidant metabolism. These physiological characteristics in ectotherms, like fishes, are impacted by habitat temperature. Exposure to cold temperature results in increased amounts of phospholipids containing polyunsaturated fatty acids (PUFA) and ethanolamine headgroups (PE). These phospholipid modifications should increase LPO susceptibility, as PUFA and PE are at an elevated risk of LPO, relative to lipids with lower degrees of unsaturation and phospholipids with choline headgroups. Furthermore, cold temperature induces mitochondrial proliferation and increases oxidative capacities, which when coupled with increased physical dissolution of oxygen at low temperatures, may enhance production of reactive oxygen species (ROS). As a result, animals at cold body temperatures are not only likely to have biological membranes which are inherently more susceptible to LPO, but also the potential to produce ROS at elevated rates, relative to animals at warm temperatures. Unless the reduction in the rates of LPO and ROS production at low temperatures counters the differences in susceptibility to LPO and the enhanced oxidative capacities, animals at cold body temperatures could experience increased LPO. In contrast, animals at warm body temperatures may be challenged by LPO and ROS generation unless lipid modifications and lower mitochondrial contents offset the elevated rates of LPO and ROS production at warm temperature.
In Chapter 1, I evaluate the effects of temperature acclimation on enzymatic indicators of oxidative capacities and antioxidant defense, phospholipid composition, and susceptibility of biological membranes from glycolytic muscle to LPO in two eurythermal fishes following temperature acclimation. Killifish (*Fundulus heteroclitus macrolepidotus*) and bluegill (*Lepomis macrochirus*) were acclimated for 9 days and 2 months, respectively, to 5° and 25°C. In order to eliminate the possibility that variable activity levels of fishes between cold- and warm-acclimation tanks confound the response to temperature acclimation, killifish were also exposed to a nine day exercise regime. Using both temperature-acclimated and exercise-trained fishes, I measured the activities of oxidative (citrate synthase - CS and cytochrome c oxidase - CCO) and antioxidant (superoxide dismutase - SOD and catalase - CAT) enzymes in glycolytic (axial skeletal) and cardiac muscle. LPO susceptibility was quantified in mitochondrial and microsomal membranes from temperature acclimated fishes 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 consider simultaneously relative contributions of thermal acclimation and variable activity level to changes in oxidative capacities, antioxidant responses, LPO susceptibility, and membrane composition of an ectotherm.

Data from Chapter 1 reveal a possible mismatch between changes in oxidative capacities and both antioxidant defenses and susceptibility of membranes to LPO. Oxidative capacities are elevated in glycolytic muscle following cold acclimation, relative to warm counterparts, which indicates an increased potential for ROS production.
in this tissue at cold temperature. In contrast, no differences in oxidative capacity are observed between temperature groups in the heart. Increased oxidative capacity in glycolytic muscle of cold-acclimated killifish is not accompanied by comparable changes in the activities of the enzymatic antioxidants CAT or SOD. Differences in the routine activity of fish among temperature treatments (tested using a controlled exercise regime) do not affect oxidative capacities or activities of antioxidant enzymes. LPO susceptibility of membranes from temperature acclimated animals, measured at an intermediate temperature (15°C) with either TBARS or C11-BODIPY, is not altered by temperature acclimation. While TBARS is widely used in the comparative literature, the current study is the first, to my knowledge, to use C11-BODIPY to quantify rates of LPO within the field of comparative physiology. This new methodology can be adopted with confidence for use with either ectothermic or other comparative model species, because the results obtained using C11-BODIPY are validated by data from TBARS assays. Also, future studies which address the effects of acclimation temperature on pro- and antioxidant metabolism need not be concerned, at least in many cases, about differences in activity level among treatment groups.

In Chapter 2, I consider how temperature influences rates of LPO directly, and how rates of LPO may vary across a range of physiological temperatures. To examine these questions, I acclimated an ectothermic model organism, the striped bass (*Morone saxatilis*) to 7° or 25°C for a period of six weeks, and prepared mitochondrial and sarcoplasmic reticular (SR) membranes from oxidative muscle fibers. Rates of LPO were quantified in both membrane types at common (16°C) and physiological (acclimation)
temperatures (7° and 25°C) using the fluorometric probe C11-BODIPY. In addition, phospholipid composition (phospholipid class and species) of biological membranes was characterized. This study complements work in Chapter 1 by considering the effects of phospholipid remodeling and temperature itself on rates of LPO in order to determine if susceptibility to LPO is conserved over a range of physiological temperatures.

Data presented in Chapter 2 reveal that while cold acclimation induces substantial remodeling of phospholipids in both mitochondrial and SR membranes, the consequences of these changes on rates of LPO vary as a function of the normalization criterion employed. Membranes prepared from cold-acclimated fish have 1.2-fold higher unsaturation indices and 1.5-fold higher PE/PC ratios, relative to animals acclimated to warm temperatures. Rates of LPO (when expressed per mg protein) are significantly elevated in membranes prepared from cold-acclimated animals when measured at common temperatures, however, a definitive explanation for the functional significance of this increase in oxidized lipid-to-protein at cold temperatures is unclear. Avenues that remain to be explored include determining whether an increase in the amount of oxidized lipid in ectotherms imparts a deleterious effect on the function of membrane proteins and if so, which proteins. In contrast, no differences are detected in LPO when rates are normalized to phospholipid content between temperature acclimation groups despite the substantial phospholipid remodeling that occurs following acclimation to low temperature which should enhance rates of LPO in these animals. While I show in Chapter 1 that activities of the primary enzymatic antioxidants (SOD and CAT) are unchanged by temperature acclimation, it remains unclear what role low molecular
weight antioxidants (i.e., vitamin E) may play in protecting PUFA- and PE-rich membranes of cold-acclimated animals from LPO. On-going work will quantify the levels of vitamin E in mitochondria and SR in order to determine what role, if any, these compounds play in protecting membranes of temperature acclimated animals.

When rates of LPO (per protein content) are considered at the respective physiological (acclimation) temperatures of these animals, rates of LPO in cold-acclimated animals are only partially conserved (12% and 22% in mitochondria and SR, respectively). As a certain ‘peroxide tone‘ (i.e., a steady-state of oxidized lipid) may be required for routine cellular processes, it is unclear whether depressed rates of LPO at cold body temperature are protective (by limiting LPO of membranes enriched in PUFA and PE) or are detrimental (by limiting the production of lipid hydroperoxides and reducing peroxide tone) for these animals at their physiological temperatures. Future work will quantify endogenous phospholipid and cholesterol hydroperoxides in order to address this question.

**Native phospholipid composition**

It is widely observed that fishes, especially those inhabiting cold water, are enriched in omega-3 fatty acids such as eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6), relative to other animals. The marine food web is anchored by primary production by phytoplankton which can synthesize these highly unsaturated fatty acids *de novo*. With few exceptions, vertebrates (including fish) lack the ability to synthesize 18:3n-3 from which EPA and DHA are metabolized, and are therefore dependent upon dietary sources to obtain this compound. From this point, some fishes
can synthesize EPA and DHA from 18:3n-3, although most marine fish species cannot produce these compounds at physiologically relevant levels because they are deficient in one or more steps of the biosynthetic pathway. While most marine fishes are dependent upon dietary sources of EPA and DHA, this is generally not a problem since in most cases the diets of marine vertebrates are enriched in omega-3 fatty acids. In contrast, diets of terrestrial vertebrates (e.g., amphibians and mammals) often lack a substantial component of omega-3 fatty acid. As a result, it is unclear 1) whether marine fishes require an increase in antioxidant defenses (i.e., GPx4) that can both protect and repair the abundant PUFA content in their tissues from LPO or 2) if vertebrate species prioritize their antioxidant defenses in response to species-specific oxidative stresses.

In Chapter 3 I test the hypothesis that the enhanced oxidizability of PUFA in marine fishes requires fortification with antioxidants, in particular GPx4, which both protect lipids from LPO and repairs oxidized lipids within biological membranes. The central objectives of Chapter 3 were to examine the activities of enzymatic antioxidants (CAT, glutathione peroxidase - GPx1, and GPx4), GPx4 protein, pro-oxidant metabolism (oxidative capacity as indicated by the activity of citrate synthase [CS]), and phospholipid composition of liver tissue from a range of vertebrates including hagfish (Myxine glutinosa), sea lamprey (Petromyzon marinus), dogfish (Squalus acanthias), killifish (F. heteroclitus), longhorn sculpin (Myxocephalus octodecemspinosus), red-spotted newt (Notopthalmus viridescens), and mouse (Mus musculus), with an emphasis on vertebrates occupying marine habitats. This study is the first to 1) quantify both GPx4 protein and enzymatic activity in ectothermic vertebrates, 2) determine the relationship
between GPx4 and phospholipid composition of vertebrates, and 3) evaluate the partitioning of enzymatic antioxidant defenses among vertebrate species.

Data collected in Chapter 3 indicate that the elevated PUFA content of marine vertebrates appears to require elevated activities and protein of GPx4, and also that vertebrates may prioritize antioxidant defenses to meet species-specific oxidative stresses. Both GPx4 protein and enzymatic activity are elevated in the majority of marine vertebrates examined, relative to values measured in terrestrial vertebrates. For example, killifish have up to 9-fold higher levels of GPx4 protein, relative to other vertebrates, including a 4.5-fold increase when compared to mouse. Further, killifish, dogfish, and hagfish (all marine fishes) show an average 7-fold higher level of GPx4 enzyme activity, when compared to mouse. Since other antioxidant enzymes (including GPx1 and CAT) are not similarly elevated, these data when taken together are particularly indicative of the importance of GPx4 in marine species. Additionally, phospholipids extracted from the livers of killifish, dogfish, and longhorn sculpin (all marine fishes) have 1.2-fold higher membrane unsaturation (an indicator of PUFA content) than those measured in terrestrial counterparts (mouse). When GPx4 activity is plotted against membrane unsaturation for all vertebrates tested, a significant correlation is detected, while no relationships are apparent between GPx4 activity and either oxidative capacity or phylogeny. Therefore PUFA content, but not oxidative capacity or phylogeny, is likely to drive the expression and activity of GPx4 in vertebrates. When activities of GPx4 are considered relative to the activities of other antioxidant enzymes (e.g., GPx1), marine fishes possess elevated GPx4/GPx1 ratios, when compared to amphibians and mammals.
Higher ratios of GPx4/GPx1 activity indicate that species with an elevated PUFA content may preferentially partition resources (e.g., selenium) more evenly between these antioxidant enzymes in order to better match antioxidant defenses to PUFA content. Future work should utilize controlled feeding studies to describe more definitively the relationship between PUFA content and GPx4 activity, as well as attempt to determine the regulatory pathways by which PUFA and GPx4 expression are linked.
CHAPTER 1: TEMPERATURE ACCLIMATION ALTERS OXIDATIVE CAPACITIES AND COMPOSITION OF MEMBRANE LIPIDS WITHOUT INFLUENCING ACTIVITIES OF ENZYMATIC ANTIOXIDANTS OR SUSCEPTIBILITY TO LIPID PEROXIDATION IN FISH MUSCLE

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 for 9 days and 2 months, respectively to 5 and 25°C. 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 polyunsaturated 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.

**INTRODUCTION**

Aerobic organisms face challenges associated with the formation of reactive oxygen species (ROS) including superoxide (O$_2^-$), hydroxyl radical (-OH), and the peroxyl radical (ROO'), 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 weight antioxidants (*e.g.* glutathione, vitamins E and C) and antioxidant enzymes (*e.g.*, superoxide dismutase and catalase). Superoxide dismutase (SOD)
catalyzes the dismutation of the superoxide radical and catalase (CAT) eliminates H$_2$O$_2$ 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/adaption 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 (2002) 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. Similarly, O‘Steen and Bennett (2003) 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). Variable activity patterns in ectotherms during temperature acclimation may be problematic for studies of 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 consider simultaneously 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*, (Linnaeus) (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). Fishes were held for two weeks in 15°C, 32 ppt seawater (killifish) or 15°C filtered, dechlorinated tap water (bluegill), to ensure all individuals were healthy, 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 hours, 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 which can experience extensive tidally-driven temperature fluctuations (8°C changes in water temperature in 30 min; Bulger, 1984), and exist over 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 O₂ saturation levels). Animals were maintained at ambient photoperiod (16 L:8 D – killifish, 12 L:12 D – bluegill) throughout the acclimatory period.

**Exercise acclimation**

During June 2007, sixty male killifish were collected from an estuarine tidal creek on Mt. Desert Island, Maine. 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 hours. Treatment fish were exercised daily for 6 hours at swimming rates of 2.25 body lengths (BL) \( \cdot \text{sec}^{-1} \), a value significantly below the species’ \( U_{\text{crit}} \) and at approximately the midpoint of the species aerobic swimming abilities (Fangue et al., 2008; exercise regime adapted from van der Meulen et al., 2006). The prescribed swimming speeds were comparable to sustained activity levels of warm-acclimated fish described above. Johnston and Moon (1980) showed that both axial glycolytic and aerobic fiber types of coalfish were active at swimming rates from 0.8–2.0 BL \( \cdot \text{sec}^{-1} \), 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 minutes at 1.15 BL \( \cdot \text{sec}^{-1} \), followed by 20 minutes at 1.75 BL \( \cdot \text{sec}^{-1} \), 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 0600-1200 and 1200-1800 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 L:8 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 mM 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 tissue-mizer (3 – five second bursts at low speed) and final homogenates were prepared in 7 mL Tenbroeck ground-glass homogenizers (5 passes). Hearts were homogenized directly in 2 mL Tenbroeck ground-glass homogenizers (3 passes). Homogenized tissues were sonicated on ice (2 – two second bursts for heart and 3 – four second 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, USA) and Pharmacia Ultrospec 3000 (Pharmacia Biotech, UK) 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, 1983), while activities of total SOD (assay modified from Crapo et al., 1978) and CAT (assay from 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 minutes 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 were used to prepare microsomal membranes as modified from Vornanen et al. (1999). For both species, skeletal muscle pieces were homogenized in 9 volumes of homogenization medium (100 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, and 20 mM 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 3100g_{av} for 30 minutes. 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 100,000g_{av} for 60 minutes. All centrifugation steps were performed at 4°C. Resulting pellets, containing microsomal membranes, were resuspended in < 1 mL 10 mM Tris-HCl (pH 7.6) using a Tenbroeck homogenizer and frozen immediately in liquid nitrogen.

Mitochondria were isolated from 2 to 3 g of skeletal muscle following a modification of Moyes (1989). Tissues were homogenized in 9 volumes of ice-cold homogenization medium (140 mM KCl, 20 mM Hepes, 10 mM EDTA, 5 mM MgCl₂, 0.5% BSA at pH 7.1) using 3 successive passes of a loose and then tight fitting Potter-Elvehjem homogenizer. Crude homogenates were centrifuged at 800g_{av} for 5 minutes.
Resulting supernatants were filtered, respun, filtered as previous, and further centrifuged at 9000g<sub>av</sub> for 10 minutes. 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 mM 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<sup>2+</sup> (as FeSO<sub>4</sub>) and ascorbic acid (AsA) (Haberland et al., 1996).

The methods described in Uchiyama et al. (1978) as modified by Pamplona et al. (1996) for TBARS were used in the current study. Briefly, bluegill microsomes were diluted to 1 mg protein·ml<sup>-1</sup> in 50 mM potassium phosphate buffer (pH 7.4). Induction was initiated by mixing membrane samples, Fe<sup>2+</sup> (0.2 mM), and AsA (1.6 mM) in a volume ratio (2:1:1). LPO was allowed to proceed for 6 hours at room temperature (approximately 23 °C) and was sub-sampled every 60 minutes. 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<sup>2</sup> ≥ 0.89 for all samples) as Δ MDA concentration · Δ minute<sup>-1</sup> were used to calculate final LPO rates with an extinction coefficient of 155 A·l·mmole<sup>-1</sup>. 
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 mM 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 mM in 100% ethanol) was diluted to 10 μM using 20 mM Tris-HCl (pH 7.4). Diluted membranes (5 mls) were mixed with a small volume of the diluted probe solution to a final probe concentration of either 150 nM (killifish) or 300 nM (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 minutes. Subsequently, LPO was induced in bluegill microsomes by adding 30 μl each of 0.2 mM Fe²⁺ and 1.6 mM AsA (final concentrations of 2.3 and 17.5 μM, respectively) and in killifish membranes by adding 2.5 μl each of the inductant solutions (final concentrations of 0.19 and 1.4 μM, respectively) to 2.5 mls 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 $\Delta$ fluorescent intensity $\cdot \Delta$ minute$^{-1}$. An extinction coefficient of 139,444 A·l·mole$^{-1}$ 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. (1970).

**Lipid extraction**

Lipids were extracted from killifish mitochondrial and microsomal preparations in the presence of chloroform and methanol according to 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. (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 0–12, with a maximum value of 12 which would represent a phospholipid containing two fatty acids chains with 6 double bonds apiece: $\text{UI} = (0 \times \text{mols}\% \text{ of fatty acids containing no double bonds}) + (1 \times \text{mols}\% \text{ of fatty acids containing one double bonds}) + (2 \times \text{mols}\% \text{ of fatty acids containing two double bonds}) \ldots (12 \times \text{mols} \% \text{ of fatty acids containing 12 double bonds})$.

**Statistical analyses**

Mean enzymatic responses (units min$^{-1}$ gram wet tissue weight$^{-1}$) and LPO susceptibility (normalized to phospholipid content) were compared between acclimation groups using parametric unpaired $t$-tests (JMP5; SAS, 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 (3 comparisons) or 0.0125 (4 comparisons). All other data were evaluated relative
to α = 0.05. Unless otherwise noted, data are presented as mean ± 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 to 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 (Figs. 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_{112} = -2.77; p = 0.016) varied significantly among
individual phospholipids of mitochondria (Table 1), the total amount of saturated phospholipids (SFA) were 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 less unsaturated PC 36:2 ($t$-test; $t_{1,6} = 2.84; p = 0.029$) relative to membranes from the cold-acclimated group (Table 1). 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 containing 2 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 UI of microsomes, were altered by temperature acclimation. When UI's 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, *Fundulus heteroclitus macrolepidotus*, 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. 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 ectothermic 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. (2003) in liver tissue from a 10°C-acclimated zoarcid, *Zoarces viviparous*. The magnitude of the observed changes 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 – Grim and Crockett, 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, and 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 1,2), as well as CAT activity measured in livers from rainbow trout, *Oncorhynchus mykiss*, acclimated to 5°C for 2 months (E. L. Crockett, unpublished), demonstrate that temperature acclimation does not
modulate antioxidant defenses. Similarly, Witas et al. (1984) and 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 in Cassini et al., 1993) be elevated in cold-adapted fishes. Speers-Roesch and Ballantyne (2005) 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. 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 currently are pursuing.

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. (2003) reported a positive relationship between CCO and both CAT and SOD in heart and skeletal muscle of cold-acclimated trout. 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, 1972 a & b; 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, 1972 a & b; 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 straight-forward 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 (Figure 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 weight, lipophilic antioxidant has been shown to be particularly abundant in mitochondrial fractions prepared from rat (Atkinson et al., 2008). The role of low molecular weight 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 the additional protection afforded 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 weight 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 phosphatidylethanolamines, mitochondria were less vulnerable to LPO than were preparations of microsomes. An explanation for these counterintuitive results may reside with low molecular weight 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 weight 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

CAT – catalase
CCO – cytochrome c oxidase
CS – citrate synthase
ePC – ether phosphatidylcholine
LPO – lipid peroxidation
PC – phosphatidylcholine
PE – phosphatidylethanolamine
PI – phosphatidylinositol
PUFA – polyunsaturated fatty acids
ROS – reactive oxygen species
SM – sphingomyelin
SOD – superoxide dismutase
TBARS – thiobarbituric acid-reactive substances
UI – unsaturation index
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Fig. 1. Activities of oxidative enzymes from thermally-acclimated killifish, Fundulus heteroclitus glycolytic (A) and cardiac muscle (B). Data are presented as mean ± s.e.m. Asterik 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 mean ± 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 mean ± s.e.m. *N*=9 (A), *N*=5 (B). CAT, catalase; SOD, superoxide dismutase.
Table 1. Phospholipid compositions of microsomal and mitochondrial membranes from glycolytic muscle of temperature-acclimated killifish

<table>
<thead>
<tr>
<th></th>
<th>Microsomes (n=4)</th>
<th>Mitochondria (n=7)</th>
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<tbody>
<tr>
<td></td>
<td>5°C</td>
<td>25°C</td>
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<tr>
<td>PC 32:1</td>
<td>1.48 ± 0.09</td>
<td>1.71 ± 0.05</td>
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<tr>
<td>PC 32:0</td>
<td>0.20 ± 0.02</td>
<td>1.13 ± 0.08*</td>
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<tr>
<td>PC 34:3</td>
<td>1.32 ± 0.34</td>
<td>1.32 ± 0.31</td>
</tr>
<tr>
<td>PC 34:2</td>
<td>5.91 ± 1.48</td>
<td>8.12 ± 1.05</td>
</tr>
<tr>
<td>PC 34:1</td>
<td>6.83 ± 1.70</td>
<td>7.81 ± 0.97</td>
</tr>
<tr>
<td>PC 36:6</td>
<td>1.51 ± 0.16</td>
<td>0.87 ± 0.10</td>
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<tr>
<td>PC 36:5</td>
<td>4.93 ± 0.25</td>
<td>4.09 ± 0.34</td>
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<tr>
<td>PC 36:4</td>
<td>4.94 ± 0.63</td>
<td>4.61 ± 0.19</td>
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<tr>
<td>PC 36:3</td>
<td>0.94 ± 0.25</td>
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<tr>
<td>PC 36:2</td>
<td>0.79 ± 0.15</td>
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<tr>
<td>PC 38:6</td>
<td>18.27 ± 0.85</td>
<td>17.45 ± 0.58</td>
</tr>
<tr>
<td>PC 38:5</td>
<td>3.78 ± 0.14</td>
<td>3.38 ± 0.49</td>
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<tr>
<td>PC 38:0</td>
<td>1.05 ± 0.04</td>
<td>0.92 ± 0.05</td>
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<tr>
<td>PC 40:8</td>
<td>1.40 ± 0.18</td>
<td>1.59 ± 0.12</td>
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<td>PC 40:7</td>
<td>3.42 ± 0.22</td>
<td>4.00 ± 0.49</td>
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<td>PC 40:6</td>
<td>1.40 ± 0.07</td>
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<td>PC 44:12</td>
<td>1.57 ± 0.28</td>
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<tr>
<td>SM 22:1</td>
<td>0.78 ± 0.12</td>
<td>1.02 ± 0.17</td>
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<tr>
<td>SM 24:1</td>
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<tr>
<td>ePC 38:6</td>
<td>1.50 ± 0.03</td>
<td>1.50 ± 0.04</td>
</tr>
<tr>
<td>ePC 38:0</td>
<td>1.81 ± 0.23</td>
<td>1.45 ± 0.05</td>
</tr>
<tr>
<td>PE 38:6</td>
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<tr>
<td>PE 38:5</td>
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<td>PE 40:8</td>
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<tr>
<td>PE 40:7</td>
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<td>PE 40:5</td>
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<td>PE 42:10</td>
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<tr>
<td>PE 44:12</td>
<td>2.58 ± 0.59</td>
<td>1.98 ± 0.31</td>
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<td>PE 44:11</td>
<td>1.76 ± 0.23</td>
<td>1.30 ± 0.12</td>
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<td>PE 44:10</td>
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<tr>
<td>PI 38:4</td>
<td>1.72 ± 0.15</td>
<td>1.40 ± 0.17</td>
</tr>
<tr>
<td>PI 40:6</td>
<td>2.05 ± 0.36</td>
<td>1.87 ± 0.36</td>
</tr>
<tr>
<td>Unsaturation Index (UI)</td>
<td>492 ± 20</td>
<td>466 ± 15</td>
</tr>
<tr>
<td>PC/PE</td>
<td>5.19 ± 0.80</td>
<td>6.07 ± 0.75</td>
</tr>
</tbody>
</table>

Diacyl lipid composition data (headgroup total # of carbons:total number of double bonds) are presented as mean mols% ± SEM. Only molecular species present in amounts ≥ 1 mols% 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 mean ± 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 mean ± s.e.m. N=5.
REFERENCES


CHAPTER 2: SUSCEPTIBILITY TO LIPID PEROXIDATION MAY BE INFLUENCED BY ACCLIMATION TEMPERATURE IN MEMBRANES FROM SKELETAL MUSCLE OF STRIPED BASS (*MORONE SAXATILIS*)

**ABSTRACT**

Cold temperature typically enhances oxidative capacities and results in elevated PUFA content and PE/PC ratios in ectotherms, which could alter the susceptibility to lipid peroxidation (LPO). While it has been hypothesized that cells may defend some level of ‘peroxide tone’ (*i.e.*, steady state of lipid hydroperoxide production), it is unclear how physiological changes at cold temperature may influence rates of LPO (and therefore peroxide tone). The current study investigates changes in mitochondria and sarcoplasmic reticular membranes from the oxidative muscle of a model ectotherm, striped bass (*Morone saxatilis*), acclimated to 7°C or 25°C for six weeks. Cold-acclimation induces an average 1.2- and 1.5-fold increase in membrane unsaturation and ratios of phosphatidylethanolamine and phosphatidylcholine, respectively. LPO rates (per mg protein) are 1.4-fold (mitochondria) and 2.3-fold (SR) higher in membranes prepared from cold-acclimated animals and measured at an intermediate temperature (16°C). No differences are observed between acclimation groups when rates of LPO are normalized to phospholipid content. Thermal sensitivity of LPO is elevated by an average of 1.3-fold in membranes prepared from warm-bodied individuals over the high range of assay
temperatures (16°C-25°C) relative to samples from cold-acclimated animals. Finally, rates of LPO are elevated by an average of 3.2- (per mg protein) and 4.3-fold (per μmole phospholipid) in membranes prepared from warm-acclimated animals at 25°C (physiological temperature) compared to membranes from cold-acclimated animals measured at 7°C. On-going work will evaluate changes in vitamin E content following temperature acclimation, and also determine whether depressed rates of LPO at low temperature serve a protective function, or if it impairs cellular processes by limiting the production of LPO products.

Introduction

Aerobic organisms constantly produce a variety of reactive oxygen species (ROS) as by-products of aerobic metabolism, including superoxide (O$_2^-$), hydroxyl radicals (·-OH), and peroxyl radical (ROO`). In most cases, ROS production is closely balanced in cells by antioxidant defenses including antioxidant enzymes (e.g., SOD, CAT, GPx) and low molecular weight antioxidants (e.g., vitamin E and glutathione). If, however, ROS production overwhelms defense mechanisms, organisms may experience bouts of oxidative stress, which may induce lipid peroxidation (LPO) (Halliwell and Gutteridge 2007). LPO is unique among the types of damage that may be inflicted upon biological macromolecules. LPO self-propagates within a membrane because oxidized lipids damage other membrane lipids unless chain-breaking antioxidants (e.g., vitamin E and phospholipid hydroperoxide glutathione peroxidase) terminate the reaction (Imai and Nakagawa 2003; Niki et al. 2005; Halliwell and Gutteridge 2007; Traber and Atkinson 2007; Niki 2009). LPO can disrupt the integrity and structures of biological membranes,
because the process of LPO adds polar hydroperoxy groups to phospholipids and cholesterol within the otherwise hydrophobic core of the membrane. These LPO-induced changes in phospholipid structure can affect lipid-lipid and lipid-protein interactions, ultimately placing membrane integrity at risk (Kagan 1988; Kühn and Borchert 2002).

The body temperatures of most fishes reflect environmental temperatures, and as such, rates of almost all physiological and biochemical processes in fishes are dictated by temperature (Hochachka and Somero 2002). Fishes undergoing temperature acclimation/adaptation maintain homeostasis in part by remodeling biological membranes (Hazel and Williams 1990) and altering their metabolic physiology (Guderley 2004). Membranes from cold-acclimated/adapted fishes are often highly unsaturated (Wodtke 1978; Crockett and Hazel 1995; Logue et al. 2000) and also possess increased ratios of phosphatidylethanolamine (PE)/phosphatidylcholine (PC) relative to warm-counterparts (Hazel and Landrey 1988). Interestingly, the extent of this phospholipid remodeling is not equal among subcellular membranes (reviewed in Hazel and Williams 1990; Hazel 1995). Mitochondrial membranes typically show a higher degree of homeoviscous efficacy than do other membrane fractions (Cossins et al. 1980; Cossins and Prosser 1982), whereas sarcoplasmic reticular membranes show no evidence of homeoviscous adaptation (Cossins et al. 1978), and modest changes in phospholipid composition (Cossins et al. 1978; Carey and Hazel 1989; Vornanen et al. 1999). Polyunsaturated fatty acids (PUFA - Holman 1954; Cosgrove et al. 1987) and PE (Wang et al. 1994) are more likely to undergo LPO when compared to less unsaturated lipids and those with choline headgroups. The increased susceptibility of LPO associated with lipid remodeling in
fishes may be exacerbated at low temperatures because cold-bodied fishes often possess elevated mitochondrial densities (Johnston and Maitland 1980; Egginton and Sidell 1989; Orczewska et al. 2010) and increased aerobic capacities (Guderley 2004) relative to animals at warm body temperatures. Although the relationship between rates of electron transport and ROS production is not linear (Brand 2000; Halliwell and Gutteridge 2007), animals at cold body temperatures may experience increased rates of ROS production when compared to warm counterparts. An increased potential for ROS production, coupled with elevated risk of LPO arising from lipid modifications, could leave cold-acclimated/adapted fishes at a greater risk of LPO relative to warm-acclimated/adapted counterparts.

One must consider the alternate prediction that higher contents of easily oxidized phospholipids and increased mitochondrial densities might not, however, be a risk for animals living at low body temperatures, because low temperature will depress rates of LPO directly and production of ROS will be slowed. The interplay between the effects of temperature and phospholipid composition on rates of LPO will determine to what extent, if any, that rates of LPO are conserved across a range of physiological temperatures. Unfortunately, no data are available to address this question in living tissue, however, work in the food sciences indicates that the relationship between temperature and LPO is exponential (Frankel 1998). It seems likely that, based on these data regarding temperature alone, rates of LPO would be elevated in tissues from warm-acclimated/adapted animals when measured at physiological temperature, relative to cold counterparts.
The deleterious effects of ROS, including the induction of LPO, are well-known (Niki et al. 2005; Halliwell and Gutteridge 2007; Niki 2009), yet there is a growing body of literature that suggests a beneficial and necessary role for LPO in organisms. Kühn and Borchert (2002) demonstrated that cells containing the 12/15 isoform of lipoxygenase (LOX) have the capability to catalyze a controlled oxidation of ester-linked fatty acids, including those of glycerophospholipids to produce phospholipid hydroperoxides (PLOOH). More recently, Kagan et al. (2004) reported that the oxidation of cardiolipin within the mitochondria (forming CLOOH) and phosphatidylserine (PS) in the plasma membrane (forming PSOOH) are necessary for apoptotic cell signaling. Production of CLOOH is important for the formation of the mitochondrial permeability transition pore and oxidation of PS externalizes these phospholipids and enables recognition by phagocytes (Kagan et al. 2004). From these results, it would appear that organisms likely defend some set-point of ‘peroxide tone‘ (i.e., steady state of lipid hydroperoxide content within a cell) in order to maintain proper cellular function (Brigelus-Flohé 1999; Halliwell and Gutteridge 2007), however, it is unclear how changes in temperature may impact the physiological levels of lipid hydroperoxides.

The current study evaluates whether the physiological changes associated with lipid remodeling result in changes to the rates of LPO in an ectothermic model, the striped bass (*Morone saxatilis*). Fish were acclimated to 7° or 25°C, and mitochondrial and sarcoplasmic reticular membranes were prepared from the oxidative muscle fibers. Rates of LPO were quantified in both membrane types at common and physiological (acclimation) temperatures using the fluorometric probe C11-BODIPY. In addition,
phospholipid composition (phospholipid class and species) of biological membranes was characterized. This study adds to previous work that considers the effects of phospholipid remodeling and altered oxidative capacities following temperature acclimation on the rates of LPO when measured at intermediate temperatures in order to determine if LPO is conserved over a range of physiological temperatures.

**Material and Methods**

*Fish maintenance and acclimation*

Juvenile striped bass, *Morone saxatilis*, were purchased from Delmarva Aquatics (Smyrna, Delaware) and housed at the Laboratory Animal Resources facility on the campus of Ohio University in two 1200-L recirculating brackish water tanks (~5 ppt) equipped with biological, chemical, and UV filtration. Water quality parameters were monitored daily, and were maintained with weekly partial water changes. Fish were fed daily to satiation with Zeigler's Finfish Gold floating pellets throughout the experiment.

Fish were maintained under ambient conditions (20 °C ± 1°C, 12:12 L:D cycle) for 12 months before being used in the acclimation experiments. Following the initial growth period, the temperature of each tank was decreased by 1°C/day to a final temperature of 17°C. This intermediate temperature set-point (relative to final acclimation temperatures of 7 and 25°C), was maintained for a period of two weeks to ensure a common thermal history of all experimental animals. After two weeks, the temperature of each tank was changed by ±1°C/day until the final acclimation temperatures (7 or 25°C) were reached. A six week acclimation period commenced once
animals had been exposed to the final acclimation temperatures for 24 hours and showed no obvious signs of stress.

**Tissue Sampling and Membrane Preparation**

Fish were stunned with a cranial blow and euthanized by cervical transection. The entire muscle mass was removed from the animal and muscle fiber types (*i.e.*, glycolytic white and oxidative red) were separated by dissection. Approximately 2-3 g (wet weight) of axial oxidative fibers were pooled from four individuals, and homogenized 10% (w/v) in a media containing 140 mM KCl, 20 mM Hepes, 10 mM EDTA, 0.1 mM EGTA, 5 mM MgCl$_2$, 0.5% BSA (pH 7.1) first with a Biospec tissuemizer (three 5 sec bursts at low speed), and followed by 5 passes of a Potter-Elvehjem homogenizer. A 1 mL aliquot of crude homogenate was saved for use in enzymatic marker analyses (see below), and the remaining volume was used to prepare mitochondrial membranes and microsomal membranes enriched in SR following the modifications of Moyes et al. (1989, 1992) and Vornanen et al (1999), respectively, described by Grim et al. (2010). Final membranes preparations were divided into 6 aliquots (5 large which were snap-frozen in LN$_2$ and stored at -70°C for further analysis and 1 smaller aliquot for marker enzyme analysis). All dissection, homogenization, and centrifugation steps were performed at 4°C to minimize sample degradation.

Activities of cytochrome *c* oxidase (CCO) for mitochondria (Wharton and Tzagoloff 1967 as modified by Hansen and Sidell 1983) and sarcoplasmic reticular Ca$^{2+}$-ATP-ase (SERCA) for SR (Simonides and van Hardeveld 1990 as modified by Riemenschneider and Sidell 2002) were measured to calculate enrichment factors in
order to ensure comparable purities of membrane preparations among acclimation groups. All enzymatic assays were conducted at 20°C using a Beckman 640 UV/VIS spectrophotometer equipped with a circulating water bath.

Susceptibility to lipid peroxidation

The susceptibility of prepared membranes to lipid peroxidation was quantified by following the oxidation rate of the fluorescent probe C11-BODIPY® 581/581 (4,4,-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid) as described previously (Grim et al 2010). C11-BODIPY is a fatty acid analogue which indexes LPO in real-time by recording shifts in probe fluorescence upon oxidation, and it is an ideal reporter for LPO of fish membranes because probe oxidation is sensitive to differences in contents of membrane PUFA (Drummen et al 2002).

Briefly, a C11-BODIPY solution (10 µM) was diluted to a final concentration of 148 nM by adding the required amount of 0.05 mg/ml membrane solution. The probe was incorporated within the membrane by stirring the mixed solution for 60 min in the dark at 4°C. Subsequently, LPO was induced in both mitochondrial and SR membranes using hydroxyl radicals produced by the Fenton reaction between Cu²⁺ (as CuSO₄) and cumene hydroperoxide (CumOOH). The induction system components, CuSO₄ and CumOOH, were added to 2.5 ml of the probe-membrane solution in a 1:4 ratio (Drummen et al 2002) to final concentrations of 13 and 52 µM. Probe oxidation was monitored for all membranes at 7, 16, and 25°C for 60 min with excitation/emission wavelengths of 568/590 nm, respectively. Linear portions of the decay slope were recorded as the rate of LPO (Δ fluorescence intensity Δ min⁻¹), and an extinction coefficient of 139,444 A l mol⁻¹
1 cm\(^{-1}\) was used in all calculations (Drummen et al. 2004). Probe oxidation was detectable in the absence of inductant for both membrane types at all assay temperatures. Seventy-percent of this background rate of oxidation was inhibited in the presence of 0.01% butylated hydroxytoluene, while the remaining 30% is unexplained. In all cases, total background rates of probe oxidation were subtracted from rates in the presence of LPO induction system in all final calculations. Quantifiable rates of LPO indicated the susceptibility to LPO in the membrane preparations. Rates of LPO were normalized to contents of phospholipid content by measuring hydrolyzable phosphate (Rouser et al 1970), mg protein (Smith et al 1985), and 3) Unit enzyme activity (CCO or SERCA) product quantified spectrophotometrically.

**Lipid extraction**

Lipids were extracted from prepared mitochondrial and sarcoplasmic reticular membranes following the methods of Bligh and Dyer (1959). Lipid extracts were sent to the Kansas State University Lipidomics Research Center for analysis of phospholipid headgroup and molecular species. Membrane unsaturation index (UI) was calculated following the modification of Hulbert et al. (2007) described in Grim et al. (2010) to account for the potential 12 double bonds present in diacyl phospholipids, relative to the 6 in individual fatty acids:  

\[
UI = (0 \times \text{mol}\% \text{ phospholipids with zero double bonds}) + (1 \times \text{mol}\% \text{ phospholipids with one double bond}) + \ldots + (12 \times \text{mol}\% \text{ phospholipids with 12 double bonds}).
\]
**Statistical Analyses**

LPO susceptibility (as Δ fluorescence intensity Δ min⁻¹ normalized to either μmole phospholipid, mg protein, or Unit of CCO/SERCA activity), enrichment factors, and all phospholipid data were compared between temperature acclimation groups using parametric unpaired *t*-tests (JMP5). Data not meeting assumptions of normality or homogeneity of variance were compared using Wilcoxon ranks sums or Welch ANOVA, respectively (JMP5; SAS Institute Inc.). All statistical conclusions were based on alpha values (α) of 0.05. Unless otherwise noted, data are presented as means ± standard error of the mean (s.e.m.).

**Results**

*Mitochondrial and SR Membrane Enrichment*

No significant differences in the relative enrichment are observed between temperature acclimation groups for either mitochondria (Wilcoxon rank sums; *z*₉₉ = 1.10; *p* = 0.27) or SR (*t*-test; *t*₁₄ = 2.069; *p* > 0.05) prepared from oxidative muscle fibers (Table 1). Enrichment factor analysis reveals that preparative centrifugation steps result in a mean 6.3-fold mitochondrial enrichment, while prepared SR were enriched by an average of 8.3-fold, relative to crude homogenates.

*Susceptibility to Lipid Peroxidation*

When normalized per mg protein, rates of LPO (corrected for autoxidation) in membrane fractions prepared from oxidative (red) muscle of cold-acclimated *M. saxatilis* are higher by an average of 1.4- and 2.1-fold in mitochondria and SR at all assay temperatures, respectively, relative to warm counterparts (Figure 1A; *t*-test; *p* < 0.05 and...
Figure 2A; t-test; p < 0.01). When susceptibility to LPO is normalized to μmole phospholipid, however, rates of lipid peroxidation are comparable between temperature acclimation groups, regardless of assay temperature (Figure 1B,2B). Susceptibilities to LPO (normalized to Unit of CCO activity) also do not vary in mitochondria prepared from the acclimation groups at any assay temperature (Figure 1C). In contrast, rates of LPO are significantly increased in SR prepared from cold-acclimated animals at 7° and 16° C assay temperatures per Unit of SERCA activity (Figure 2C).

**Thermal Sensitivity of Lipid Peroxidation**

Trends in the thermal sensitivities of LPO, as expressed by the temperature coefficient ($Q_{10}$), are similar in both mitochondria and SR. No statistical difference is observed in $Q_{10}$ values of mitochondria from cold- and warm-acclimated individuals between assays temperatures of 7° and 16°C, at a mean value for mitochondria of 2.2 (Wilcoxon rank sums; $z_{44} = -1.33; p = 0.18$; Figure 3A). $Q_{10}$ values of SR are also similar over that same temperature range (7°-16°C) for membranes prepared from cold- and warm-acclimated animals with a mean of 3.0 ($t$-test; $t_{13} = 0.801; p > 0.05$; Figure 3B), but are elevated relative to values in mitochondria. $Q_{10}$ values of both mitochondria and SR over the higher range of assay temperatures (16°-25°C), however, vary significantly between temperature treatment groups. $Q_{10}$ values of mitochondria prepared from warm-acclimated *M. saxatilis* are 1.3-fold higher relative to those from cold-acclimated animals ($t$-test; $t_{13} = 3.822; p = 0.002$; Figure 3A). Similarly over the same temperature range, SR prepared from warm-acclimated animals have thermal sensitivities that are elevated by
1.2-fold when compared with values from cold-acclimated fish \((t\text{-test}; t_{13}=2.235; p < 0.05; \text{Figure 3B})\).

**Phospholipid Composition**

Temperature acclimation induces significant remodeling of phospholipid headgroups in both mitochondria and SR (Table 2). Although phospholipid compositions of mitochondria and SR are dominated by phospholipids with PE and PC headgroups, eleven other phospholipid “classes” are identifiable in both membranes, and many of these vary in response to changing temperatures (Table 2). As expected, mitochondrial and SR membranes prepared from cold-acclimated individuals are enriched in phospholipids with ethanolamine headgroups, relative to warm-acclimated counterparts (Table 2), which is accompanied by a concomitant decrease in the relative amount of PC. Consequently, mitochondrial membranes from cold-acclimated animals have ratios of PE-to-PC that are 1.6-fold higher relative to warm-acclimated individuals \((t\text{-test}; t_{14}=12.069; p < 0.0001)\), and similarly, SR membranes following cold-acclimation show a 1.3-fold increase in this ratio \((t\text{-test}; t_{12}=5.325; p < 0.0002)\).

A range of phospholipid species varying in contents of saturated and unsaturated fatty acids are impacted by temperature acclimation in both mitochondria and SR from oxidative muscle, with membranes from cold-acclimated animals generally showing significant enrichment in PUFA (Table 3). Mitochondria prepared from cold-acclimated animals are enriched by 1.3 to 1.8-fold in PC and PE with polyunsaturated fatty acyl chains including PC38:6, PC40:7, PE38:6, PE40:8, PE40:7, PE40:6, AND PE42:7 \((p < 0.001; \text{Table 3})\). SR undergoes a quantitatively similar, yet less diverse, enrichment in PC.
and PE with polyunsaturated fatty acyl chains cold-acclimation, as amounts of PC38:6, PC40:7, PE38:6, PE40:8, and PE40:7 are increased by 1.3 to 1.9-fold in membranes from cold-acclimated animals ($p < 0.0001$; Table 3).

Increasing amounts of PUFA in both mitochondria and SR following cold-acclimation significantly impact the unsaturation index (UI) of the membranes. The unsaturation of nine of the eleven phospholipid classes are affected by temperature acclimation (Table 4), with a 1.4-fold increase in UI of PE phospholipids from mitochondria following cold-acclimation ($t$-test; $t_{14}=24.707; p < 0.0001$), leading to a total increase in UI of 1.2-fold mitochondria prepared from cold-acclimated animals ($t$-test; $t_{13}=36.157; p < 0.0001$; Table 4). SR membranes from cold-acclimated animals also have higher UI values for nine of the eleven phospholipid types, with 1.2- and 1.3-fold increases in UI for PC and PE, respectively ($p < 0.0001$; Table 4), which results in an overall increase of 1.2-fold in SR from cold-acclimated individuals ($t$-test; $t_{13}=16.167; p < 0.0001$; Table 4).

**Discussion**

*Effects of acclimation temperature on the susceptibility to LPO*

It is clear from the current study that interpretations of LPO results can be affected by the normalization criterion employed, and as a result, it is best to examine rates of LPO relative to several membrane components. For example, rates of LPO normalized to mg protein are elevated in membranes prepared from cold-acclimated animals, relative to warm counterparts. Elevated rates of LPO (normalized to the content of membrane-associated proteins) indicate that for a given protein content, membranes from cold-
acclimated animals undergo increased oxidation. Although Kagan (1988) summarized more than a decades’ worth of work in which he and co-authors demonstrated changes in SERCA activity with LPO, further work is necessary to determine how the change in the balance of oxidized lipid/protein may affect the function of membrane-associated proteins in membranes from temperature-acclimated animals.

Rates of LPO (normalized to total membrane-associated protein) in mitochondria and SR prepared from cold-acclimated fish are 1.4- and 2.1-fold higher, respectively, than those from warm-acclimated counterparts when compared at common temperatures. In contrast, rates of LPO for the phospholipid portion of the membrane are similar for cold- and warm-acclimated animals at all assay temperatures. Finally, rates of LPO per Unit of CCO activity are comparable in mitochondrial membranes prepared from cold- and warm-acclimated individuals, while LPO rate (per Unit SERCA) is elevated by 1.7-fold in SR of cold-bodied animals, relative to warm-bodied counterparts. Comparisons of LPO rates (normalized to phospholipid content), at common temperatures indicate a possible fortification of membrane-associated antioxidant defenses at cold temperature, especially in mitochondrial membranes where ROS production may be highest.

When rates of LPO are evaluated relative to phospholipid content, no difference is observed between acclimation groups in either mitochondria or SR. These results may be explained in part by increased lipid-to-protein values following acclimation to cold temperature, relative to warm-acclimated counterparts, which is not surprising as increased intracellular and membrane phospholipid content is a widely-reported response in ectothermic animals exposed to cold temperature (Egginton and Sidell 1989; Hazel and
Williams 1990; Storelli et al. 1998). Nonetheless, per μmole phospholipid, animals at cold body temperatures still possess more PUFA and PE, relative to warm counterparts, which should result in elevated rates of LPO in membranes of these animals, yet rates of LPO are comparable between acclimation groups. While these paradoxical results reveal that membrane oxidation cannot be explained by phospholipid composition alone, they do indicate that other membrane components (i.e., vitamin E) may be enhanced in membranes of cold-acclimated animals.

While conclusions regarding the effects of temperature on rates of LPO are generally consistent within other normalization criterion, rates of LPO relative to Unit of enzyme activity reveal that mitochondria and SR may not be affected comparably. Rates of LPO (per Unit enzyme) were elevated in SR (activity of SERCA), but not mitochondria (activity of CCO), prepared from cold-acclimated animals, relative to warm counterparts. Comparable rates of LPO in mitochondria from cold- and warm-acclimated animals likely reflect the well-known mitochondrial proliferation and increase in oxidative capacity that accompanies cold temperature maintenance of metabolism (Egginton and Sidell 1989; Guderely 2004; Grim et al. 2010; Orczewska et a. 2010). Further, these data reveal a potential disconnect between the primary source of ROS production in tissues (i.e., oxidative phosphorylation) and the process of LPO in mitochondria. Although the relationship between electron flux and ROS production is not linear in nature (Brand 2000), it seems reasonable to assume that enhanced oxidative capacities in cold-acclimated animals should result in elevated ROS production, and
subsequently increased rates of LPO in these animals, relative to warm counterparts, yet no increase in LPO is observed (Figure 1C).

When rates of LPO are compared at physiological (acclimation) temperatures of 7°C and 25°C, however, rates of LPO (normalized to protein content) are elevated in warm-bodied animals measured at 25°C relative to cold counterparts measured at 7°C. This observation supports the hypothesis of Crockett (2008) that in vivo rates of LPO would likely be higher at warm temperatures. These rates can be used to calculate the degree to which cold-acclimated fish counter the reduction of rates of LPO that occurs at cold body temperatures. Compensation in rates of LPO for the current study are expressed as % compensation calculated by:

\[
\frac{(\text{Rate of LPO}_{\text{cold acc}} - \text{Rate of LPO}_{\text{warm acc}}) \text{ measured at 7 C}}{(\text{Rate of LPO}_{\text{warm acc}} \text{ measured at 25°C}} - \text{Rate of LPO}_{\text{warm acc}} \text{ measured at 7°C}) \times 100
\]

Mitochondria prepared from cold-acclimated animals display 12% compensation in rates of LPO (per mg protein), while LPO (per mg protein) is compensated to a greater extent (22%) in SR prepared from cold-bodied fish (Table 5). According to Precht’s definitions of compensation (1958), the compensatory response in both mitochondria and SR at cold-acclimation temperatures is only partial.

It is unclear why membrane fractions display different degrees of compensation for LPO (normalized to protein content) in the face of temperature change, however, as discussed below, a higher degree of compensation of LPO in SR may be necessary to maintain Ca^{2+} cycling (and thereby muscle contraction) at reduced body temperatures. The significant remodeling of both membrane unsaturation and PE/PC in SR following cold-acclimation (Tables 2-4), likely contributes to the partial compensation in LPO, as
these lipids are more oxidizable than membranes that are less unsaturated and have lower PE/PC values.

Although several studies have reported differences in LPO following temperature change by measuring LPO endproducts (Viarengo et al. 1991; Abele et al. 1998; Estevez et al. 2002; Malek et al. 2004; Bagnyukova et al. 2007; Heise et al. 2007), there is no consensus on whether temperature acclimation/acclimatization actually increases or decreases the extent of LPO (reviewed by Crockett 2008). Discrepancies in the responses of LPO to changing temperatures likely arise from different experimental approaches (e.g., acute vs. chronic exposure and acclimation vs. acclimatization), the use of tissues (e.g., liver) with highly complex ROS metabolism (e.g., ROS production from mitochondria and cytochromes P450), and the utilization of various assays (e.g., TBARS, isoprostanes, or C11-BODIPY). Thus far, only a few studies have addressed the effects of controlled temperature acclimation on the extent (Malek et al. 2004) and rates of LPO (Grim et al. 2010) in fish muscle. Results from aerobic muscle in the current study are consistent with previous work (Grim et al. 2010) that showed temperature acclimation generally does not impact the susceptibility of LPO when normalized to phospholipid content in muscular tissues between fish acclimated to either 5° or 25°C. Significantly increased rates of LPO per mg protein detected with C11-BODIPY in the current study, however, conflict with Malek et al. (2004), which reports no differences in malondialdehyde production (a secondary LPO product) per mg protein between 18° and 28°C-acclimated zebrafish (Danio rerio).
LPO is both beneficial and necessary for routine cellular processes of organisms, and as a consequence, it has been hypothesized that organisms may defend some level of ‘peroxide tone’, or lipid hydroperoxide (LOOH) content in their cells and tissues (Brigelius-Flohé 1999; Halliwell and Gutteridge 2007). The production of LOOH by LPO has been linked to a variety of physiological processes such as eicosanoid production, cell differentiation and maturation, and cell signaling including apoptosis (Brigelius-Flohé 1999; Kühn and Borchert 2002). More recently, Kagan et al (2004) described specific roles for controlled oxidation of mitochondrial (cardiolipin) and cytosolic (phosphatidylserine) phospholipids in intrinsic and extrinsic apoptosis. If one assumes that rates of LOOH production are in direct proportion with rates of LPO, then data from the current study would indicate that ‘peroxide tone’, or a steady concentration of LOOH, is not conserved in either mitochondrial or SR membranes over the range of physiological temperatures considered. It is unclear whether lower rates of LPO in cold-bodied individuals at physiological temperatures actually imparts some imbalance in routine cellular homeostasis by limiting the production of LOOH and thereby decreasing peroxide tone. It is possible, however, that depressed rates of LPO in these animals may actually serve a protective function by limiting apoptosis (via a reduction in LOOH production) or by defending the activity of membrane-associated proteins, as the activity of integral membrane proteins (e.g., sarcoplasmic reticulum Ca^{2+}-ATPase) can decrease with LPO (Kagan 1988; Dinis et al. 1993). There are no data available with which to discuss the former supposition, however, the latter is supported by data which show activation of SERCA at low concentrations of phospholipid hydroperoxides (PLOOH).
and subsequent decreases in the activity of this enzyme complex at high PLOOH concentrations (Kagan et al. 1988). As a consequence, it appears that a higher degree of compensation of LPO in SR may be necessary to maintain the sufficient rates of PLOOH production required for the activation of the enzyme, while limiting excessive PLOOH production to prevent an inactivation of SERCA and subsequent imbalance in the Ca$^{2+}$/ATP.

Interestingly, the process of LPO in membranes prepared from warm-acclimated animals is affected to a greater extent by temperature than membranes from cold-bodied individuals. Over the lower temperature range (7°-16°C), $Q_{10}$ values of LPO in both mitochondria and SR were statistically similar between acclimation groups, however, $Q_{10}$ are significantly elevated in membranes from warm-acclimated animals at higher assay temperatures (16°-25°C). While these data indicate that the process of LPO is affected by temperature in all membranes ($Q_{10} > 1$), they also reveal that both mitochondria and SR from cold-acclimated animals (between 16° and 25°C) are more resistant to oxidation in the face of changing temperature than are those from warm-acclimated individuals. The relative thermal insensitivity of membranes prepared from the tissues of cold-acclimated animals may reflect either an increased concentration of vitamin E in the membranes of these animals, or perhaps the accumulation of different vitamin E isoforms by cold- and warm-acclimated animals.

Increased thermal stability of LPO in mitochondria ($Q_{10} \sim 2$ regardless of temperature range), and $Q_{10}$ values in mitochondria that are lower than all values reported for SR, may be explained by differences in the vitamin E content of these membranes.
Although changes in the vitamin E content following temperature acclimation have yet to be considered, there is evidence for elevated vitamin E content in plasma (Gieseg et al. 2000.), but not muscle (Yamamoto et al. 2001) of cold-adapted fish relative to temperate/tropical counterparts. Additionally, vitamin E tends to concentrate in membranes with the greatest potential for ROS generation (Gavazza and Catalá 2006). Finally, Stillwell et al. (1996) reported that vitamin E preferentially associates with phospholipids containing the PUFA 22:6, and also that vitamin E protects model membranes from oxidation by two distinct mechanisms: 1) the traditional free radical scavenging mechanism which can delay the onset of detectable LPO and 2) a membrane structural function which decreases the availability of oxygen within the hydrophobic core and creates steric hinderance of radical chain reactions. On-going work will evaluate whether the increased thermal stability of LPO in mitochondria from cold-acclimated animals, and in mitochondria relative to SR may be explained by changes in the content of low molecular weight antioxidants (e.g., vitamin E) in these membranes following temperature change.

*Temperature-induced remodeling of biological membranes*

While mitochondria and SR in the current study show similar magnitudes of increase in both UI and PE/PC, it has been shown previously that not all membrane fractions respond similarly to cold-temperatures (Hazel and Williams 1990; Hazel 1995). The 1.6-fold increase in PE/PC of mitochondrial membranes from cold-acclimated animals in the current study aligns closely with the 1.5-fold change in PE/PC for reported for oxidative muscle from cold-acclimated carp (Wodtke 1981). The 1.3-fold difference in PE/PC
following cold acclimation in SR, however, is higher than previously reported for fish glycolytic muscle (Cossins et al. 1978; Carey and Hazel 1989). The changes in PUFA content of mitochondria from cold-acclimated striped bass in the current study are consistent with reports of high homeoviscous efficacy in this membrane fraction (Cossins et al. 1980; Cossins and Prosser 1982; Hazel and Williams 1990). In comparison, Cossins et al. (1978) reported only modest changes in PUFA content in SR prepared from glycolytic muscle of thermally-acclimated goldfish (increased PC 18:2 at the expense of 18:1 in cold-acclimated animals), which they indicate did not alter membrane fluidity. In contrast, SR prepared from cold-acclimated striped bass in the current study display a more balanced modification of PUFA, as a number of both PC and PE phospholipid species are elevated by cold temperature. Nonetheless, it appears that membrane unsaturation is only slightly increased by cold temperature in the current study (1.2-fold), relative to previous reports (1.1-fold, calculated from Cossins et al. 1978). As a result, it remains unclear whether the breadth of phospholipid remodeling in the SR of striped bass results in a change of membrane fluidity at cold temperature in this species, and if so, what the functional significance of this phenomenon may be.

Conclusions
Striped bass acclimated to 7°C undergo a significant phospholipid remodeling of their mitochondrial and sarcoplasmic reticular membranes relative to warm counterparts that should increase the susceptibility to LPO, yet the effects of temperature acclimation on rates of LPO vary as function of the normalization criterion employed. Rates of LPO are significantly elevated in mitochondria and SR from cold-acclimated fish when considered
relative to membrane-associated protein. The functional significance of an increased amount of oxidized lipid per mg protein in these membranes remains unclear, and should be addressed in future studies. In contrast, the observation that rates of LPO (normalized to the amount of phospholipid) are comparable between temperature acclimation groups in both mitochondria and SR, is likely a function of increased phospholipid content relative to membrane protein following cold acclimation. Finally, rates of LPO (per Unit enzyme activity) are elevated in SR but not mitochondria from cold-acclimated individuals, which indicate that activities of mitochondrial enzymes may be altered to a greater extent by temperature acclimation than those of the SR. When rates of LPO at physiological (acclimation) temperatures are compared between acclimation groups, rates are significantly lower in the cold-bodied animals, relative to warm-acclimated counterparts. As of now, it is unclear whether depressed rates of LPO at low temperature may represent a protective function, or if the resulting low levels of LOOH production may actually impair routine cellular process in these animals. These data indicate that the increased risk of LPO following cold-temperature induced remodeling of biological membranes is, in part, offset by the rate depressing effects of low temperature on LPO itself. Changes in low molecular weight antioxidants (e.g., vitamin E) following temperature acclimation, however, may also play a role in mitigating oxidative stress at low temperature. On-going work will quantify changes in vitamin E content in both mitochondria and SR used in the current study in order to address this question.
Acknowledgements

I would like to thank Tammy Mace for her invaluable efforts in fish maintenance. The assistance of Ms. Liz Simonik during fish dissection and membrane preparation was invaluable. This work was supported by a National Science Foundation (NSF) award to Elizabeth L. Crockett (IOS 0842624).
Figure 1. Rates of lipid peroxidation in mitochondrial membranes prepared from oxidative muscle of temperature acclimated striped bass, *Morone saxatilis*. LPO was induced at all assay temperatures (7, 16, and 25°C) with hydroxyl radicals produced by Fenton chemistry. * indicates significant difference between temperature treatment groups at a given assay temperature ($p < 0.05$). Minimum $n = 7$. Data are presented as mean ± s.e.m.
Figure 2. Rates of lipid peroxidation in sarcoplasmic reticular membranes prepared from oxidative muscle of temperature acclimated striped bass, *Morone saxatilis*. LPO was induced at all assay temperatures (7, 16, and 25°C) with hydroxyl radicals produced by Fenton chemistry. * indicates significant difference between temperature treatment groups at a given assay temperature ($p < 0.05$). Minimum $n = 7$. Data are presented as mean ± s.e.m.
Figure 3. Thermal sensitivity of LPO in mitochondrial (A) and sarcoplasmic reticular (B) membranes prepared from oxidative muscle of temperature acclimated striped bass, *Morone saxatilis*. LPO was induced at all assay temperatures (7, 16, and 25°C) with hydroxyl radicals produced by Fenton chemistry. * indicates significant difference between temperature treatment groups over a given range of assay temperatures (*p* < 0.05). Minimum *n* = 7. Data are presented as mean ± s.e.m.
Table 1: Enrichment factor analysis of mitochondrial and SR membrane preparations

<table>
<thead>
<tr>
<th></th>
<th>Relative Enrichment Factors</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7°C</td>
<td>25°C</td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>5.50 ± 2.63</td>
<td>6.99 ± 1.26</td>
<td></td>
</tr>
<tr>
<td>SR</td>
<td>9.27 ± 1.61</td>
<td>7.29 ± 2.17</td>
<td></td>
</tr>
</tbody>
</table>

Note. *n* = 8. Data are presented as mean ± s.e.m.
Table 2: Relative amounts of phospholipid classes in mitochondrial and sarcoplasmic reticular membranes from temperature acclimated striped bass, *Morone saxatilis*

<table>
<thead>
<tr>
<th></th>
<th>Mitochondria 7°C</th>
<th>Mitochondria 25°C</th>
<th>Sarcoplasmic Reticula 7°C</th>
<th>Sarcoplasmic Reticula 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyso PC</td>
<td>0.55 ± 0.09</td>
<td>0.31 ± 0.03*</td>
<td>0.10 ± 0.00</td>
<td>0.12 ± 0.01*</td>
</tr>
<tr>
<td>SM/DSM</td>
<td>2.00 ± 0.10</td>
<td>2.85 ± 0.07*</td>
<td>3.08 ± 0.06</td>
<td>4.81 ± 0.14*</td>
</tr>
<tr>
<td>ePC</td>
<td>3.72 ± 0.08</td>
<td>5.26 ± 0.08*</td>
<td>4.42 ± 0.07</td>
<td>5.56 ± 0.17*</td>
</tr>
<tr>
<td>PC</td>
<td>44.03 ± 0.84</td>
<td>53.45 ± 0.34*</td>
<td>57.29 ± 0.63</td>
<td>59.21 ± 0.40+</td>
</tr>
<tr>
<td>Lyso PE</td>
<td>0.62 ± 0.05</td>
<td>0.28 ± 0.01*</td>
<td>0.15 ± 0.01</td>
<td>0.13 ± 0.00*</td>
</tr>
<tr>
<td>ePE</td>
<td>2.98 ± 0.05</td>
<td>2.26 ± 0.02*</td>
<td>2.32 ± 0.05</td>
<td>1.94 ± 0.05*</td>
</tr>
<tr>
<td>PE-cer</td>
<td>0.01 ± 0.00</td>
<td>0.00 ± 0.00*</td>
<td>0.01 ± 0.00</td>
<td>0.00 ± 0.00*</td>
</tr>
<tr>
<td>PE</td>
<td>42.58 ± 0.46</td>
<td>31.58 ± 0.26*</td>
<td>26.69 ± 0.64</td>
<td>21.66 ± 0.55*</td>
</tr>
<tr>
<td>PI</td>
<td>2.81 ± 0.13</td>
<td>2.86 ± 0.07</td>
<td>4.63 ± 0.07</td>
<td>5.54 ± 0.11*</td>
</tr>
<tr>
<td>ePS</td>
<td>0.01 ± 0.00</td>
<td>0.03 ± 0.00*</td>
<td>0.03 ± 0.00</td>
<td>0.06 ± 0.00*</td>
</tr>
<tr>
<td>PS</td>
<td>0.23 ± 0.05</td>
<td>0.73 ± 0.04*</td>
<td>0.95 ± 0.03</td>
<td>1.50 ± 0.05*</td>
</tr>
<tr>
<td>PA</td>
<td>0.05 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.25 ± 0.02</td>
<td>0.30 ± 0.02*</td>
</tr>
<tr>
<td>PG</td>
<td>0.31 ± 0.02</td>
<td>0.35 ± 0.01*</td>
<td>0.13 ± 0.00</td>
<td>0.16 ± 0.01*</td>
</tr>
</tbody>
</table>

**Total** | **100%**          | **100%**          | **100%**                  | **100%**                  |

Note. Minimum \(n=7\). Data are presented as mean mols% ± s.e.m. + indicates a significant difference between temperature treatment groups within a membrane \((p < 0.05)\) and * indicates a significant difference between temperature treatment groups within a membrane \((p < 0.01)\).
Table 3: Relative amounts of phospholipid species in mitochondrial and sarcoplasmic reticular membranes from temperature acclimated striped bass, *Morone saxatilis*

<table>
<thead>
<tr>
<th></th>
<th>Mitochondria 7°C</th>
<th>Mitochondria 25°C</th>
<th>Sarcoplasmic Reticula 7°C</th>
<th>Sarcoplasmic Reticula 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC 32:1</td>
<td>1.20 ± 0.07</td>
<td>1.45 ± 0.03*</td>
<td>2.07 ± 0.07</td>
<td>2.25 ± 0.08</td>
</tr>
<tr>
<td>PC 34:3</td>
<td>1.87 ± 0.02</td>
<td>2.28 ± 0.06*</td>
<td>2.79 ± 0.11</td>
<td>2.30 ± 0.04*</td>
</tr>
<tr>
<td>PC 34:2</td>
<td>9.24 ± 0.23</td>
<td>12.53 ± 0.20*</td>
<td>12.72 ± 0.16</td>
<td>13.67 ± 0.29*</td>
</tr>
<tr>
<td>PC 34:1</td>
<td>2.40 ± 0.04</td>
<td>8.42 ± 0.20*</td>
<td>3.69 ± 0.13</td>
<td>9.75 ± 0.18*</td>
</tr>
<tr>
<td>PC 36:5</td>
<td>7.01 ± 0.14</td>
<td>5.14 ± 0.11*</td>
<td>8.51 ± 0.12</td>
<td>5.31 ± 0.20*</td>
</tr>
<tr>
<td>PC 36:4</td>
<td>2.17 ± 0.07</td>
<td>3.01 ± 0.03*</td>
<td>2.65 ± 0.04</td>
<td>2.80 ± 0.08</td>
</tr>
<tr>
<td>PC 36:3</td>
<td>3.86 ± 0.12</td>
<td>4.95 ± 0.09*</td>
<td>5.06 ± 0.10</td>
<td>4.79 ± 0.08*</td>
</tr>
<tr>
<td>PC 36:2</td>
<td>1.27 ± 0.03</td>
<td>2.34 ± 0.05*</td>
<td>1.76 ± 0.04</td>
<td>2.75 ± 0.06*</td>
</tr>
<tr>
<td>PC 38:6</td>
<td>6.20 ± 0.15</td>
<td>4.61 ± 0.14*</td>
<td>7.44 ± 0.11</td>
<td>5.02 ± 0.09*</td>
</tr>
<tr>
<td>PC 38:5</td>
<td>1.55 ± 0.03</td>
<td>1.57 ± 0.04</td>
<td>1.96 ± 0.03</td>
<td>1.67 ± 0.04*</td>
</tr>
<tr>
<td>PC 40:7</td>
<td>2.06 ± 0.056</td>
<td>1.22 ± 0.05*</td>
<td>2.22 ± 0.04</td>
<td>1.19 ± 0.06*</td>
</tr>
<tr>
<td>SM 16:0</td>
<td>--</td>
<td>--</td>
<td>0.38 ± 0.02</td>
<td>1.00 ± 0.07*</td>
</tr>
<tr>
<td>SM 24:0</td>
<td>1.15 ± 0.04</td>
<td>1.41 ± 0.04*</td>
<td>1.84 ± 0.05</td>
<td>1.78 ± 0.04</td>
</tr>
<tr>
<td>ePC 38:0</td>
<td>1.01 ± 0.034</td>
<td>0.91 ± 0.02*</td>
<td>1.14 ± 0.02</td>
<td>0.81 ± 0.02*</td>
</tr>
<tr>
<td>PE 34:2</td>
<td>1.98 ± 0.08</td>
<td>1.73 ± 0.02*</td>
<td>1.26 ± 0.05</td>
<td>1.34 ± 0.05</td>
</tr>
<tr>
<td>PE 36:5</td>
<td>2.49 ± 0.08</td>
<td>1.27 ± 0.01*</td>
<td>1.77 ± 0.07</td>
<td>1.07 ± 0.05*</td>
</tr>
<tr>
<td>PE 36:4</td>
<td>1.29 ± 0.04</td>
<td>1.03 ± 0.01*</td>
<td>1.03 ± 0.04</td>
<td>0.90 ± 0.03*</td>
</tr>
<tr>
<td>PE 36:3</td>
<td>1.48 ± 0.08</td>
<td>0.99 ± 0.02*</td>
<td>1.59 ± 0.07</td>
<td>1.01 ± 0.02*</td>
</tr>
<tr>
<td>PE 36:2</td>
<td>0.60 ± 0.03</td>
<td>1.06 ± 0.02*</td>
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<td>--</td>
</tr>
<tr>
<td>PE 38:6</td>
<td>11.62 ± 0.13</td>
<td>6.83 ± 0.06*</td>
<td>6.37 ± 0.18</td>
<td>4.24 ± 0.15*</td>
</tr>
<tr>
<td>PE 38:5</td>
<td>3.83 ± 0.06</td>
<td>2.82 ± 0.02*</td>
<td>2.21 ± 0.07</td>
<td>1.77 ± 0.06*</td>
</tr>
<tr>
<td>PE 38:4</td>
<td>0.88 ± 0.02</td>
<td>1.02 ± 0.01*</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PE 40:8</td>
<td>1.62 ± 0.02</td>
<td>1.23 ± 0.02*</td>
<td>1.10 ± 0.01</td>
<td>0.87 ± 0.02*</td>
</tr>
<tr>
<td>PE 40:7</td>
<td>5.11 ± 0.07</td>
<td>2.91 ± 0.04*</td>
<td>3.02 ± 0.06</td>
<td>1.89 ± 0.01*</td>
</tr>
<tr>
<td>PE 40:6</td>
<td>3.93 ± 0.07</td>
<td>4.25 ± 0.08*</td>
<td>2.18 ± 0.06</td>
<td>2.24 ± 0.06</td>
</tr>
<tr>
<td>PE 40:5</td>
<td>1.00 ± 0.02</td>
<td>1.07 ± 0.01*</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PE 42:7</td>
<td>1.34 ± 0.03</td>
<td>0.75 ± 0.02*</td>
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<td>--</td>
</tr>
<tr>
<td>PI 38:5</td>
<td>--</td>
<td>--</td>
<td>1.15 ± 0.02</td>
<td>1.36 ± 0.03*</td>
</tr>
<tr>
<td>PI 40:6</td>
<td>--</td>
<td>--</td>
<td>1.10 ± 0.02</td>
<td>1.28 ± 0.03*</td>
</tr>
</tbody>
</table>
Note. Minimum \( n = 7 \). Data are presented as mean mols\% ± s.e.m. \( + \) indicates a significant difference between temperature treatment groups within a membrane \( (p < 0.05) \) and \( * \) indicates a significant difference between temperature treatment groups within a membrane \( (p < 0.01) \). Data for phospholipids less abundant that 1.0 mols\% not shown.
Table 4: Unsaturation indices of individual phospholipid classes in mitochondrial and sarcoplasmic reticular membranes from temperature acclimated striped bass, *Morone saxatilis*

<table>
<thead>
<tr>
<th></th>
<th>Mitochondria 7°C</th>
<th>Mitochondria 25°C</th>
<th>Sarcoplasmic Reticula 7°C</th>
<th>Sarcoplasmic Reticula 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyso PC UI</td>
<td>1.03 ± 0.12</td>
<td>0.48 ± 0.05*</td>
<td>0.14 ± 0.00</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>SM/DSM UI</td>
<td>0.08 ± 0.01</td>
<td>0.12 ± 0.00*</td>
<td>0.10 ± 0.00</td>
<td>0.16 ± 0.00*</td>
</tr>
<tr>
<td>ePC UI</td>
<td>9.92 ± 0.26</td>
<td>14.28 ± 0.16*</td>
<td>11.76 ± 0.21</td>
<td>13.79 ± 0.14*</td>
</tr>
<tr>
<td>PC UI</td>
<td>169.91 ± 3.88</td>
<td>166.40 ± 1.64</td>
<td>211.02 ± 2.04</td>
<td>174.67 ± 2.86*</td>
</tr>
<tr>
<td>Lyso PE UI</td>
<td>2.09 ± 0.17</td>
<td>0.82 ± 0.05*</td>
<td>0.37 ± 0.01</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>ePE UI</td>
<td>7.38 ± 0.11</td>
<td>6.58 ± 0.07*</td>
<td>4.83 ± 0.11</td>
<td>4.95 ± 0.09</td>
</tr>
<tr>
<td>PE- cer UI</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00*</td>
</tr>
<tr>
<td>PE UI</td>
<td>243.37 ± 2.29</td>
<td>173.88 ± 1.63*</td>
<td>149.194 ± 3.39</td>
<td>116.73 ± 2.85*</td>
</tr>
<tr>
<td>PI UI</td>
<td>14.39 ± 0.66</td>
<td>13.44 ± 0.36</td>
<td>24.02 ± 0.36</td>
<td>26.67 ± 0.49*</td>
</tr>
<tr>
<td>ePS UI</td>
<td>0.03 ± 0.01</td>
<td>0.10 ± 0.01*</td>
<td>0.09 ± 0.01</td>
<td>0.16 ± 0.01*</td>
</tr>
<tr>
<td>PS UI</td>
<td>1.23 ± 0.23</td>
<td>3.44 ± 0.17*</td>
<td>4.70 ± 0.12</td>
<td>6.38 ± 0.25*</td>
</tr>
<tr>
<td>PA UI</td>
<td>0.15 ± 0.03</td>
<td>0.32 ± 0.02</td>
<td>0.93 ± 0.06</td>
<td>0.88 ± 0.05</td>
</tr>
<tr>
<td>PG UI</td>
<td>0.49 ± 0.02</td>
<td>0.67 ± 0.02*</td>
<td>0.21 ± 0.01</td>
<td>0.31 ± 0.02*</td>
</tr>
<tr>
<td><strong>Total UI</strong></td>
<td><strong>450.75 ± 1.39</strong></td>
<td><strong>378.67 ± 1.42</strong></td>
<td><strong>407.13 ± 2.58</strong></td>
<td><strong>347.29 ± 2.63</strong></td>
</tr>
</tbody>
</table>

Note. Minimum $n = 7$. Data are presented as mean mols% ± s.e.m. * indicates a significant difference between temperature treatment groups within a membrane ($p < 0.05$) and ** indicates a significant difference between temperature treatment groups within a membrane ($p < 0.01$). UI calculated following the modifications of Hulbert et al (2007) by Grim et al (2010).
Table 5: % Compensation in the rate of LPO at cold physiological temperatures following temperature acclimation

<table>
<thead>
<tr>
<th>% Compensation in the rate of LPO at cold physiological temperatures</th>
<th>Δ FI</th>
<th>Δ FI</th>
<th>Δ FI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ min·mg protein</td>
<td>Δ min·μmole PL</td>
<td>Δ min·Unit enzyme</td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>12%</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>SR</td>
<td>22%</td>
<td>---</td>
<td>15%</td>
</tr>
</tbody>
</table>

Note. Minimum \( n = 7 \). Data are presented as % compensation with three different normalization criteria (protein content, phospholipid content, and Unit of enzyme activity). See text for calculation details. Compensation cannot be calculated for rates of LPO normalized to phospholipid content (in either membrane) or Unit of CCO (mitochondria) because no differences were detected between acclimation groups when compared at common temperatures. FI/Δ min = slope of fluorescence decay of C11-BODIPY following induction of LPO.
Literature Cited


CHAPTER 3: ELEVATED POLYUNSATURATED FATTY ACIDS IN MARINE FISHES ARE PROTECTED BY VERY HIGH ACTIVITIES AND PROTEIN OF THE SELENOPROTEIN GPx4

Abstract

Biological membranes are protected from lipid peroxidation, in part by enzymatic antioxidants including catalase (CAT) and glutathione peroxidases (GPx1; GPx4). GPx4 is unique among them because it can directly detoxify phospholipid hydroperoxides independent of phospholipase A2. To date no studies have considered whether elevated levels of highly oxidizable polyunsaturated fatty acids (PUFA) in marine fishes requires enhanced GPx4, or how vertebrates in general may prioritize activities of antioxidant enzymes to meet species-specific stresses. The current study addresses these questions by examining the activities of pro- (citrate synthase) and antioxidant enzymes (CAT, GPx1, and GPx4) in livers of vertebrate taxa. In addition, GPx4 protein and phospholipid composition also were quantified. Vertebrates were sampled including hagfish (Myxine glutinosa), sea lamprey (Petromyzon marinus), spiny dogfish shark (Squalus acanthias), marine teleost fishes (saltmarsh killifish - Fundulus heteroclitus and longhorn sculpin - Myxocephalus octodecemspinosus), red-spotted newt (Notophthalmus viridescens), and mouse (Mus musculus). F. heteroclitus had 4.5-fold more GPx4 protein than mouse, and GPx4 activities were higher in 3 out of 5 marine taxa compared to mouse. In contrast,
activities of CAT and GPx1 were highest in red-spotted newt and mouse, respectively. GPx4 activity for all vertebrates was positively correlated with membrane unsaturation, but not oxidative capacity or phylogeny. GPx4/GPx1 activity ratios approached 0.5 and were elevated in the majority of marine fishes (approaching 0.5), relative to mouse. In total, these data support my hypothesis that elevated GPx4 in marine vertebrates is a function of high PUFA contents of these species, and by partitioning resources (i.e., selenium) among GPx isozymes, defenses are likely prioritized to meet species-specific demands.

**Keywords:** glutathione peroxidases, GPx4, enzymatic antioxidants, membrane unsaturation, membrane composition, polyunsaturated fatty acids

Most animals require a constant supply of oxygen, and as a result their cells regularly produce reactive oxygen species (ROS) as byproducts of metabolism. ROS can damage the phospholipids that make up the matrix of biological membranes by initiating lipid peroxidation (21). Lipid peroxidation (LPO) is a self-propagating process in which ROS-damaged lipids can themselves damage other lipids making the risk of LPO to lipids unique among ROS-induced damage to biological molecules. The potential for threat of lipid peroxidation is further confounded by the fact that lipids are not equally susceptible to LPO. Phospholipids which contain polyunsaturated fatty acids (PUFA) are at an elevated risk of LPO compared to phospholipids with lower degrees of unsaturation (10, 23). Unless unbalanced by cellular antioxidants, LPO can threaten membrane integrity (37), and can even expedite cell death (8).

Cells are protected from ROS-induced damage by a variety of antioxidant defenses including antioxidant enzymes. Both catalase (CAT) and a family of
selenoproteins called glutathione peroxidases (GPx1/4) can catalyze the detoxification of hydroperoxides (e.g., $\text{H}_2\text{O}_2$). While the latter also detoxifies organic hydroperoxides (e.g., cumene hydroperoxide), GPx4 is unique among all antioxidant enzymes because it can also directly repair phospholipid hydroperoxides within biological membranes (52) without cleavage of the damaged fatty acid via phospholipase A$_2$ (PLA$_2$). Kinetic modeling has demonstrated that the flux of phospholipid hydroperoxides through GPx4 is $10^4$-fold greater than through the PLA$_2$ pathway, and consequently it is believed that GPx4 is more efficient for neutralizing lipid hydroperoxides in membranes and protecting the integrity of biological membranes from LPO (1).

GPx4 enzymatic activity, protein, tissue-specific expression and stability have been characterized in cells and tissues of mammals including mice (38), humans (36, 52), and pigs (53). Biosynthesis of GPx4 is preferentially retained over other glutathione peroxidases in mammalian brain, testes, heart, and liver when selenium (a component of the active site in of GPx enzymes) is limiting (6). Perhaps not surprisingly, these same tissues contain high levels of PUFA (16, 17, 25). Together, these data emphasize the importance of GPx4 in mammals and provide evidence for a link between GPx4 and PUFA levels in these animals.

Tissues of marine fishes contain high levels of PUFA (12,30), relative to other vertebrate taxa, and consequently these animals may require additional protection from LPO afforded by GPx4. In contrast with the relatively large body of work in mammalian tissues and cells, GPx4 has not been characterized in other vertebrate groups. Basal expression of two GPx4 genes ($\text{gp}x4\text{a/b}$) have been reported for zebrafish (51) and carp
(22), yet, no studies have examined how gene expression may be linked to protein or enzymatic activity of GPx4 in tissues from fishes. Furthermore, no study has probed a possible relationship between elevated PUFA content of marine fish and GPx4 activity or protein.

I hypothesize that the enhanced oxidizability of PUFA in marine fishes may require additional antioxidant protection compared to the less unsaturated lipids in other vertebrate groups. The objectives of the current study were to examine the activity of enzymatic antioxidants (CAT, GPx1, and GPx4), GPx4 protein, pro-oxidant metabolism (oxidative capacity as indicated by the activity of citrate synthase [CS]), and phospholipid composition of liver tissue from a range of vertebrates including seven species representing a continuum from basal (jawless fishes) to more derived representatives (mice), with an emphasis on vertebrates occupying marine habitats. This study is the first to 1) quantify both GPx4 protein and enzymatic activity in ectothermic vertebrates, 2) determine the relationship between GPx4 and phospholipid composition of vertebrates, and 3) evaluate the partitioning of enzymatic antioxidant defenses among vertebrate species.
METHODS

**Tissue collection.** Livers were collected from seven vertebrate species spanning the entire vertebrate clade. Hagfish (*Myxine glutinosa*), sea lamprey (*Petromyzon marinus*), dogfish shark (*Squalus acanthias*), saltmarsh killifish (*Fundulus heteroclitus macrolepidotus*), and longhorn sculpin (*Myoxocephalus octodecemspinosus*) were sampled opportunistically from on-going research at Mount Desert Island Biological Laboratory (MDIBL). Red-spotted newt (*Notophthalmus viridescens*) and mouse (*Mus musculus*) in the C57B1/6J background were obtained from researchers at Ohio University. All animals were treated according to institutional IACUC protocols. Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until analyses.

**GPx4 protein.** Frozen liver tissues were homogenized 10% (w/v) in an ice-cold extraction medium consisting of Chelex®-treated PBS (pH 7.5) with 0.3% Triton-X 100 and 1 Complete-Mini® protease inhibitor tablet (Roche Diagnostics, Indianapolis, IN) for every 10 mls of extraction medium. Crude homogenates were divided into two aliquots for assays of GPx4 protein and enzyme assays (described below). Homogenates were prepared for immunoblotting by sonicating the homogenized material on ice (10 sec burst) to ensure all subcellular compartments were lysed. Samples were subsequently centrifuged for 10 min at a low speed (600g_{av}) to pellet cellular debris, and resulting supernatants were subjected to a final high speed spin (100,000g_{av}) for 60 min. All centrifugation steps were performed at 4°C.

GPx4 protein was quantified in all vertebrate groups using slight modifications of standard immunoblot techniques (27, 28). Briefly, the protein content of final
supernatants were determined using the Bradford Assay (BioRad, Hercules, CA). Initially, samples were diluted to 2.4 mg/ml protein with 10 mM Tris (pH 7.4). Finally, samples were diluted 2 parts sample to 1 part Laemmli sample buffer (containing 5% β-mercaptoethanol), and were heated to 95°C for 10 min. Six to twenty micrograms of total protein, depending on the species, and 15 μl of purified GPx4 were separated on SDS-page, 12% Tris-HCl pre-cast polyacrylamide gels (BioRad, Hercules, CA) for 30 min at 60 V followed by 90 mins at 100 V. Proteins were transferred to Immuno-blot PVDF membrane (BioRad, Hercules, CA) and membranes were blocked overnight at 4°C in 1% (w/v) Casein in TBS (Pierce Scientific, Rockford, IL). Membranes were then incubated for 60 min in anti-GPx4 primary rabbit polyclonal antibody purified from E. coli (human - ab16800) (Abcam Inc, Cambridge, IL) diluted to 1/1000 in 0.05% Casein solution. Subsequently, membranes were rinsed 3 times in TBS-T (0.1% Tween-20, pH 7.4), and incubated with alkaline-phosphatase-conjugated goat anti-rabbit IgG (1/3000 in 0.05% Casein, BioRad, Hercules, CA). Chemiluminescent signal (Immun-star chemiluminescent kit, BioRad, Hercules, CA) was detected by exposing membranes to Amersham-Pharmacia Hyperfilm (Pitaskaway, NJ) for a period of 2 min. Preliminary troubleshooting was required to ensure that the loaded volumes of both sample and purified GPx4 protein would not produce saturated bands. Purified GPx4 was run as a positive control on all gels, and was used to normalize data between membranes. Negatives were scanned and antibody binding was quantified using Quantity One® (BioRad, Hercules, CA). Recorded protein binding was normalized to both quantity of loaded sample protein and antibody binding to purified GPx4.
**Enzyme activity.** Whole liver tissue was homogenized as described above. Crude homogenates were kept ice-cold for a period of one hour during which samples were vortexed thoroughly every 15 mins to ensure cell compartments were lysed. Following this incubation period, homogenates were centrifuged for 10 min at 1,700 (CS and CAT) or 6600 g_{av} (GPx1/4), and resulting supernatants were used in all subsequent enzyme assays.

Enzyme activities were assayed with Beckman DU640 UV/VIS and Pharmacia Ultrospec 3000 spectrophotometers fitted with circulating water baths and a temperature-controlled cell holder. Activities of GPx1 and GPx4 (assay modified from 14, 33, respectively) and CAT (assay from 3) represented the enzymatic antioxidant response. Cumene-hydroperoxide and phosphatidylcholine-hydroperoxide (PCOOH) were used as substrates in assays for GPx1 and GPx4, respectively. PCOOH was prepared as previously described (36). CS activity was assayed according to Srere et al. (47) as modified by Hansen and Sidell (20). All enzyme activities were determined from assays performed in either duplicate or triplicate. GPx1, CAT, and CS were measured at 25°C for mice and 15°C for all other vertebrate groups. GPx4 was measured at 25°C for all species. Enzyme activities were compared directly at a common temperature (15°C) by adjusting enzyme activities measured at 25°C using a conservative Q_{10}=2. In addition to comparisons of individual enzyme activities between taxa, enzymatic activities were also used to calculate ratios of GPx4/GPx1 activity and GPx4/CAT activity for all taxa. GPx4/GPx1 and GPx4/CAT were used in order to test hypotheses regarding the prioritization of antioxidant defenses among taxa in response to species-specific stresses.
**Phospholipid composition.** Lipids were extracted from liver homogenates of samples in the presence of chloroform and methanol according to Bligh and Dyer (5). No newt liver samples remained for lipid extraction after other analyses were completed. Consequently, amphibian lipid class and fatty acid composition were meta-analyzed from Gili and Alonso (18) and Berner et al. (4), respectively. Phospholipid class and molecular species composition of total lipid extracts of all other species were analyzed by triple-quadrupole mass spectrometry by the Kansas State University Lipidomics Research Center. Unsaturation index (UI), which represents the average number of double bonds per 100 fatty acids, was calculated as modified from Hulbert et al. (26) to account for the total number of double bonds present in diacyl phospholipids, rather than individual fatty acids. Therefore double-bond number ranges from 0-12 with the maximum double bond number of 12 corresponding to a phospholipid containing two fatty acids chains with 6 double bonds in each. UI = (0 * mols% containing zero double bonds) + (1 * mols% containing one doubles bonds) + (2 * mols% containing two double bonds)…(12 * mols % containing 12 double bonds). All lipid composition data are reported as mols %, and only diacyl phospholipids more abundant than 2 mols % were included in analyses.

**Statistical analyses.** Species-specific mean GPx4 protein (units mg loaded protein\(^{-1}\))\(^1\), mean enzymatic responses (units min\(^{-1}\) mg total protein\(^{-1}\)), GPx4 activity/GPx1 activity, GPx4 activity/CAT activity, and phospholipid metrics (UI, PE/PC, and relative amounts of each lipid class) were compared using ANOVA or Kruskal-Wallis (JMP5, SAS Institute Inc, Cary, NC). Significant ANOVA/Kruskal-Wallis results were followed by Tukey’s Post-Hoc analysis to identify statistically
distinct subsets of data. Regression analysis was used to examine relationships between GPx4 protein and activity, and links between GPx4 activity with both UI and CS activity were investigated using correlation analysis. All statistical conclusions on enzyme data were based on Bonferroni-adjusted alpha values of 0.0125 (4 comparisons). All other data were evaluated using an alpha value of 0.05. Unless otherwise noted, data are presented as mean ± standard error of the mean (s.e.m.).

RESULTS

Pro- and antioxidant enzyme activities and GPx4 protein. Both GPx4 protein and enzymatic activity are elevated in most, but not all marine vertebrate livers sampled (Figs. 1A,B), relative to mouse. Western blot analysis revealed that killifish liver contain up to 9-fold higher levels of GPx4 protein than other vertebrates sampled, including a 4.5-fold increase relative to mouse (Welch ANOVA; \( F_{5,10.025} = 15.64; p < 0.001 \); Fig. 1A). Similarly, enzymatic activity of GPx4 is between 1.3- and 14-fold greater in killifish liver when compared to other vertebrates, with values 9-fold higher than mice (Welch ANOVA; \( F_{6,11.335} = 11.34; p < 0.0001 \); Fig. 1B). A positive relationship exists between GPx4 protein and enzymatic activity among the vertebrates examined (\( R^2 = 0.50 \); ANOVA; \( F_{1,33} = 32.62; p < 0.001 \); Fig. 1C). GPx4 protein is, however, undetectable in longhorn sculpin. Enzymatic activity of GPx4 in vertebrate liver is positively correlated with the unsaturation index (UI) of tissue lipids (Pearson correlation=0.65; \( p < 0.0001 \); Fig. 4).

Activities of other antioxidant enzymes are highest in livers sampled from red-spotted newts and mice. CAT activity is 1.7- to 6-fold higher in livers from red-spotted
newts, relative to all other vertebrates (Welch ANOVA; $F_{6,13.19}=15.00; p < 0.001$; Fig. 2A), while GPx1 is increased in mice by 6- to 64-fold (Welch ANOVA; $F_{5,8.86}=36.13; p < 0.001$; Fig 2B). Oxidative capacity (as indicated by CS activity) is highest in mice, killifish, and sea lampreys relative to all other vertebrates (Welch ANOVA; $F_{5,14.04}=144.73; p < 0.0001$; Fig. 3).

Ratios of GPx4 to either GPx1 or CAT activities vary significantly among the vertebrates tested (Figs. 5A,B). GPx4/GPx1 activity is elevated, relative to mouse, in 4 out of 5 marine vertebrates tested (Fig. 5A; $p < 0.0001$), and ranged from 0.385 (sea lamprey) to 0.004 (mouse). GPx4/CAT, in contrast, is extremely low in all vertebrates ($4.1 \times 10^{-4}$–$1.3 \times 10^{-5}$), and at maximum, GPx4 activity is 1/2500 of CAT (in killifish; Fig. 5B). Nonetheless, GPx4/CAT is significantly increased in killifish relative to all other vertebrates. In all marine vertebrates, GPx4/CAT is greater or equal to values reported for mice (Fig. 5A; $p < 0.001$).

**Phospholipid composition.** Vertebrate livers vary in distributions of phospholipid classes and individual phospholipid species (Tables 1 and 2, respectively). Lipid profiles of all vertebrates are dominated by phospholipids containing either choline or ethanolamine headgroups (Table 1), although the proportions of phospholipid classes vary among species. In the two jawless fishes, sea lampreys and hagfish, PE is largely replaced by ether-linked phosphatidylcholines (ePC – PC plasmalogenes) which comprise 43% and 37% of the total lipid extract from liver of hagfish and lamprey, respectively. The ePC contents in these groups are elevated by an average of 3.8-fold relative to other marine vertebrates and 31-fold greater than levels in mouse tissue (Welch ANOVA;
**F**_{5,8.50}=388.54; p < 0.001; Table 1). PE/PC values are highest in dogfish shark relative to other the other vertebrates sampled (Kruskal-Wallis; \( \chi^2 = 24.77; p < 0.001 \); Table 1).

Thirty-six phospholipid species are present in amounts \( \geq 2 \) mols\% for at least one vertebrate group (Table 2), and of these phospholipids, 27 contain polyunsaturated fatty acids. Seventy percent of these PUFAs are more abundant in the lipid extracts from livers of marine than terrestrial vertebrates. Elevated levels of PUFA lead to increased UI values (double-bond content) in 3 of the 5 species of marine vertebrates tested relative to mouse, including a maximum 1.3-fold higher UI observed in killifish, relative to mouse (Welch ANOVA; \( F_{5,10.94}=42.60; p < 0.001 \) Table 2). Although UI of newt could not be determined in the current study, UI values for two Australian frog species, *Cyclorana alboguttata* (296) and *C. australis* (233), as reported by Berner et al. (2009) would likely place amphibians (*i.e.*, newt) in the low UI group of the current study.

**DISCUSSION**

Capacities for glutathione-dependent reduction of phospholipid hydroperoxides (GPx4) in liver appear to be a function, at least partially, of the PUFA content of vertebrate animals. Marine fishes (including representatives from the Agnatha, Chondrichthys and Teleostei) contain high levels of GPx4 protein compared with the relatively low levels of GPx4 in terrestrial animals (Amphibia and Mammalia). This can be explained, in part, by the positive relationship between lipid unsaturation index (UI) and GPx4 enzymatic activity. Although the broad phylogenetic scope in the current study precludes the use of a predictive phylogenetic-based statistical approach, it appears that neither GPx4 activity nor UI are confounded by phylogeny, as mice group
statistically in all assays (excluding GPx1 enzyme activity) with a changing subset of more basal vertebrates. Additionally, no relationship is observed between oxidative capacity (activity of CS) and GPx4 activity (Pearson’s correlation=0.32) among vertebrates. These data, when interpreted together, imply a link between GPx4 and UI, but not between GPx4 and either oxidative capacity or taxonomic status.

It would appear that robust GPx4 may be requisite in tissues, organs and even organisms that are enriched in PUFA in order to counter the damage to biological membranes that would otherwise be particularly vulnerable to LPO. Comparisons of tissue-specific PUFA contents with GPx4, together with several in vitro feeding studies, indicate a coupling between membrane unsaturation and GPx isozymes, providing further support for our hypothesis that high levels of GPx4 in marine fishes represent a mechanism to protect the highly oxidizable PUFA in these animals. GPx4 activities in rat testes (17), retina (50), and adrenal gland (24) mirrors the abundance of PUFA of these tissues (43, 58, 43, respectively; summarized in Table 3). While results from feeding studies are equivocal, several reports demonstrate that mammals undergoing prolonged consumption of feeds fortified with fish oil have increased hepatic GPx enzymatic activity (likely to represent total GPx activity as H₂O₂ is a substrate of all GPx isozymes (6)), and also increased tissue levels of ω-3 PUFA including 20:5 and 22:6 (32, 45, 46, 55). More specifically, a 1.4-fold increase is reported in hepatic GSH-Px (glutathione peroxidase) activity in monkeys fed marine oil, relative to animals consuming corn oil diets (32). Similarly, GSH-Px activity of mouse liver is increased by an average of 1.4-fold following consumption of diets enriched in krill and fish oil,
relative to those with corn oil (55). On the other hand, reduced activities of hepatic GPx have been found after prolonged marine oil feeding (40, 56, 57), and no clear explanation rectifies the conflicting reports of GPx activity in feeding studies, even by the same authors (55-57). Since GPx1 and GPx4 can both detoxify soluble hydroperoxides (e.g., H$_2$O$_2$ which is commonly used in assays of GPx1 enzyme activity), it is unclear to which GPx isozyme the measured changes in these studies can be attributed. Because GPx4 in marine fishes is high, in the absence of similarly high levels of GPx1 activity (current study), it seems likely that the reported increases in GPx activity following marine oil feeding may reflect elevations in GPx4 activity.

Comparisons of GPx4 with GPx1 across a range of vertebrates may also shed light on how different species prioritize their defenses against oxidative stress. In the current study GPx4/GPx1 ratios are highest in many of the marine fishes compared to those in mouse (Fig.5A). Elevated GPx4/GPx1 ratios in marine fishes, compared with the lower ratios found in more derived vertebrates, indicate disparate partitioning of resources among GPx isozymes. The physiological requirements for different GPx isozymes become more evident when the stability of the isozymes is considered in the face of selenium depletion. When selenium is limiting, GPx4 (and to a lesser extent GPx1) is (are) preferentially retained, relative to other GPx isozymes, in both mammalian brain and testis (6), indicating selective partitioning of resources amongst GPx isozymes. Organisms may benefit from prioritizing the maintenance of different GPx isozymes, especially given the redundancy in selenium requirement and overlap in substrate compatibility between GPx1 and GPx4. Therefore, the vulnerability of PUFA in marine
fishes may warrant a more balanced partitioning of resources (e.g., selenium) between GPx4 and GPx1. This would provide protection against H$_2$O$_2$, while simultaneously mitigating lipid peroxidation associated with phospholipid hydroperoxides.

In contrast to the fundamental similarities between GPx isozymes GPx4 and GPx1, (e.g. selenium incorporation and significant substrate overlap), GPx4 and CAT have little in common aside from the ability to detoxify H$_2$O$_2$. Therefore, low GPx4/CAT values (Fig. 5B) for all vertebrates tested is likely not a metric for potential resource partitioning among species, but rather reflects the primary localization of catalase to peroxisomes, which are particularly abundant in liver (11). It has been hypothesized, however, that GPx1 and CAT work cooperatively in vivo to detoxify endogenous H$_2$O$_2$ with GPx1 (by virtue of its relatively small $K_m$ for H$_2$O$_2$) responding to eliminate lower concentrations of H$_2$O$_2$, while CAT (high $K_m$) is recruited to assist at higher concentrations of H$_2$O$_2$ (21). More recently, this relationship has been expounded upon by Baud et al. (2), who have proposed that GPx1 protects CAT in oligodendrocytes from irreversible inactivation by maintaining [H$_2$O$_2$] < 100 μM. It is unclear what role GPx4 plays relative to CAT, in vivo in other cells types, or how a more balanced complement of GPx isozymes in marine fishes may potentially affect the catalytic cooperativity between GPx1 and CAT.

While there is no immediately obvious explanation for why the marine teleost longhorn sculpin (Myxocephalus octodecimspinosus) possesses such low levels of GPx4 protein/activity in contrast with other marine fishes, there are several points that should be made. First, low GPx4 does not appear to be offset by elevated activities of other
enzymatic antioxidants CAT and GPx1. Second, preliminary work indicates that endogenous levels of a LPO product (malondialdehyde) is extremely low in sculpin compared with other teleosts examined (Crockett, unpublished). These data, in combination, indicate that the relatively high complement of unsaturated fatty acids in this species may be protected and/or repaired by one or more unmeasured antioxidants (either of low molecular weight or other antioxidant enzymes) or PLA2, respectively. Because longhorn sculpin are a relatively derived marine teleost (35), and duplicate GPx4 genes have been reported in teleosts (22, 51), there exists the possibility that a novel GPx4 splice variant (undetected by our methods) may be occupying the role traditionally associated with GPx4. In light of the apparent relationship between membrane unsaturation and GPx4, future studies may consider how the elevated levels of highly oxidized phospholipids of sculpin are protected against oxidation since GPx4 does not appear to occupy this antioxidant niche.

**Perspectives and Significance**

*Additional GPx4 function(s) in vertebrates*

In addition to protecting membranes from oxidative stresses, GPx4 has been implicated in wide-variety of biological functions in vertebrates. Although the earliest reports of GPx4 focused on its ability to detoxify complex lipid hydroperoxides (52, 53), several more recent reports have recognized additional functions for this protein, including roles as a structural protein during spermatogenesis, and as a regulator of apoptosis, eicosanoid production, and redox signaling (6, 9, 15, 29, 39, 49, 54). Many of the recently described additional functions for GPx4 are dependent, however, upon the
basic enzymatic activity of this protein, indicating that the antioxidant function of GPx4 is paramount to the suite of complex processes now linked to the protein. At the same time, not much is known about the fundamental mechanism(s) which drive(s) the range of GPx4 expression within tissues of an organism and/or between organisms within a taxonomic group. Results from the current study demonstrate a strongly correlative relationship between GPx4 and membrane unsaturation, perhaps indicating these other functions attributed to GPx4 may have evolved secondarily in response to the elevated need for antioxidant protection in PUFA-rich tissues (and organisms).

Conclusion

The broad substrate specificity of GPx4, including complex lipid hydroperoxides, suggests that elevated GPx4 activity may be necessary to protect the high content of PUFA in marine organisms from excess lipid peroxidation. The proposed link between GPx4 and PUFA content among vertebrates is strengthened by the strong correlation between GPx4 activity and UI among vertebrates in the current study. Since no phylogentic trends are apparent in either GPx4 protein or activity, we suggest that phylogeny plays little role in driving expression and activity of GPx4 in vertebrate organisms.

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Figure 1. GPx4 protein (A), GPx4 activity (B), and the relationship between GPx4 activity and protein (C) in livers from different vertebrate taxa. Killifish (FH), dogfish shark (SA), Atlantic hagfish (MG), red-spotted newt (NV), sea lamprey (PM), mouse (MM), and longhorn sculpin (MO); bars for all marine vertebrates are patterned throughout. Means ± SEM plotted (minimum n = 4 per species). Bars not connected by horizontal lines in 1(A) and 1(B) are significantly different as determined by Tukey post-hoc analysis ($p < 0.05$).
Figure 2. CAT (A) and GPx1 activity (B) among vertebrate taxa. Killifish (FH), dogfish shark (SA), Atlantic hagfish (MG), red-spotted newt (NV), sea lamprey (PM), mouse (MM), and longhorn sculpin (MO); bars for all marine vertebrates are patterned throughout. Means ± SEM plotted (minimum n = 4 per species). Bars not connected by horizontal lines are significantly different as determined by Tukey post-hoc analysis (p < 0.05).
Figure 3. CS activity among vertebrate taxa. Killifish (FH), dogfish shark (SA), Atlantic hagfish (MG), red-spotted newt (NV), sea lamprey (PM), mouse (MM), and longhorn sculpin (MO); bars for all marine vertebrates are patterned throughout. Means ± SEM plotted (minimum n = 4 per species). Bars not connected by horizontal lines are significantly different as determined by Tukey post-hoc analysis (p < 0.05).

Figure 4. Correlation between GPx4 activity and UI among vertebrate taxa. Killifish (FH), dogfish shark (SA), Atlantic hagfish (MG), sea lamprey (PM), mouse (MM), and longhorn sculpin (MO); minimum n = 4 per species.
Figure 5. GPx4/GPx1 (A) and GPx4/CAT (B) and enzyme activity among vertebrate taxa. Killifish (FH), dogfish shark (SA), Atlantic hagfish (MG), red-spotted newt (NV), sea lamprey (PM), mouse (MM), and longhorn sculpin (MO); bars for all marine vertebrates are patterned throughout. Means ± SEM plotted (minimum n = 4 per species). Bars not connected by horizontal lines are significantly different as determined by Tukey post-hoc analysis ($p < 0.05$).
Table 1. Relative abundance of phospholipid classes in vertebrate liver

<table>
<thead>
<tr>
<th></th>
<th>Myxine glutinosa</th>
<th>Petromyzon marinus</th>
<th>Squalus acanthias</th>
<th>Fundulus heteroclitus</th>
<th>Myoxocephalus octodeccemspinosus</th>
<th>Bufo arenarum*</th>
<th>Mus musculus</th>
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<td>n</td>
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<td>4</td>
<td>4</td>
<td>8</td>
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<td>31 ± 1.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>37 ± 5.9&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>36 ± 0.3&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>56 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55</td>
<td>59 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>% PE</td>
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<td>9 ± 1.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>27 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24 ± 1.0&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td>% PI</td>
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<td>6 ± 1.0&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>% PS</td>
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<tr>
<td>% SM</td>
<td>3.7 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.6 ± 0.6&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>3.6 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.1 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4</td>
<td>3.3 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>% PC plasmalogen</td>
<td>43 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37 ± 9.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.3 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>1.2 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.24 ± 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.73 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.38 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.54 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.46</td>
<td>0.38 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
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Mols %, total of 100% when lysoPC, lysoPE, ePE, PE-cer, and PA are included (data not shown). Levels not connected by the same letters are significantly different (Tukey’s post hoc). * Amphibian values measured in frog oocyte mitochondria and whole oocytes (Gili and Alonso, 2004).
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<th>Mus musculus</th>
<th>Fundulus heteroclitus</th>
<th>Myxine glutinosa</th>
<th>Myoxocephalus octodecemspinosus</th>
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<td>0.73 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.00 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.99 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.23 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.88 ± 1.09&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>PC 34:2</td>
<td>6.75 ± 0.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.46 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.30 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>3.82 ± 0.41&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.27 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.19 ± 0.02&lt;sup&gt;c,d&lt;/sup&gt;</td>
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<td>0.37 ± 0.07&lt;sup&gt;c,d&lt;/sup&gt;</td>
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<td>1.52 ± 0.26&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.86 ± 0.42&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.38 ± 0.05&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>0.09 ± 0.01&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>PE 40:6</td>
<td>2.13 ± 0.32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.87 ± 0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.11 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.11 ± 0.30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.41 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.09 ± 0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PE 42:7</td>
<td>0.05 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.46 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.10 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.47 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.17 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.07 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PI 38:5</td>
<td>0.35 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.60 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.82 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.97 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.36 ± 0.17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.87 ± 0.15&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PI 38:4</td>
<td>4.76 ± 0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.59 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.06 ± 0.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.60 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.85 ± 0.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.81 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Unsaturation Index (UI)</td>
<td>371 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>485 ± 8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>360 ± 23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>449 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>437 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>368 ± 22&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PC/PE</td>
<td>0.38 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.38 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.30 ± 0.01&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>0.54 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.73 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.24 ± 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>
Diacyl lipid composition data (headgroup total # of carbons:total number of double bonds) are presented as mean mols % ± SEM. Only molecular species present in amounts ≥ 2 mols% for at least one of the vertebrate species sampled are shown.

Unsaturation index (UI) was from Hulbert et al. (2007) as follows:  
\[ UI = (0 \times \text{mols}\% \text{ of fatty acids containing no double bonds}) + (1 \times \text{mols}\% \text{ of fatty acids containing one double bonds}) + (2 \times \text{mols}\% \text{ of fatty acids containing two double bonds}) \ldots (12 \times \text{mols}\% \text{ of fatty acids containing 12 double bonds}). \]

Significant ANOVA results were followed by Tukey’s post-hoc analysis. Superscript letters identify statistically distinct subsets of data.
### Table 3. Reported GPx4 enzyme activities and membrane unsaturation in rat tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>GPx4 (mU/mg protein)</th>
<th>UI</th>
<th>Sat/Unsat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testes</td>
<td>311</td>
<td>234</td>
<td>0.45</td>
</tr>
<tr>
<td>Retina</td>
<td>31.9-66.7#</td>
<td>243</td>
<td>0.93</td>
</tr>
<tr>
<td>Liver</td>
<td>23.8</td>
<td>139</td>
<td>0.77</td>
</tr>
<tr>
<td>Adrenal</td>
<td>11.49</td>
<td>196</td>
<td>0.74</td>
</tr>
<tr>
<td>Kidney</td>
<td>7.4</td>
<td>194</td>
<td>0.8</td>
</tr>
<tr>
<td>Heart</td>
<td>4.4</td>
<td>135</td>
<td>0.7</td>
</tr>
<tr>
<td>Lung</td>
<td>3.7</td>
<td>116</td>
<td>0.82</td>
</tr>
<tr>
<td>Brain</td>
<td>2</td>
<td>154</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Enzymatic activity of GPx4 estimated in selenium-adequate animals.

UI: Unsaturation Index (sum of percentages of individual fatty acids * # of double bonds).

Sat/Unsat: mols% saturated fatty acid/mols% unsaturated fatty acids from published phospholipid profiles.

(Data from: 17, 24, 43, 50, 58)
REFERENCES


47) **Srere PA, Brazil H, Gonen L.** The citrate condensing enzyme of pigeon breast muscle and moth flight muscle. *Acta Chem Scand* 17, S219-234.


Perspectives

LPO and Temperature

The susceptibility of biological membranes to LPO depends on more than the indices of phospholipid composition alone. Results in Chapters 1 and 2 indicate that neither fatty acid unsaturation nor ratios of PE/PC can be used to predict the rank order of LPO rates (when normalized to phospholipid content) in biological membranes from temperature-acclimated fishes. Based on previous studies using model lipids, biological membranes with high levels of PUFA and PE would be expected to oxidize more readily than membranes enriched in fewer unsaturated fatty acids and lower levels of PE (Holman 1954; Cosgrove et al. 1987; Wang et al. 1994). Yet the data presented herein indicate that when working with biological membranes, other membrane elements, in addition to phospholipid composition, must be taken into account in order to make successful predictions on membrane susceptibility to LPO. As shown in Chapter 1, rates of LPO (normalized to phospholipid) measured at common temperatures are actually lower in mitochondria, relative to microsomes, in spite of the observation that mitochondria contain higher levels of PUFA and PE. Similarly, data presented in Chapter 2 indicate that while both mitochondria and SR from oxidative muscle of cold-acclimated striped bass have higher membrane unsaturation and PE/PC ratios, rates of LPO (per phospholipid content) are unchanged from values measured in warm counterparts that are less unsaturated and have less PE. In total, these data show that in order to draw conclusions about the relationships between membrane oxidizability and higher-order physiological functions, rates of LPO in biological membranes must be measured directly, and not estimated from phospholipid composition.
It seems possible that the inaccuracies in the predicted oxidizability of biological membranes to LPO, based on lipid composition, may reflect the fact that most model membrane systems are constructed of model lipids alone. As a result, these estimates of membrane susceptibility cannot account for the contributions of other membrane components such as membrane-associated antioxidant defenses (*i.e.*, vitamin E), to membrane oxidizability. While on-going work will consider what role, if any, acclimation temperature plays in dictating vitamin E levels, it has been shown that adaptation to cold temperature results in higher levels of vitamin E relative to warm-bodied counterparts (Gesing et al. 2000). If effects of cold acclimation mirror those of cold adaptation, difference in vitamin E level between membranes in cold and warm acclimation groups may explain, in part, the comparable rates of LPO (measured at common temperatures) in Chapters 1 and 2 which reflect the apparent discrepancy between measured and predicted susceptibilities of membranes to LPO.

Data from Chapters 1 and 2 indicate that rates of LPO are not conserved across a range of body temperatures. Rates of LPO (regardless of normalization criterion) are on average 4.2- and 3.2-fold higher in mitochondrial and SR membranes, respectively, in warm-acclimated striped bass than cold-acclimated counterparts when rates are compared at respective physiological temperatures. Decreased rates of LPO at cold body temperatures (Chapter 2) seem to correspond with lower activities of antioxidant enzymes CAT and SOD in tissues at cold temperatures (inferred from enzyme activities measured at a common temperature in Chapter 1). Together these data would seem to indicate that cold-bodied animals may experience less oxidative stress, at physiological
temperatures, than animals at warmer temperatures. While it seems clear that temperature influences rates of LPO (and oxidative stress) at physiological temperatures, it has yet to be determined whether lower rates of LPO in cold-bodied animals are protective or detrimental. It has been proposed that a certain peroxide tone is necessary for the maintenance of routine cellular function. If this is true, it would indicate that animals at cold temperature may be unable to maintain comparable rates of cellular function, relative to warm counterparts, at physiological temperatures. On-going work will evaluate rates of endogenous phospholipid and cholesterol hydroperoxide production among cold- and warm-acclimated animals in order to address the question. If differences in LPO do indeed result in variable rates of endogenous lipid hydroperoxide production, then future work will be necessary to examine what, if any, physiological processes are negatively impacted by the reduced hydroperoxide production.

**Native Phospholipid Composition**

Although the antioxidant function of GPx4 has been recognized since the early 1990’s, several more recent reports have described additional functions for this protein, including roles as a structural protein during spermatogenesis, and as a regulator of apoptosis, eicosanoid production, and redox signaling (Ursini et al. 1999; Seiler et al. 2008; Liang et al. 2009; see reviews by Imai and Nakagawa 2003; Conrad et al. 2007; Flohé 1999, 2007). Many of the recently described alternative functions for GPx4 are dependent, however, upon the enzymatic activity of this protein. This would indicate that the antioxidant function of GPx4 is paramount to the suite of complex processes now linked to the protein, yet little is known about the fundamental mechanism(s) which
drive(s) the range of GPx4 activity within tissues of an organism and/or between organisms within a taxonomic group. Data collected in Chapter 3 of my dissertation explore one possible candidate – PUFA content. Unsaturation index (an indicator of PUFA content), as well as GPx4 activities and protein are higher in liver tissues of marine fishes, when compared to terrestrial vertebrates. More importantly, perhaps, a strongly correlative relationship was detected between GPx4 and the extent of unsaturation in biological membranes. While these data do not provide with certainty a cause and effect relationship, the work does link PUFA content of vertebrates with GPx4 protein and activity. Future studies could use these data as a starting point to examine more directly the relationship between PUFA and GPx4, while working toward determining the mechanistic pathways responsible for governing the expression level and activities of GPx4 among animals. Furthermore, a closer examination of alternative functions linked to GPx4, in light of the data from Chapter 3, would seem to indicate the possibility that these other functions attributed to the protein may have evolved secondarily following the need for additional antioxidant protection in PUFA-rich tissues (and organisms).

Note: See respective chapters for full citations.
Appendix A: BCA Protein Analysis

BCA Protein Analysis
(from Thermo Scientific kit)

Check stock BSA solution to ensure the starting concentration = 2 mg/ml

<table>
<thead>
<tr>
<th>[BSA] mg/ml</th>
<th>Components</th>
<th>Final Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>300 µl stock + 300 µl H₂O</td>
<td>200 µl</td>
</tr>
<tr>
<td>0.75</td>
<td>200 µl 1 + 67 µl H₂O</td>
<td>267 µl</td>
</tr>
<tr>
<td>0.5</td>
<td>200 µl 1 + 200 µl H₂O</td>
<td>200 µl</td>
</tr>
<tr>
<td>0.25</td>
<td>200 µl 0.5 + 200 µl H₂O</td>
<td>300 µl</td>
</tr>
<tr>
<td>0.1</td>
<td>100 µl 0.25 + 150 µl H₂O</td>
<td>250 µl</td>
</tr>
<tr>
<td>0</td>
<td>200 µl H₂O</td>
<td>200 µl</td>
</tr>
</tbody>
</table>

1) Label tubes

2) Make up standards as described above and keep on ice

3) Make/thaw sample homogenates

4) Vortex and pipette 50 µl of each standard/sample into each tube (replicate x 2)

5) 6 stds x 2 reps = 12

6) Make BCA working reagent 50 parts: 1 part blue (need 1 ml/tube, so calc vol)

7) Add 1.0 ml working reagent to 50 µl standard/sample tubes

8) Vortex thoroughly

9) Incubate @ 37 C for 30 min (or RT for 2 hours)

10) Remove from heat block and allow to cool to room temp

11) Turn on Spec – set wavelength to 562 nm

12) Read absorbance of standards/samples

13) Make standard curve in Excel and calculate the concentration of the samples
Appendix B: Phosphate Analysis for Total Phospholipid Content

**Phosphate Analyses for Total Phospholipid Content**
(from Rouser *et al.*, 1970; Lipids 5: 494-496.)

Total [phospholipid] is quantified as acid-hydrolyzable phosphate (P\textsubscript{i}).

**Preparation of Standards**

P\textsubscript{i} standard (stock): approximately 0.2500 g of KH\textsubscript{2}PO\textsubscript{4}/250 ml water (FW = 136.06). Use a volumetric flask and weigh KH\textsubscript{2}PO\textsubscript{4} to 4 decimal places. Weighing exactly 0.2500 g is not important; just aim for between 0.23g and 0.27g. Record weight in order to calculate [standards] for standard curve. (Stock = 1 mg/ml OR 7.35 mM)

<table>
<thead>
<tr>
<th>mM</th>
<th>mg/ml</th>
<th>Stock Dilution</th>
<th>Dilution Made</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.35</td>
<td>1</td>
<td>----</td>
<td>Stock made as described above</td>
</tr>
<tr>
<td>1.47</td>
<td>0.2</td>
<td>5 x</td>
<td>0.5 ml stock + 2.0 ml water - not used in std. curve</td>
</tr>
<tr>
<td>0.294</td>
<td>0.04</td>
<td>25 x</td>
<td>0.5 ml 5x + 2.0 ml water - not used in std. curve</td>
</tr>
<tr>
<td>0.163</td>
<td>0.022</td>
<td>45 x</td>
<td>0.2 ml 5x + 1.6 ml water</td>
</tr>
<tr>
<td>0.147</td>
<td>0.02</td>
<td>50 x</td>
<td>0.2 ml 5x + 1.8 ml water</td>
</tr>
<tr>
<td>0.1225</td>
<td>0.017</td>
<td>60 x</td>
<td>0.2 ml 5x + 2.2 ml water</td>
</tr>
<tr>
<td>0.105</td>
<td>0.014</td>
<td>70 x</td>
<td>0.2 ml 5x + 2.6 ml water</td>
</tr>
<tr>
<td>0.086</td>
<td>0.012</td>
<td>85 x</td>
<td>0.2 ml 5x + 3.2 ml water</td>
</tr>
<tr>
<td>0.0735</td>
<td>0.01</td>
<td>100 x</td>
<td>0.5 ml 25x + 1.5 ml water</td>
</tr>
<tr>
<td>0.0598</td>
<td>0.008</td>
<td>125 x</td>
<td>0.5 ml 25x + 2.0 ml water</td>
</tr>
<tr>
<td>0.0490</td>
<td>0.006</td>
<td>150 x</td>
<td>0.5 ml 50x + 1.0 ml water</td>
</tr>
<tr>
<td>0.03675</td>
<td>0.005</td>
<td>200 x</td>
<td>0.5 ml 100x + 0.5 ml water</td>
</tr>
<tr>
<td>0.029</td>
<td>0.004</td>
<td>250 x</td>
<td>0.5 ml 50x + 2.0 ml water</td>
</tr>
<tr>
<td>0.0147</td>
<td>0.002</td>
<td>500 x</td>
<td>0.5 ml 250x + 0.5 ml water</td>
</tr>
<tr>
<td>----</td>
<td>-----</td>
<td>----</td>
<td>1.0 ml water</td>
</tr>
</tbody>
</table>

**Sample Hydrolysis (standards do not need to hydrolyzed)**

Using hot plate (with heat block on top) to achieve 180°C set to 3 (? – I haven’t done this in a while). Do this is hood. (This will take hours to heat up so set it up first.) 50 µl of membrane sample should be used. This volume of sample is added to 0.35 ml HClO\textsubscript{4} (full strength) – be careful – this acid is very, very strong. A blank should be done using water or the buffer used to resuspend membranes (50 µl) and 0.35 ml HClO\textsubscript{4}. Samples are heated for approximately two hours (they will turn black but later they will clarify - contents should appear “clear” when they are fully hydrolyzed). This should be done in culture tube using marble on top (reflux) to limit evaporation. I found that I needed to account for evaporation (recording weight of tube and its contents before heating and correct for concentrating effect due to evaporation).

Once hydrolysis is complete (samples have clarified) they should be cooled to room temperature.
**Incubation with Color Reagent (standards just develop, no hydrolysis)**

*Stock Solutions:* 2.5% ammonium molybdate (store at room temperature)
6 N H₂SO₄ (26 ml full strength acid + 124 ml H₂O)
10 % ascorbate (store refrigerated for 2 weeks only)

*Color Reagent:* The color reagent should be made up on day of assay only. To make up 75 ml of color reagent (enough for > 75 assay; 0.8 ml required for each):

30 ml H₂O
15 ml H₂SO₄ (6N)
15 ml ammonium molybdate (2.5%)
15 ml ascorbate

10, 25, or 50 µl of acid hydrolysate is then added to either 190, 175 or 150 µl of water (for total volume of 200 µl)….before adding 0.8 ml of color reagent (see below). (How much acid hydrolysate is needed for detection of Pᵢ will depend on concentration of phospholipids in original sample. Using more than 50 µl of acid hydrolysate is problematic as too much HClO₄ will interfere with color development.

Incubate for 7 minutes at 60°C. Alternatively, these can be incubated overnight at room temperature. If using the standard protocol (60°C) the samples should be return to room temperature before reading absorbance at 820 nm.
Appendix C: Enzyme Assay Protocols

Catalase (CAT)
(from Beers and Sizer, 1952; J Biol Chem 195: 133-140)

Buffer – 50 mM Phosphate (pH 7.24 @15C)

Roughly 2.39 g K$_2$HPO$_4$ in 500 mL
1.53 g KH$_2$PO$_4$

Better to make two 50 mM solutions and titrate against each other to desired pH.

Sol A – Buffer
Sol B – 12 ml Buffer + 0.5 ml 30% H$_2$O$_2$

Procedure:
Add 1.0 ml Sol A to 3 quartz cuvettes – 1 for blank and 2 for sample
Bring to desired assay temperature
Add homogenate to all cuvettes (10 or 25 µl)
Autozero spec at 240 nm
Add 33 µl Sol B to initialize rxn (only to samples – VORTEX BEFORE!!!!)
Monitor ΔA over ≈ 0.55-0.35A.

Factors:
25 µl sample (* 2752.3) = U/ml
10 µl sample (* 6880.7) = U/ml
Appendix C: Enzyme Assay Protocols cont…

**Citrate Synthase (CS)**


Extraction medium: Sol A (below) without DTT

Assay Medium:

<table>
<thead>
<tr>
<th>[Final]</th>
<th>FW</th>
<th>g/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 mM Tris</td>
<td>121.1</td>
<td>0.908 g</td>
</tr>
<tr>
<td>0.5 mM oxaloacetate</td>
<td>132.07</td>
<td>6.60 mg</td>
</tr>
<tr>
<td>0.24 mM DTNB</td>
<td>396.4</td>
<td>9.91 mg</td>
</tr>
<tr>
<td>[5,5'-dithiobis(2-nitrobenzoic acid)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4 mM acetyl CoA</td>
<td>876.46</td>
<td>35.06 g</td>
</tr>
</tbody>
</table>

pH 8.0 @ 15°C

Initiate reaction by addition of OAA. Control = omit OAA (subtract from initiated)

Protocol (for 40 assays):

**Solutions:**

Solution A: 4.96 mg DTNB, 17.03 mg Acetyl CoA brought to final volume of 45 ml with 75 mM Tris; re-pH as necessary

Solution B: 3.30 mg OAA dissolved in 5 ml of 75 mM Tris

**Assay:**

To a 1.4 ml (semimicro) cuvette, add:

- 0.9 ml Solution A
- 10-50 μl homogenate

Stir and monitor change in A₄₁₂ (if any) for 3-5 minutes = control

Add 100 μl Solution B; stir to initiate and record A₄₁₂ for 3-5 mins

**Notes:**

Preferable to make up excess Tris/DTNB and add volume required to desired amount of Acetyl CoA

Controls must be run with all samples since background deacylase activity is significant

EmM (412 nm) of DTNB (reduced) = 13.6

Stable in tissues frozen for several months
Appendix C: Enzyme Assay Protocols cont…

**Cytochrome Oxidase (CCO)**

(from Hansen and Sidell 1983; AJP 244: R356-362; modified from Wharton and Tzagoloff 1967; Meth Enzymology 10: 245-260)

Extraction Medium: Sol A (below) with or without DTT OR 10-50 mM Phosphate

Assay Medium:

- 10 mM K$_2$HPO$_4$ / KH$_2$PO$_4$
- 0.65% (w/v) reduced Fe$^{2+}$) cytochrome c = 52.8 μM
- pH 7.0 @ 15°C

Preparation of substrate:

Add 1.25 mg/ml ascorbate to a 1% (w/v) solution (250 mg on weigh paper in 25 ml) of cytochrome c in 10 mM phosphate buffer (see above)

Dialyze against 3 changes (~900 ml) of 10 mM phosphate buffer (stir slowly in cold) – Soak dialysis tube in 100 mL or 10 mM KPhos for 20 mins before use

Store frozen under N$_2$ gas

Assay Protocol:

To a 1.4 ml (semimicro) cuvette add:
- 67 μl reduced cytochrome c (1% w/v) stock
- 0.93 ml 10 mM phosphate buffer

Stir and record baseline A$_{550}$ against a reference cuvette containing:
- 67 μl reduced cytochrome c (1% w/v) stock
- 0.93 ml 10 mM phosphate buffer
- 14 μl 0.1 M K$_3$Fe(CN)$_6$ – in distilled water (will oxidize cytochrome c)

**Blank once daily with this cuvette**

Add 10-50 μl homogenate to initiate the reaction, stir rapidly

Record decrease in A$_{550}$ for 1-3 mins or until deviation from linearity*

Notes:
Reaction rapidly deviates from linearity, must record initial rate!
To determine if cytochrome c (reduced) substrates is still adequately reduced (has not oxidized during storage):

Read $A_{550}$ and $A_{565}$; $A_{550}/A_{565}$ should be $> 8-10$ (Initially preparation should show ~14)

EmM (@550) for reduced-oxidized cytochrome c = 18.5

Must be assayed in fresh tissues
Appendix C: Enzyme Assay Protocols cont…

Glutathione Peroxidase 1 (GPx1)

\[
\text{ROOH} + 2 \text{GSH} \rightleftharpoons \text{GSSG} + \text{ROH} + \text{H}_2\text{O} \quad (\text{GPx reaction})
\]
\[
\text{GSSG} + \text{NADPH} \rightleftharpoons \text{GSH} + \text{NADP}^+ \quad (\text{GR reaction})
\]

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (µl)</th>
<th>[Final]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M Tris-HCl, pH 8</td>
<td>710 – x sample volume (e.g., 685 µl if adding 25 µl of sample)</td>
<td></td>
</tr>
<tr>
<td>20 mM EDTA</td>
<td>50</td>
<td>1 mM EDTA</td>
</tr>
<tr>
<td>2 mM DFO</td>
<td>50</td>
<td>0.1 mM DFO</td>
</tr>
<tr>
<td>60 mM GSH</td>
<td>50</td>
<td>3 mM GSH</td>
</tr>
<tr>
<td>4 mM NADPH</td>
<td>50</td>
<td>0.2 mM NADPH</td>
</tr>
<tr>
<td>4 U/ml GR</td>
<td>50</td>
<td>0.2 U/ml</td>
</tr>
<tr>
<td>5% Triton X-100</td>
<td>20</td>
<td>0.1% Triton X-100</td>
</tr>
<tr>
<td>10 mM cumene hydroperoxide</td>
<td>20</td>
<td>200 µM cumene hydroperoxide</td>
</tr>
<tr>
<td>sample (tissue supernatant)</td>
<td>x (25 µl ?)</td>
<td></td>
</tr>
</tbody>
</table>

**Substrate preparation (cumene hydroperoxide):** Cumene-OOH is somewhat soluble in water (e.g., 1.5 g/100 ml; density of commercial stock = 1.06). Concentration of commercial stock = 6.77 M. To prepare a working solution of 10 mM cumene-OOH, make a 677-fold dilution by mixing 10 µl of commercial stock in 6.77 ml of Tris-HCl. Vortex for several minutes to mix well.

**Tissue Preparation:** Homogenize in 2ml Ten-Broeck homogenizer as 10% w/v in Chelex-treated PBS (PBS = 125 mM NaCl, 25 mM K-phosphate, pH 7.5; make up 25 mM of each and then mix approximately 3 parts K_2HPO_4 to 2 parts KH_2PO_4) with 0.3 % Triton-X-100 (added right before; e.g., 30 µl of stock to 10 ml of buffer) and 1 tablet Complete-Mini per 10 ml of solution.

Allow cells to lyse for 1 hour (on ice) with vortexing every 15 minutes. Centrifuge at 16,000g (approximately 13,000 rpm) for 10 minutes.

**Assay Protocol:** Add reagents to 1.5 ml (semi-micro) plastic cuvettes and bring to desired temperature (15°C). Before adding substrate, measure blank (everything except the substrate, cumene-OOH) for 10 minutes. Also measure blank with substrate (no sample). Both of these blanks will need to be summed and the sum subtracted from ΔA/Δt with all reagents and sample. Initiate with substrate. Follow disappearance of NADPH at 340 nm.
Appendix C: Enzyme Assay Protocols cont…

**Glutathione Peroxidase 4 (GPx4)**
(from the Girotti lab)

\[
ROOH + 2 \text{ GSH} \rightleftharpoons \text{GSSG} + \text{ROH} + \text{H}_2\text{O} \quad \text{(GPx reaction)}
\]

\[
\text{GSSG} + \text{NADPH} \rightleftharpoons \text{GSH} + \text{NADP}^+ \quad \text{(GR reaction)}
\]

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume (µl)</th>
<th>[Final]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M Tris-HCl, pH 8</td>
<td>70 – x sample volume (e.g., 60 µl if adding 10 µl of sample)</td>
<td></td>
</tr>
<tr>
<td>20 mM EDTA</td>
<td>5</td>
<td>1 mM EDTA</td>
</tr>
<tr>
<td>2 mM DFO</td>
<td>5</td>
<td>0.1 mM DFO</td>
</tr>
<tr>
<td>60 mM GSH</td>
<td>5</td>
<td>3 mM GSH</td>
</tr>
<tr>
<td>4 mM NADPH</td>
<td>5</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>NADPH</td>
<td>4 U/ml GR</td>
<td>0.2 U/ml</td>
</tr>
<tr>
<td>5% Triton X-100</td>
<td>2</td>
<td>0.1% Triton</td>
</tr>
<tr>
<td>POPC-OOH</td>
<td>2.5</td>
<td>100 µM</td>
</tr>
<tr>
<td>tissue supernatant*</td>
<td>x (10 µl ?)</td>
<td></td>
</tr>
</tbody>
</table>

**Tissue Preparation:** Homogenize in Dounce homogenizer as 10% w/v (loose- followed by tight-fitting pestle) in

Chelex-treated PBS (PBS = 125 mM NaCl, 25 mM K-phosphate, pH 7.5; make up 25 mM of each and then mix approximately 3 parts K$_2$HPO$_4$ to 2 parts KH$_2$PO$_4$) with 0.3 % Triton-X-100 (added right before; e.g., 30 µl of stock to 10 ml of buffer) and 1 tablet Complete-Mini per 10 ml of solution.

Allow cells to lyse for 1 hour (on ice) with vortexing every 15 minutes.

Centrifuge at 16,000g (approximately 13,000 rpm) for 10 minutes.

**Assay Protocol:** Add reagents to 100 µl (submicro) cuvettes and bring to desired temperature (15°C) Before adding substrate, measure blank (everything except substrate, POPC-OOH) for 10 minutes. Also will need to measure a second blank which will represent ΔA/Δt that will be due to substrate alone (there is usually a small decrease over time). Initiate with POPC-OOH. Follow disappearance of NADPH at 340 nm.
Appendix C: Enzyme Assay Protocols cont…

Sarcoplasmic reticular ATPase (SERCA)
(from Riemenschneider and Sidell 2002; JEZ 292: 231-240; modified from Simonides and van Hardeveld 1990; Anal Biochem 191: 321-331)

Homogenization: 10% (w/v) in 50 mM Tris-HCL, 0.3 M sucrose, 0.5 mM EGTA, 0.05% Triton X-100, pH 8.0. Dilutions made (approx 5-fold for white muscle)

Reaction Mixture:

[Final]
25 mM MOPS, pH 7.3, 200 mM KCl, 5 mM MgCl₂, 5 Mm NaN₃, 1 mM DTT, 0.5 mM EGTA, 20 U/ml LDH, 10 U/ml PK, 3 mM PEP, 0.2 Mm NADH, 0.5 mM CaCl₂

Reaction initiated with addition of ATP; [final] = 1 mM

Solution A: 25 mM MOPS, 400 mM KCl, 10 mM MgCl₂, 10 mM NaN₃, 2 mM DTT, 1 mM EGTA, 40 U/ml LDH, 20 U/ml PK, 6 mM PEP. Add PEP before linking enzymes, adjust pH if necessary before adding enzymes (7.3)

Solution B (MOPS): 25 mM MOPS, pH 7.3

Solution C (NADH): 2 mM NADH in 25 mM MOPS

Solution D (CaCl₂): 5 mM CaCl₂ in 25 mM MOPS

Solution E: (ATP): 10 mM ATP in MOPS

Thapsigargin: 20 μM in DMSO – [final] = 100 nM

To semi-micro cuvette add:
0.5 ml Solution A
0.1 ml Solution B
0.1 ml Solution C
Bring to desired temperature
Add 0.1 ml of sample (enzyme) and 5 μl thapsigargin or DMSO. Incubate for 5 minutes
Add 0.1 ml each of Solutions D and E.
Stir and record ΔA₃₄₀
Superoxide dismutase (SOD)

Coupled Assay:
Buffer 1: 50 mM potassium phosphate, 0.1 mM EDTA – pH 7.8

Per liter:
- 6.52 g KH\textsubscript{2}PO\textsubscript{4}
- 1.70 g KH\textsubscript{2}PO\textsubscript{4}
- 0.037 g EDTA

Reagents:
- Xanthine – Stock 0.5 mM (Final 0.05 mM)
- Cytochrome c acetylated – Stock 0.1 mM (Final 0.01 mM)
- KCN – Stock 1 mM (Final 0.01 mM) – for assay of total SOD
- Stock 1 100 mM (Final 1 mM) – for assay of mitochondrial form (MnSOD)
- XO – Dilute 1 ul stock in 999 ul Buffer 1 (depends on day and stock)

In semi-micro cuvette – **Total**: (low dose KCN)
- 680 ul Buffer 1
- 0.1 ml cytochrome c acetylated
- 0.1 ml xanthine
- 10 ul KCN
- 60 ul XO

- Monitor rate of increase absorbance at 550 nm – Rate should be near 0.02 OD/min
  
  **NOTE:** If rate is not within the desired range, the concentration of XO stock should be adjusted to alter the background rate.

If rate is within appropriate range, add 50 ul sample + monitor rate of increase at 550 nm

In semi-micro cuvette – **Mn form**: (high dose KCN)
- 680 ul Buffer 1
- 0.2 ml cytochrome c acetylated
- 0.2 ml xanthine
- 10 ul KCN – Mn form
- 60 ul XO

- Monitor rate of increase absorbance at 550 nm – Rate should be near 0.02 OD/min
NOTE: If rate is not within the desired range, the concentration of XO stock should be adjusted to alter the background rate.
If rate is within appropriate range, add 50 ul sample + monitor rate of increase at 550 nm.

Notes:
In both assays, you want between 40-60% inhibition of the rate of increase in Abs$_{550}$

One unit of SOD = 50% inhibition (calculate units of SOD based on measured inhibition).
Appendix D: Measures of Lipid Peroxidation (LPO)

C-11 BODIPY
NOTE: ALL PROBE TRANSFERS DONE IN DARK

☐ PROBE SOLUTION A. Thaw BODIPY and determine concentration of stock solution (Probe Solution A)
  - Blank spectrophotometer with EtOH (1 ml).
  - Place 25 μl Probe Solution A in 975 μl EtOH and read A_{581}.
    - Multiply absorbance by extinction coefficient (139444A l) and correct for 40x dilution in cuvette.
    - e.g. 0.2733A × \frac{1mol}{139444A·l} = 2μM × 40x(\text{dilution}) = 80μM

☐ PROBE SOLUTION B. Make a working solution of probe in Tris so [probe] = 10 μM. Step 1 is essential in determining [PROBE SOLUTION A] to determine dilution.

☐ MEMBRANE RESUSPENSION B. Thaw membranes and dilute to either 1 (freshwater fishes) or 0.5 (marine fishes) mg/ml protein with 20 mM Tris (pH 7.4)—this makes up membrane solution B, which will be diluted further.

☐ MEMBRANE RESUSPENSION C. Make Membrane Resuspension C by diluting Membrane Resuspension B to a final membrane [protein] of either 0.1 (freshwater) or 0.5 (marine) mg/ml.

☐ PROBE SOLUTION C. Dilute Probe Solution B (10 μM) by 67.6x in Membrane Resuspension C to achieve a concentration of either 300 nM (freshwater) or 150 nM (marine).

☐ DISPERSE PROBE IN MEMBRANES. Stir combined membrane/probe solutions (Probe Solution C) slowly in dark (in amber bottle covered with foil) at cold temperature for at least 60 minutes.

POTENTIAL INDUCTANT SYSTEMS: FeSO₄/Ascorbate, CuSO₄/Cumene hydroperoxide (both produce hydroxyl radicals via Fenton Chemistry), AAPH, or AMVN (are azo-initiators which mainly produce peroxyl radicals, but also others). CuSO₄/CumOOH was shown by Drummen et al. (2002) to be more effective than CuSO₄/H₂O₂, so the former should be used if possible. CuSO₄/CumOOH has been used successfully to induce LPO across a wide range (1-25°C) of assay temperatures. FeSO₄/Ascorbate has also been used effectively with this method at 15°C. ROS production by azo-initiators (AAPH and AMVN) is dependent on thermal decomposition of the molecules, so initial trials reveal that even at assay temperatures of 25°C these compounds are ineffective at initiating LPO, and most certainly do not function sufficiently at lower temperatures. As a result, they are not likely to be useful in the Crockett lab.

☐ MEASURE FLUORESCENCE INTENSITY. Excitation (568 nm); Emission (590 nm). Probe Solution C alone (in cuvette 3.0 ml Tris buffer +45 μl Probe Solution B) (67.6 of Probe Solution B).
LPO INDUCTION. In cuvette, add 2.5 ml membrane with probe dispersed. Monitor overtime to confirm stable baseline.

- If using a two part induction system, add one part (i.e., CuSO4) with a volume of Tris so that total addition = 120 μl. Monitor for 5 minutes
- If using a two part induction system, add second part (i.e., CumOOH) with a volume of Tris so that total addition = 120 μl at 15 mins elapsed time. Follow decay slope until no longer linear or FI = 0.
- If using a one part induction system, add inductant (i.e., AAPH) with a volume of Tris so that total addition = 240 μl at 10 mins elapsed time. Follow decay slope until no longer linear or FI = 0.

NOTES:

Linear rates of fluorescence decay indicate rates of LPO

EmM A581=139,444

All solutions can be made with cold buffer and maintained on ice except CumOOH which falls out of solution at cold temperature. Make in RT buffer and store at RT day of. All inductant solutions and Probe Solution B must be made fresh day of use. Store C11-BODIPY stock under N2 gas at -70°C.

Final concentrations of FeSO4/Ascorbate used in Chapter 1 were 2.3/17.5 μM (bluegill) and 0.19/1.4 μM (killifish)

Final concentrations of CuSO4/CumOOH used in Chapter 2 were 13/52 μM (striped bass)

- Final concentrations of inductants in both Chapter 1 and 2 were titrated through trial and error to determine what minimum inductant concentration was necessary to induce the highest rate of probe oxidation (i.e., at what point adding additional inductants did not significantly increase the rate of probe oxidation, but instead just caused increasingly large drops in FI upon addition of the substance)

- Titration steps were conducted at 25°C in order to maximize the rate of probe oxidation at this temperature. Ideally, linearity would be retained for at least 5 mins to ensure accuracy of slope measurements, but 3-5 mins is acceptable. Maximizing probe oxidation at 25°C allowed me to add the same volume of inductant to the cuvettes over a range of assay temperatures and still have significant probe oxidation at all assay temperatures. This approach allows for a standardization of inductant load among assay temperatures.
Appendix D: Measures of Lipid Peroxidation (LPO) cont…

TBARS

Reagents
- 50 mM Phosphate buffer, pH 7.4 @ 25°C
  - 1.64 g H₂KPO₄
  - 6.61 g HK₂PO₄
  - 1 Liter water
- 1% Phosphoric acid (lasts one week)
- 0.6% TBA in water (prepare day of)
- FeSO₄ 0.2 mM
  - 5.6 mg in 100 ml 50 mM Phosphate buffer
- 1.6 mM ascorbic acid
  - 7 mg in 25 ml 50 mM Phosphate buffer (cover with aluminum foil to prevent oxidation)

Method (For membrane preparations)
- Turn on heat block
- Use 16 mm culture tubes
- Dilute membrane preparation to 1 mg/ml
- Need a blank and a time 0 (*in situ* peroxidation)
  - Blank: 140 μl phosphate buffer + 1 ml cold 1% phosphoric acid
  - Time zero: 140 μl sample + 1 ml cold 1% phosphoric acid.
  - Cover with parafilm and place in the fridge
- Need a control for each time point (1:1 ratio of sample to phosphate buffer)
  - e.g. 100 μl sample + 100 μl phosphate buffer
- For the incubation add in a proportion of 2:1:1 (as low 100 μl of sample has been used successfully—so 100 μl sample, and 50 μl each of FeSO₄ and AsA)
  - 2 sample (quantity depends on how much you have)
  - 1 FeSO₄
  - 1 Ascorbic Acid
- Mix everything well and incubate for chosen temperatures-agitate the entire time.
- Take out the samples and add 140 μl to 1 ml of cold phosphoric acid and cover with parafilm and place in fridge.
- When all of the samples are incubated, begin the color development part.
- For this add 300 μl of TBA (for color development)
- Vortex and boil for 45 minutes, covered
- Measure at 535 nm and 520 nm with spectrophotometer. The difference represents MDA.
NOTE: With whole tissue homogenates:

- After heating for 45 minutes, add 1.4 ml of butanol, vortex thoroughly, and centrifuge at 4000 rpm for 15 minutes.
- Measure the absorbance of the organic phase (collect with Pasteur pipette without disturbing the intermediate layer—there will be three layers).
Appendix E: Preparation of Mitochondrial and Microsomal/SR Membranes

Preparation of mitochondrial and microsomal membranes from teleost skeletal muscle

(modified from Moyes et al 1989, 1992; Leary SC et al 2003; Vornanen et al., 1999; Carey and Hazel, 1989; Li et al., 2004)

Isolate glycolytic and aerobic muscle fibers from representative animals

Dice muscle fibers with scissors on ice-cold stage

Homogenize sample 10% (w/v) in isolation buffer containing 140 mM KCl, 20 mM Hepes, 10 mM EDTA, 5 mM MgCl₂, 0.5% BSA (pH 7.1), 0.1 mM EGTA in Tissuemizer

Transfer crude homogenate to a PE homogenizer and make 5 passes

Aliquot final crude homogenate for marker enzyme analyses (if necessary)

Prepare mitochondria and microsomes:

SPIN 1 - Centrifuge at 1000 g (2600 rpm) for 5 minutes (SS-34 in Sorvall high speed)
Collect supernatant, filter through 8 layers of cheesecloth and spin #2

SPIN 2 - Centrifuge at 1000 g (2600 rpm) for 5 minutes (SS-34 in Sorvall high speed).
Collect supernatant, filter through 8 layers of cheesecloth and spin #3

SPIN 3 - Centrifuge at 9000 g (8700 rpm) for 10 minutes (SS-34 in Sorvall high speed).
Collect pellet (save supernatant for microsomes), break-up with glass rod, and resuspend in 0.5 mLs (w/Pasteur pipette) in Buffer A (minus BSA), and then in 20 mLs of Buffer A (minus BSA) and spin #4

SPIN 4 - Centrifuge at 9000 g (8700 rpm) for 10 minutes (SS-34 in Sorvall high speed).
Collect pellet (save supernatant for microsomes) and resuspend (w/ Tenbroeck) in 10 mM Tris-HCl (pH 7.3) (about 10 drops)
Freeze in LN2

SPIN 5- Combine supernatants from Spins 3+4 above and centrifuge at 33K (100xg) for 1 hour. Collect pellet and resuspend in ~12 drops of 10 mM Tris (w/Tenbroeck)
Appendix F: Quantification of GPx4 Protein using Western Blot Analysis

**Extraction Medium**
Must make fresh (for 10 ml)
9.4 ml Chelex PBS
0.6 ml 5% Triton X
1 Complete-Mini® protease inhibitor tablet (Roche)
pH 7.5

1. Homogenize tissue 10% (w/v) with a Tenbroeck homogenizer
2. Sonicate crude homogenate (50% output – 1 ten sec burst)
3. Remove debris and nuclei with a 300 g spin for 10 min (this step not really necessary)
4. Collect supernatant and centrifuge at 100 x g for 60 min (All centrifugation steps at 4°C)
5. Quantify proteins using Bradford Assay (BioRad) or BCA Assay (preferred see Appendix 1) – sample dilutions will vary depending on the tissue and species being used
6. Combine 50 μl β-mercaptoethanol to 950 μl Laemmli Sample Buffer (BioRad). Must be made fresh
7. Make final sample dilutions in Laemmli/ β-ME (2:1vol:vol)
8. Heat all samples for 10 minutes at 95°C, can use PCR machine.
9. Load 6-20 μg of protein (amount species dependent), standards (5 ul), or purified GPx4 enzyme stock (15 ul) on a 12% Tris-HCl minigel (depends on protein size), and run at 60 volts for 30 minutes, and then 100 volts until the BPB is close to the bottom of the gel. See notes on setup. Ensure bands (both samples and purified GPx4) are not saturated by preliminary trials – ideally you would like to see barely visible (faint) bands.
10. Make transfer buffer (2.64 g Tris, 13 g glycine, 900 ml H2O, 300 ml MeOH – make fresh) Store in the fridge.
11. Activate PVDF membranes as per the manufacturer’s instructions.
12. Transfer the proteins to a Biorad Immun-Blot PVDF membrane (162-0174), 90V for five hours at 4°C. See transfer notes.
13. If storing overnight, rinse in dH2O and block overnight in full-strength TBS-Casein (1% w/v) (Pierce) at cold-temp
14. Following blocking, blot dry with Kimwipe (touching only corner of blot)
15. Incubate with 1° antibody (Abcam – ab16800) diluted to 1/1000 in 0.05% Casein solution – time for primary antibody rinse will vary (a few hours to overnight
16. Make TBS-T (0.6 g Tris, 4 g NaCl in 500 ml pH 7.6 + ½ ml Tween 20)
17. Remove blot and save primary
18. Dab blot on Kimwipe as previous
19. Rinse 3x in TBS-T (15 min each)
20. Incubate with 2° antibody (GAR-AP Immun-star chemiluminescent kit – BioRad) diluted 1/3000 in 0.05% Casein solution (1 hr)
21. Rinse blot 3x in TBS-T (15 min each)
22. Add 0.5 ml substrate (from kit in Step 20), cover in Saran Wrap and incubate for 5 mins (must be the real stuff here – cheaper plastic wrap seems to enhance background signal)
23. Detect by film or ChemiDoc (BioRad).
24. Quantify bands using Quantity One (BioRad) – basic version is a free download from their website
Appendix F: Quantification of GPx4 Protein using Western Blot Analysis cont…

Notes: (modified from Dr. K. Hyndman)

Gel

1. Gel set up:
2. Use vertical box
3. Short plate on gel faces inward on box
4. Place gel in frame and then squeeze the frame evenly from all side. This ensures that current won’t flow around the gel, but instead will only flow down it.
5. If only running one gel, place the plastic gel mimic in other frame to simulate a gel.
6. Fill outside first with 1X electrophoresis buffer (1 g SDS, 6 g Tris, 28.5 g glycine in 1 L), fill 1/3 of the way and get rid of any bubbles that are in the bottom by tipping or use the bent syringe.
7. Then fill ½ cm above short plate on the inside and fill the rest of the outside.
8. Find lid that fits box, make sure negatives and positives are correct
9. Plug electrodes in biorad machine

PDVF transfer

1. Membranes are white - cut bottom left corner off and handle on upper right corner only
2. Wet membranes in a little methanol, then a water wash and then in a little transfer buffer
3. Pour a little transfer buffer into a tray
4. On the PDVF frame, put a white scotch pad, roll with a 50ml tube to get out any bubbles, then place a wetted filter paper (the same size as the membrane) on top of it.
5. Next open up the gel and remove the top part (follow line) with a razor, be careful not to rip the gel. Put in the transfer buffer and remove it. Wear nonpowdered gloves.
6. Place the gel on the black side of the PDVF frame, make sure to place the gel with the left standards on the right, put it near the bottom of the frame
7. Place the membrane on top, with the cut corner on the right
8. Place another piece of filter paper on top, roll to remove air
9. Place a scotch pad on top and roll again
10. Close frame and place in box, Black side facing black box, and white side facing red box.
11. Put on electrodes, MAKE SURE THEY ARE CORRECT
12. Fill box with all of the transfer buffer
Appendix G: Lipid Extraction from Biological Membranes of Fishes

Extraction and purification of total lipids

Solvents: Solvents used are HPLC grade.

Solutions:
Solution A: MeOH/CHCl$_3$ (2:1), BHT (40 ml MeOH, 20 ml CHCl$_3$, 6 mg BHT – final [BHT] = 0.01%)
Solution B (storage only): CHCl$_3$/MeOH (2:1) (30 ml CHCl$_3$, 15 ml MeOH)

Prep:
- Rinse all glassware with solvent (CHCL$_3$) and allow to dry overnight
- “Make water” – Good distilled water + chelex; calculate water volume needed for all extractions and transfer to Erylenmeyer flask and add Chelex to dialysis tubing and add to water (5 g/100 ml). Leave to set overnight.
- BHT is tough to get into solution – add 6mg to 40 ml MeOH and allow to stir; once completely dissolved, add CHCL$_3$.

1. Label (and weigh) Wheaton vials for final lipid storage.
2. Set up, preweigh, and label round-bottom (or pointed bottom) ground glass tubes (cover labels with scotch tape to prevent labels from being removed).
3. Add sample to tube (assume virtually all water, 1g sample = 1 ml H$_2$O) – if using membranes, this sample volume will likely vary based on what is left of the sample.
4. Measure tubes + samples. Subtract this weight from the tube weight in Step #2 to obtain sample volume weight.
5. For every 1 ml of sample, add 1.25 ml of CHCL$_3$ and 2.50 ml of MeOH (or 3.75 ml of Solution A).
6. Gas with nitrogen, cap tube and let stand for 15 minutes (more if done cold maybe 30 mins), vortexing every 3 minutes.
7. Add 1.25 ml CHCL$_3$ (per 1 ml of original sample), VORTEX and 1.25 ml water.
8. Spin for ten minutes in clinical centrifuge until two phases formed (Max speed for 5 minutes).
9. Collect bottom (organic) phase (Do not put tubes on ice following centrifugation or phase separation will be lost).
10. Dry under nitrogen or in Speedvac (Wheaton vials can be placed directly in the Speedvac rotor and will be fine).
11. Weight Wheaton vial with dried lipids and subtract from weight in Step #1 to obtain lipid weights
Appendix G: Lipid Extraction from Biological Membranes of Fishes cont…

<table>
<thead>
<tr>
<th>Sample</th>
<th>0.2 ml</th>
<th>0.5 ml</th>
<th>1 ml</th>
<th>1.5 ml</th>
<th>2 ml</th>
<th>3.5 ml</th>
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</thead>
<tbody>
<tr>
<td>1:2 CHCL$_3$:MeOH</td>
<td>0.750</td>
<td>1.9</td>
<td>3.75</td>
<td>5.7</td>
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<tr>
<td>CHCL$_3$</td>
<td>0.250</td>
<td>0.625</td>
<td>1.25</td>
<td>1.875</td>
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<tr>
<td>H$_2$O</td>
<td>0.250</td>
<td>0.625</td>
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<td>1.875</td>
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<tr>
<td>Total Volume</td>
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<td>10.95</td>
<td>14.50</td>
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