The Journal of Experimental Biology 214, 3751-3759
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doi:10.1242/jeb.058214

RESEARCH ARTICLE

Relationship between oxidizable fatty acid content and level of antioxidant glutathione peroxidases in marine fish

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Accepted 25 August 2011

SUMMARY

Biological membranes can be protected from lipid peroxidation by antioxidant enzymes including catalase (CAT) and selenium-dependent glutathione peroxidases 1 and 4 (GPx1 and GPx4). Unlike GPx1, GPx4 can directly detoxify lipid hydroperoxides in membranes without prior action of phospholipase A_2. We hypothesized that (1) GPx4 is enhanced in species that contain elevated levels of highly oxidizable polyunsaturated fatty acids (PUFA) and (2) activities of antioxidant enzymes are prioritized to meet species-specific oxidative stresses. In this study we examined (i) activities of the oxidative enzyme citrate synthase (CS) and antioxidant (CAT, GPx1 and GPx4) enzymes, (ii) GPx4 protein expression, and (iii) phospholipid composition in livers of five species of marine fish (*Myxine glutinosa*, *Petromyzon marinus*, *Squalus acanthias*, *Fundulus heteroclitus* and *Myoxocephalus octodecemspinosus*) that contain a range of PUFA. GPx4 activity was, on average, 5.8 times higher in *F. heteroclitus* and *S. acanthias* than in the other three marine fish species sampled. Similarly, activities of CAT and GPx1 were highest in *S. acanthias* and *F. heteroclitus*, respectively. GPx4 activity for all species correlates with membrane unsaturation, as well as oxidative activity as indicated by CS. These data support our hypothesis that GPx4 level in marine fish is a function, at least in part, of high PUFA content in these animals. GPx1 activity was also correlated with membrane unsaturation, indicating that marine species partition resources among glutathione-dependent defenses for protection from the initial oxidative insult (e.g. H_2O_2) and to repair damaged lipids within biological membranes.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/22/3751/DC1

Key words: glutathione peroxidases, enzymatic antioxidants, membrane unsaturation, membrane composition, polyunsaturated fatty acids.

INTRODUCTION

Most animals require a constant supply of oxygen, and as a result their cells 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 (Halliwell and Gutteridge, 2007). Lipid peroxidation (LPO) is a self-propagating process in which ROS-damaged lipids can initiate peroxidation of other lipids, making the risk of LPO unique among ROS-induced damage to biological molecules. Not all lipids, however, are equally susceptible to LPO. Phospholipids that contain polyunsaturated fatty acids (PUFA) are at an elevated risk of LPO compared with phospholipids with lower degrees of unsaturation (Cosgrove et al., 1987; Holman, 1954). Unless balanced by cellular antioxidants, LPO can threaten membrane integrity (Kühn and Borchert, 2002), and can even expedite cell death (Choudhary et al., 2002).

Hydrogen peroxide and organic hydroperoxides can induce oxidative damage in organisms; however, cells are protected from such damage by a variety of antioxidant defenses including antioxidant enzymes such as catalase (CAT) and a family of selenoproteins called glutathione peroxidases (GPx). While GPx isozyme 1 (GPx1) and isozyme 4 (GPx4) are both able to catalyze the reduction of H_2O_2 and small organic hydroperoxides (e.g. cumene hydroperoxide or fatty acid hydroperoxide), GPx4 is unique because it can also directly reduce phospholipid and cholesterol hydroperoxides to their corresponding alcohols within biological membranes (Thomas et al., 1990). Kinetic modeling has demonstrated that the flux of phospholipid hydroperoxides through GPx4 is 10^4-fold greater than that through the phospholipase A_2 (PLA_2)/GPx1 pathway, and consequently GPx4 is considered to be more efficient at eliminating lipid hydroperoxides in membranes and protecting the integrity of biological membranes from LPO (Antunes et al., 1995).

GPx4 enzymatic activity, and tissue-specific expression and stability of GPx4 protein and mRNA have been characterized in cells and tissues of mammals including mice (Liang et al., 2007), humans (Thomas et al., 1990; Kriska and Girotti, 2005) and pigs (Ursini et al., 1982). Biosynthesis of GPx4 is preferentially retained over that of other glutathione peroxidases in mammalian brain, testes, heart and liver when selenium (a component of the active site in of GPx enzymes) is limiting (Brigelius-Flohé, 1999). These same tissues contain constitutively high levels of PUFA (Floyd, 1999; Hulbert et al., 2002; Gavazza and Catalá, 2006). These findings emphasize the importance of GPx4 in protecting mammalian lipids from LPO and suggest that the risk of oxidative damage with increasing PUFA levels may be reduced by elevated GPx4 expression and activity.

In contrast to the relatively large body of work in mammalian tissues and cells, GPx4 has not been well studied in other vertebrate

groups. Tissues of marine fish contain high levels of PUFA (Sargent et al., 1999), relative to other vertebrate taxa. As a result, these animals may require additional protection from LPO. Expression of two GPx4 genes (*gpx4a/b*) has been reported during the time course of development in zebrafish (Thisse et al., 2003), following acute exposure to both cold temperature and cadmium in carp (Hermesz and Ferencz, 2009), and in various tissues from farmed southern bluefin tuna (Thompson et al., 2010). In spite of the growing interest in GPx4 of fish, thus far no studies have systematically examined the protein levels or enzymatic activity of GPx4 in their tissues. Further, to our knowledge, no study has probed a possible relationship between GPx4 and elevated PUFA content of tissues from marine fish.

We hypothesized that the enhanced oxidizability of lipids in marine fish may require additional antioxidant protection in the form of GPx4. To test this hypothesis, we sampled liver tissue from an array of marine fish whose membranes contain a range of PUFA. The species investigated are widely used in physiological studies as model organisms for their respective taxonomic classes (e.g. Edwards et al., 2001; Yang et al., 2002; Hyndman and Evans, 2009). The objectives of the current study were to quantify (i) the activities of enzymatic antioxidants (CAT, GPx1 and GPx4), (ii) GPx4 protein level, (iii) oxidative activity as indicated by citrate synthase (CS) activity, and (iv) phospholipid composition of liver tissue in order to assess the relationship(s) between PUFA content and pro-oxidant *vs* antioxidant processes of marine fish. Our study is the first to utilize a range of marine fish species to explore the potential relationships between GPx4, and other antioxidant enzymes, with the level of membrane unsaturation.

MATERIALS AND METHODS

Animal maintenance and status

Hagfish (*Myxine glutinosa*, Linnaeus), sea lamprey (*Petromyzon marinus*, Linnaeus), dogfish shark (*Squalus acanthias*, Linnaeus) and longhorn sculpin [*Myoxocephalus octodecemspinosus* (Mitchill)] were wild-caught and maintained at Mount Desert Island Biological Laboratory (MDIBL) until sampling. Wild-caught saltmarsh killifish [*Fundulus heteroclitus macrolepidotus* (Linnaeus)] from Aquatic Research Organisms (Hampton, NH, USA) were maintained at Ohio University. All animals were held between 12 and 15°C on an ambient photoperiod. Holding times varied among species, and all animals were fed *ad libitum* with widely used commercial feeds that are representative of native diets. The exceptions were the sea lamprey, which were sampled during their upstream migration and do not feed during this life history stage. We acknowledge that differences in the age of the study species may represent a potentially confounding variable in our analyses, and others have encountered similar problems in studies of oxidative stress with a large phylogenetic scope (Lambert et al., 2007). Values for the morphometric characteristics of all animals sampled are shown in Table 1.

Tissue collection and preparation

All animals were fasted for 24 h prior to sampling. Animals were killed and liver tissue was quickly removed and immediately frozen in liquid nitrogen, and stored at –80°C until analyses. All animal procedures were approved by Ohio University and MDIBL Institutional Animal Care and Use Committees. Liver was chosen for the current study because it has been shown to have a robust GPx4 expression in embryonic fish (Thisse et al., 2003).

Frozen liver tissues were homogenized (10% w/v) in an ice-cold extraction buffer consisting of Chelex®-treated phosphate-buffered saline (138 mmol l^{-1} NaCl, 2.6 mmol l^{-1} KCl, 1.5 mmol l^{-1} KH_2PO_4, 10 mmol l^{-1} Na_2HPO_4, pH 7.5) with 0.3% Triton-X 100 and one Complete-Mini® protease inhibitor tablet (Roche Diagnostics, Indianapolis, IN, USA). Crude homogenates were divided into two aliquots for GPx4 protein measurements and enzyme activity assays.

GPx4 protein

Samples were prepared by sonicating the homogenates on ice (10 s burst). Cellular debris was pelleted at a low speed (600 ***g***) for 10 min, and resulting supernatants were subjected to a final high-speed spin (100,000 ***g***) for 60 min. All centrifugation steps were performed at 4°C.

GPx4 protein was quantified in all marine fish using slight modifications of standard immunoblot techniques (Hurst et al., 2001; Hyndman et al., 2006). Briefly, the protein content of final supernatants was determined using the Bradford Assay (BioRad, Hercules, CA, USA). Samples were initially diluted with 10 mmol l^{-1} Tris (pH 7.4); samples were subsequently diluted 2 parts sample to 1 part Laemmli sample buffer (BioRad) which contained 5% β-mercaptoethanol, and were heated to 95°C for 10 min. Ten to 35 μg of total protein, depending on the species, and 10 ng of purified GPx4 were separated by SDS-PAGE on 12% Tris-HCl pre-cast polyacrylamide gels (BioRad) for 30 min at 60 V followed by 90 min at 100 V. GPx4 protein was purified as described previously (Kernstock and Girotti, 2008). Proteins were transferred to Immuno-blot polyvinylidene fluoride membrane (BioRad) and membranes were blocked overnight at 4°C in 1% (w/v) casein in TBS (Pierce Scientific, Rockford, IL, USA). Membranes were then incubated for 60 min in anti-GPx4 primary rabbit polyclonal antibody purified from *E. coli* (human, ab16800; Abcam Inc., Cambridge, MA, USA) diluted to 1/1000 in 0.05% casein solution. The choice of a human antibody in non-mammlian vertebrates was based, in part, on the relatively high (average of 64%) conservation of GPx4 protein sequences between fish (*Dania rerio* GenBank accession no. NP_001025241/ACF72883, *Cyprinus carpio* GenBank accession no. ACR33822, and *Salmo salar* GenBank accession no. ACH86324) and humans (GenBank accession no. NP_002076). Following incubation in the primary antibody, membranes were rinsed 3 times in TBS-T (10 mmol l^{-1} Tris, 137 mmol l^{-1} NaCl, 0.1% Tween-20, pH 7.4), and incubated

Table 1. Morphometric data for all study species

Species	SL (mm)	TL (mm)	Mass (g)
Myxine glutinosa	–	370–480	130–245
Petromyzon marinus	–	630–710	400–815
Squalus acanthias	–	–	1000–3000
Fundulus heteroclitus	58–72	71–92	3.2–4.7
Myoxocephalus octodecemspinosus	–	–	225–271

Myxine glutinosa, Atlantic hagfish; *Petromyzon marinus*, sea lamprey; *Squalus acanthias*, dogfish shark; *Fundulus heteroclitus*, killifish; and *Myoxocephalus octodecemspinosus*, longhorn sculpin. SL, standard length; TL, total length.

with alkaline phosphatase-conjugated goat anti-rabbit IgG (1/3000 in 0.05% casein, BioRad). Chemiluminescent signal of an Immun-star chemiluminescent kit (BioRad) was detected by exposing membranes to Amersham-Pharmacia Hyperfilm (Piscataway, NJ, USA) for a period of 2 min. Purified GPx4 protein was run as a positive control on all gels, and was used to normalize data between membranes. Preliminary experiments were run to ensure that the loaded volumes of both sample and purified GPx4 protein would not produce saturated bands. Negatives were scanned and antibody binding was quantified using Quantity One® (BioRad). All samples were standardized to the quantity of loaded protein and were normalized to antibody binding to the purified GPx4 protein, obtained by densitometry.

Assays of enzyme activity

Whole liver tissue was homogenized as described above for GPx4 protein. Crude homogenates were kept ice-cold for a period of 1 h, during which samples were vortexed thoroughly every 15 min to ensure cell compartments were lysed. After this incubation period, homogenates were centrifuged for 10 min at 1700 ***g*** (CS and CAT) or 6600 ***g*** (GPx1 and GPx4), and the resulting supernatants were used in all subsequent enzyme assays.

Enzyme activity was assayed with Beckman DU640 UV/VIS and Pharmacia Ultrospec 3000 spectrophotometers fitted with circulating waterbaths and a temperature-controlled cell holder. CAT, GPx1 and GPx4 activities represented the enzymatic antioxidant response. CAT activity (see Beers and Sizer, 1952) was quantified in a reaction mixture containing 50 $\mathrm{mmol\,l^{-1}}$ potassium phosphate (pH 7.24) and 12.5 $\mathrm{mmol\,l^{-1}}$ H_2O_2. GPx1 (modified from Flohé and Gunzler, 1984) and GPx4 (Kernstock and Girotti, 2008) activities were assayed in a cuvette containing 1 $\mathrm{mmol\,l^{-1}}$ EDTA, 0.1 $\mathrm{mmol\,l^{-1}}$ DFO (desferrioxamine), 3 $\mathrm{mmol\,l^{-1}}$ reduced glutathione, 0.2 $\mathrm{mmol\,l^{-1}}$ NADPH, 0.2 $\mathrm{U\,ml^{-1}}$ glutathione reductase, 0.1% Triton X-100, and either 200 $\mathrm{\mu mol\,l^{-1}}$ cumene hydroperoxide (GPx1) or 100 $\mathrm{\mu mol\,l^{-1}}$ phosphatidylcholine hydroperoxide (GPx4). Phosphatidylcholine hydroperoxide substrate was prepared as described previously (Kriska and Girotti, 2005). CS activity (a measure of oxidative capacity) was assayed as described elsewhere (Srere et al., 1963) [as modified by Hansen and Sidell (Hansen and Sidell, 1983)], using a reaction mixture containing 40 $\mathrm{mmol\,l^{-1}}$ Hepes, 0.5 $\mathrm{mmol\,l^{-1}}$ oxaloacetate, 0.25 $\mathrm{mmol\,l^{-1}}$ 5,5′-dithio-bis(2-nitrobenzoic acid) and 0.4 $\mathrm{mmol\,l^{-1}}$ acetyl CoA (pH 8.0 at 10°C). All assays of enzyme activity were performed at least in duplicate. GPx1, CAT and CS activities were measured at 15°C, while GPx4 activity was measured at 25°C. Enzyme activities were compared directly at a common temperature (15°C) by adjusting enzyme activity measured at 25°C (GPx4) using a conservative Q_{10}=2. In addition to comparisons of individual enzyme activities between taxa, enzyme activities were also used to calculate ratios of GPx4/GPx1 activity and GPx4/CAT activity for all taxa. These ratios were used to test hypotheses regarding the prioritization of antioxidant defenses among taxa in response to species-specific stresses (e.g. differences in PUFA content or oxidative activity).

Phospholipid composition

Lipids were extracted from liver homogenates in the presence of chloroform and methanol (see Bligh and Dyer, 1959). Phospholipid class and molecular species composition of total lipid extracts of all other species were analyzed by triple-quadrupole mass spectrometry by the Kansas Lipidomics Research Center. Unsaturation index (UI), which represents the average number of double bonds per 100 fatty acids, was calculated as described before (Grim et al., 2010) [modified from Hulbert et al. (Hulbert et al., 2007)] to account for the total number of double bonds present in diacyl phospholipids, rather than individual fatty acids. Therefore double bond number ranges from 0 to 12 with the maximum double bond number of 12 corresponding to a phospholipid containing two fatty acids chains with six double bonds each:

$$\mathrm{UI} = \sum_{n=12}^{n=0} n \times \text{mol\% of fatty acids containing } n \text{ double bonds.} \quad (1)$$

All lipid composition data are reported as mol%, and only diacyl phospholipids more abundant than 2 mol% were included in the analysis of individual pospholipids.

Statistical analyses

Species-specific mean GPx4 protein (units $\mathrm{mg^{-1}}$ loaded protein normalized to purified GPx4), mean enzymatic activity ($\mathrm{U\,mg^{-1}}$ total protein), GPx4/GPx1 activity, GPx4/CAT activity, and phospholipid metrics [UI, phosphatidylcholine/phosphatidylethanolamine (PE/PC), and relative amounts of each lipid class] were compared using either an ANOVA or Kruskal–Wallis test (Graphpad Prism, Graphpad Software Inc., La Jolla, CA, USA). Significant ANOVA or Kruskal–Wallis results were followed by Tukey's or Dunn's multiple comparison test, respectively, to identify statistically distinct subsets of data. Regression analysis was used to examine relationships between GPx4 protein and activity, and also links between antioxidant enzyme activity and both UI and CS activity (Graphpad Prism). A Bonferroni-adjusted critical α-value of 0.0125 was used in all ANOVA/Kruskal–Wallis analyses of enzyme data

Table 2. Relative abundance of phospholipid classes in marine fish liver

	Myxine glutinosa *N*=5	*Petromyzon marinus* *N*=4	*Squalus acanthias* *N*=4	*Fundulus heteroclitus* *N*=8	*Myoxocephalus octodecemspinosus* *N*=4
%PC	31±1.1^{c}	37±5.9^{b,c}	36±0.3^{b,c}	56±1.1^{a}	45±1.1^{b}
%PE	9±0.3^{c}	9±1.9^{c}	27±0.3^{a}	21±1.0^{b}	24±1.0^{a,b}
%PI	6±0.3^{b}	6±1.0^{b}	13±0.7^{a}	7±0.2^{b}	6±0.4^{b}
%PS	3.5±0.2^{a}	2.3±0.2^{b}	1.9±0.3^{b}	2.4±0.2^{b}	3.6±0.1^{a}
%SM	3.7±0.4^{b}	2.6±0.6^{b,c}	3.6±0.1^{b}	2.1±0.2^{c}	7.4±0.2^{a}
%PC plasmalogen	43±1.3^{a}	37±9.4^{a}	14±1.3^{b}	8.3±0.2^{b}	10±0.8^{b}
PE/PC	0.30±0.01^{c,d}	0.24±0.02^{d}	0.73±0.01^{a}	0.38±0.03^{c}	0.54±0.03^{b}

Data are presented as mol%; total of 100% when minor lipid components are included (data not shown). Significant ANOVA and Kruskal–Wallis results were followed by Tukey's or Dunn's *post hoc* analysis, respectively. Levels not connected by the same letters are significantly different.

Myxine glutinosa, Atlantic hagfish; *Petromyzon marinus*, sea lamprey; *Squalus acanthias*, dogfish shark; *Fundulus heteroclitus*, killifish; and *Myoxocephalus octodecemspinosus*, longhorn sculpin. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

to control for multiple comparisons (four enzymes). All *post hoc* analyses and other statistical analyses were evaluated using an α-value of 0.05. Unless otherwise noted, data are presented as means ± s.e.m.

RESULTS

Phospholipid composition

In order to calculate the UI and relative abundance of the different phospholipid classes in the livers of the studied animals, we analyzed the distribution of phospholipid classes and individual phospholipid species (Tables 2 and 3, respectively). Lipid profiles of all species were dominated by phospholipids containing either choline or ethanolamine headgroups (Table 2), although the proportions of phospholipid classes vary among species.

Thirty-five phospholipid species were present in amounts ≥2 mol% for at least one marine species (Table 3) and, of these, 26 contained PUFA. UI values (double bond content) were elevated 1.3 times in killifish, longhorn sculpin and dogfish shark, relative to hagfish and sea lamprey (Kruskal–Wallis; H_4=20.61; P<0.001; Table 3).

Enzyme activity and protein level of GPx4

GPx4 protein and enzyme activity in liver samples of different marine fish were determined by immunoblot and coupled enzyme assay, respectively. The GPx4 antibody produced bands of the expected molecular mass (22 kDa) in all samples (killifish replicates shown) and positive controls of purified GPx4 protein (supplementary material Fig. S1). Western blot analysis revealed that marine fish contain comparable levels of GPx4 protein (ANOVA; $F_{3,21}$=1.96; P>0.05; Fig. 1A). The activity of GPx4, however, was nearly 6 times higher in killifish and dogfish shark, relative to the other species (ANOVA; $F_{4,24}$=23.11; P<0.0001; Fig. 1B). GPx4

Table 3. Diacyl phospholipid composition of five vertebrate species

	Myxine glutinosa (*N*=5)	*Petromyzon marinus* (*N*=4)	*Squalus acanthias* (*N*=4)	*Fundulus heteroclitus* (*N*=8)	*Myoxocephalus octodecemspinosus* (*N*=4)
PC 32:1	1.00±0.06^{b}	2.88±1.09^{a}	0.23±0.02^{b}	0.73±0.05^{b}	0.99±0.14^{b}
PC 34:2	1.30±0.16^{a,b}	2.35±1.06^{a,b}	0.86±0.04^{b}	2.46±0.06^{a}	1.04±0.10^{a,b}
PC 34:1	2.20±0.17	2.26±0.84	2.27±0.15	3.54±0.24	3.82±0.41
PC 36:6	0.19±0.02^{c}	0.37±0.07^{c}	0.48±0.04^{c}	1.12±0.12^{b}	2.19±0.15^{a}
PC 36:5	1.52±0.26^{c}	2.13±0.28^{c}	10.63±0.29^{a}	2.13±0.15^{c}	8.86±0.42^{b}
PC 36:4	0.76±0.09^{c}	1.75±0.33^{b,c}	3.16±0.37^{a}	1.94±0.12^{b}	1.53±0.36^{b,c}
PC 36:3	0.38±0.05^{b,c}	2.44±1.03^{a}	0.03±0.02^{d}	1.62±0.08^{a,b}	0.09±0.01^{d}
PC 36:2	3.71±0.98^{a}	1.36±0.61^{b}	0.30±0.03^{b}	1.90±0.23^{a,b}	0.43±0.11^{b}
PC 38:6	2.31±0.18^{d}	4.49±0.28^{c}	6.98±0.29^{b}	12.67±0.41^{a}	13.35±0.92^{a}
PC 38:5	3.68±0.39^{a}	2.80±0.12^{a,b}	1.82±0.07^{b}	3.18±0.18^{a}	1.79±0.23^{b}
PC 38:0 or ePC 40:7	0.68±0.14^{b}	2.93±1.27^{a}	0.95±0.05^{a,b}	0.67±0.03^{b}	1.83±0.25^{a,b}
PC 40:7	1.58±0.41^{b,c}	0.85±0.13^{c}	1.05±0.07^{c}	7.61±0.27^{a}	2.53±0.39^{b}
PC 40:6	2.87±0.14^{a,b}	1.06±0.11^{b}	1.23±0.13^{b}	5.26±0.90^{a}	0.76±0.26^{b}
SM 14:0	0.03±0.01^{d}	5.53±0.79^{a}	1.41±0.11^{b,c}	2.01±0.10^{b}	0.43±0.08^{c,d}
SM 22:1	2.11±0.29^{a}	0.00±0.00^{b}	0.59±0.04^{b}	0.05±0.04^{b}	2.65±0.39^{a}
SM 24:1	0.69±0.10^{c}	0.02±0.02^{c}	1.81±0.06^{b}	1.62±0.12^{b}	4.27±0.52^{a}
ePC 32:1	2.10±0.23^{a}	0.51±0.09^{b}	0.31±0.04^{b}	0.08±0.01^{b}	0.23±0.02^{b}
ePC 34:1	4.54±0.39^{a}	0.91±0.17^{b}	0.74±0.07^{b}	0.26±0.01^{b}	0.30±0.03^{b}
ePC 36:5	3.14±0.38^{a}	2.27±0.93^{a,b}	1.49±0.07^{b,c}	0.18±0.00^{c}	0.48±0.08^{c}
ePC 36:4	1.95±0.33^{a,b}	2.56±0.99^{a}	0.29±0.01^{b,c}	0.16±0.02^{c}	0.08±0.03^{c}
ePC 36:2	2.27±0.35^{a}	1.50±0.25^{a}	0.37±0.04^{b}	0.25±0.02^{b}	0.19±0.02^{b}
ePC 38:6	3.76±0.28^{a,b}	7.86±3.21^{a}	3.87±0.25^{a,b}	1.37±0.07^{b}	1.43±0.25^{b}
ePC 38:5	10.39±0.62^{a}	7.70±2.60^{a}	1.48±0.12^{b}	0.29±0.03^{b}	0.66±0.16^{b}
ePC 38:4	0.51±0.18^{b}	3.41±0.53^{a}	0.34±0.02^{b}	0.13±0.02^{b}	0.20±0.09^{b}
ePC 38:0	0.07±0.02^{c}	0.68±0.16^{c}	0.36±0.00^{c}	2.49±0.09^{b}	3.53±0.48^{a}
ePC 40:6	6.95±0.40^{a}	4.70±2.00^{a,b}	0.91±0.06^{c}	1.31±0.09^{c}	1.31±0.25^{b,c}
ePC 40:5	2.46±0.18^{a}	0.69±0.25^{b}	0.68±0.11^{b}	0.32±0.02^{b}	0.37±0.08^{b}
PE 38:6	0.69±0.10^{d}	0.80±0.24^{d}	4.75±0.17^{b}	2.78±0.20^{c}	7.01±1.04^{a}
PE 38:5	3.02±0.12^{a}	1.69±0.25^{b}	2.80±0.10^{a}	1.63±0.25^{b}	1.73±0.07^{b}
PE 38:4	0.92±0.18^{b,c}	1.89± 0.60^{a,b}	2.39±0.26^{a}	1.27±0.10^{b,c}	0.32±0.06^{c}
PE 40:7	0.17±0.03^{d}	0.66±0.21^{c,d}	1.73±0.16^{c}	3.71±0.33^{b}	7.18±0.39^{a}
PE 40:6	1.11±0.09^{c}	1.09±0.13^{c}	7.41±0.18^{a}	4.87±0.53^{b}	2.11±0.30^{c}
PE 42:7	0.10±0.01^{c}	0.07±0.02^{c}	2.17±0.15^{a}	0.46±0.07^{b}	0.47±0.06^{b}
PI 38:5	1.82±0.19^{a}	0.87±0.15^{b,c}	1.36±0.17^{a,b}	1.60±0.08^{a}	1.97±0.25^{a}
PI 38:4	2.06±0.42	1.81±0.21	2.85±0.42	2.59±0.09	2.60±0.23
UI	360±23^{b}	368±22^{b}	437±3^{a}	485±8^{a}	449±6^{a}

Diacyl lipid composition data (headgroup total no. of carbons:total no. of double bonds) are presented as mean mol% ± s.e.m. Only molecular species present in amounts ≥2 mol% for at least one of the species sampled are shown. Unsaturation index (UI) was calculated as:

$$\mathrm{UI} = \sum_{n=12}^{n=0} n \times \text{mol\% of fatty acids containing } n \text{ double bonds.}$$

Significant ANOVA and Kruskal–Wallis results were followed by Tukey's or Dunn's *post hoc* analysis, respectively. Superscript letters identify statistically distinct subsets of data.

Myxine glutinosa, Atlantic hagfish; *Petromyzon marinus*, sea lamprey; *Squalus acanthias*, dogfish shark; *Fundulus heteroclitus*, killifish; and *Myoxocephalus octodecemspinosus*, longhorn sculpin.

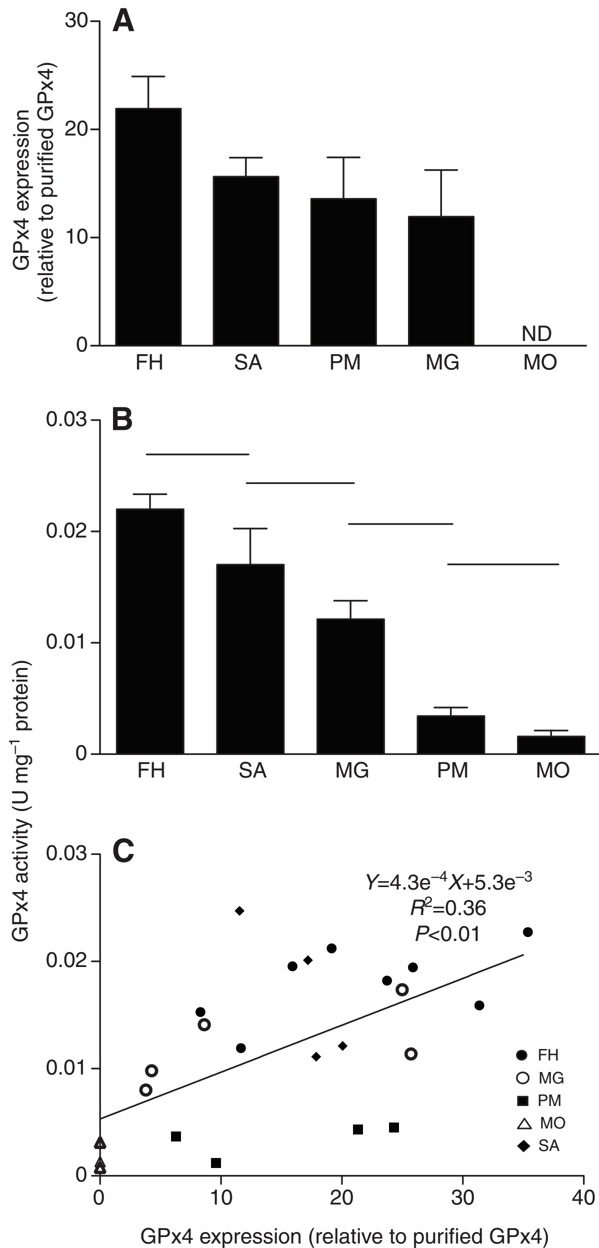

Fig. 1. Glutathione peroxidase 4 (GPx4) protein (A), GPx4 activity at 15°C (B), and the relationship between GPx4 activity and protein (C) in livers from different species of marine fish: killifish (FH), dogfish shark (SA), Atlantic hagfish (MG), sea lamprey (PM) and longhorn sculpin (MO). Means and s.e.m. are plotted (minimum N=4 per species). Bars not connected by horizontal lines in A and B are significantly different. ND, not detectable.

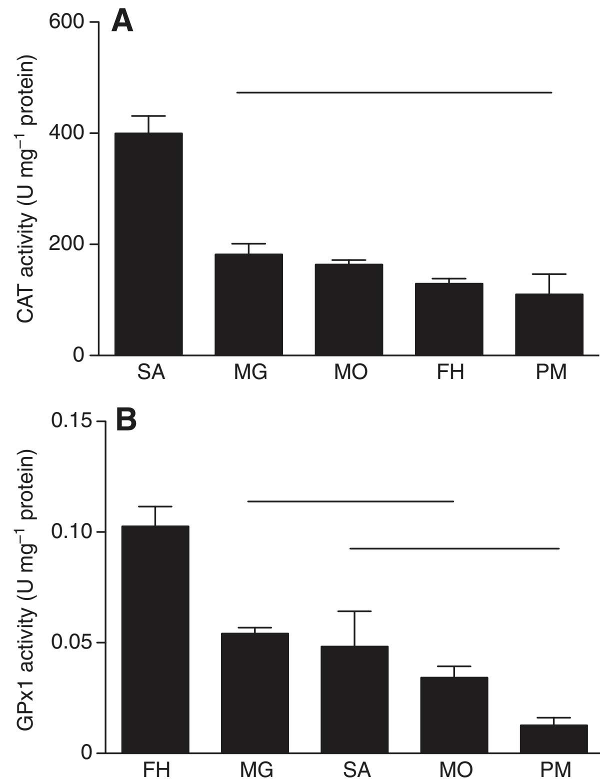

Fig. 2. Comparison of catalase (CAT, A) and GPx1 activity (B) among marine fish at 15°C: killifish (FH), dogfish shark (SA), Atlantic hagfish (MG), sea lamprey (PM) and longhorn sculpin (MO). Means and s.e.m. are plotted (minimum N=4 per species). Bars not connected by horizontal lines are significantly different.

protein was undetectable in longhorn sculpin, while GPx4 activity was very low but measurable. A positive relationship exists between GPx4 protein and activity (R^2=0.36; ANOVA; $F_{1,33}$=12.94; P<0.01; Fig. 1C).

Activities of pro-oxidant and other antioxidant enzymes

In order to obtain a more complete picture of the enzymatic antioxidant defenses of marine fish, we also compared the activities of CS, CAT and GPx1. CAT activity was 2.7 times higher in livers from dogfish shark relative to all other species (ANOVA; $F_{4,28}$=22.12; P<0.0001; Fig. 2A), while GPx1 was elevated 2.8 times in killifish (ANOVA; $F_{4,17}$=17.70; P<0.0001; Fig. 2B). Oxidative activity as indicated by CS was highest in killifish and sea lamprey relative to sculpin (ANOVA; $F_{3,21}$=13.80; P<0.0001; Fig. 3), and was undetectable in dogfish shark.

Relationship between UI, oxidative activity and enzymatic antioxidants

We used linear regression analyses to examine the relationship(s) between the content of PUFA and oxidative and anti-oxidative enzymes in all species tested. A positive relationship exists between GPx4 enzyme activity in the liver of marine fish and the UI of tissue lipids (R^2=0.30; ANOVA; $F_{1,23}$=10; P< 0.01; Fig. 4A). GPx4 activity was also related, to a lesser extent, to the activity of CS (R^2=0.28; ANOVA; $F_{1,23}$=8.77; P<0.01; Fig. 4D). GPx1 activity was related to both UI (R^2=0.24; ANOVA; $F_{1,21}$=6.8; P< 0.05; Fig. 4B) and the activity of CS (R^2=0.24; ANOVA; $F_{1,21}$=6.6; P< 0.05; Fig. 4E), although less strongly than was observed for relationships involving GPx4 activity. In contrast to the glutathione peroxidases, there was no relationship between CAT activity and UI (Fig. 4C), and CAT activity was negatively related to CS activity (R^2=0.43; ANOVA; $F_{1,23}$=17.41; P< 0.001; Fig. 4F).

Ratios of antioxidant enzyme activity

Ratios of GPx4 activity to either GPx1 or CAT activity were generally similar among marine fish (Fig. 5). GPx4/GPx1 activity is elevated, relative to that in longhorn sculpin, in sea lamprey, dogfish shark and killifish (ANOVA; $F_{4,17}$=6.00; P<0.01; Fig. 5A), and ranged

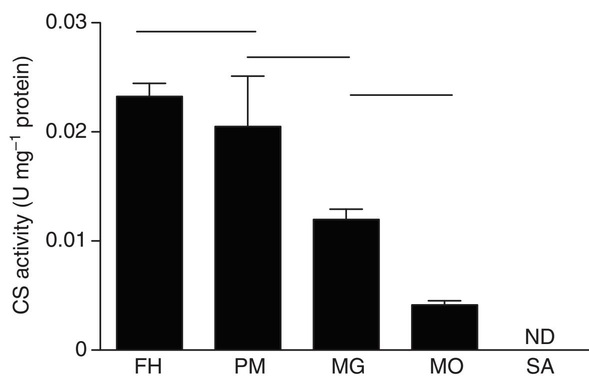

Fig. 3. Comparison of citrate synthase (CS) activity among marine fish at 15°C: killifish (FH), dogfish shark (SA), Atlantic hagfish (MG), sea lamprey (PM) and longhorn sculpin (MO). Means and s.e.m. are plotted (minimum *N*=4 per species). Bars not connected by horizontal lines are significantly different. ND, not detectable.

from 0.385 (sea lamprey) to 0.05 (longhorn sculpin). GPx4/CAT, in contrast, is extremely low in all species ($2.0e^{-4}$ to $9.4e^{-6}$), and at the maximum in killifish, GPx4 activity is 1/5000 of CAT activity (Kruskal–Wallis; H_5=20.28; P<0.001; Fig. 5B).

DISCUSSION

Our results suggest that both the PUFA content and the oxidative activity of animals contribute to the capacity for GPx4-catalyzed reduction of phospholipid hydroperoxides. A high degree of membrane unsaturation and elevated oxidative activity are accompanied by robust GPx4 activity in marine fish (including representatives of Agnatha, Chondrichthys and Teleostei), as indicated by the positive regressions between GPx4 and both UI and CS activity. At the same time, preliminary data (J.M.G. and E.L.C., unpublished) indicate that marine fish (which have on average 1.3 times higher unsaturation indices than some terrestrial vertebrates, including mouse and newt) also have nearly 10 times more GPx4 activity in liver than terrestrial vertebrates when compared at a common temperature. The activity of GPx1 was also positively related to both UI and oxidative activity in marine fish, although to a lesser degree. These data, when interpreted together, imply a strong link between both membrane unsaturation and oxidative activity with glutathione-dependent peroxidase activity in livers of marine fish. Additionally, these data reveal that marine fish likely defend against initial oxidative insults (primarily using GPx1), as well as increase repair mechanisms (i.e. GPx4 activity) in order to counteract damage that may occur as a result of having an elevated PUFA content.

While the fish species sampled span the entire marine fish lineage from Agnatha to Teleostei, it appears that the data are not confounded by phylogeny or differences in diet or activity among the studied species. Ages of the studied species represent a variable that could not be controlled in the current study. None of the datasets, including enzyme activity, protein level and UI, included a phylogenetic signal [as indicated by Blomberg's *K* using the phylogeny published by Takezaki et al. (Takezaki et al., 2003) and the 'phytools' package in R (R Development Core Team, 2008); P>0.05]. While we cannot definitively eliminate a role for variable activity levels in the differences in the antioxidant enzymes among the studied species (Wilhelm-Filho et al., 1993), the impact of locomotor activity level should be insignificant, because it has been reported that exhaustive exercise under controlled laboratory conditions has no effect on antioxidant enzymes in fish liver (Aniagu et al., 2006). While it is possible that the metamorphic, fasting state of sea lamprey sampled may have influenced the relationship(s) between antioxidant enzymes and either UI or CS activity (presented in Fig. 4), all other species used in the current work were maintained on diets that mimic the natural complement of lipids present in the native diets. Therefore, lipid profiles for all experimental animals should reflect those of wild-caught animals at the time of sampling, and represent a range of PUFA content with which to test the relationship(s) between GPx4 (and other antioxidant defenses) and PUFA.

Comparisons of tissue-specific PUFA content and GPx4 activity, as well as *in vitro* feeding studies in mammals indicate a link between unsaturation level of tissue lipids and the activity of GPx isozymes in other vertebrate groups. For example, high GPx4 activity has been found in rat testes (Roveri et al., 1992), retina (Wang et al., 1994) and adrenal gland (Roveri et al., 1994) – tissues that contain abundant PUFA (Gavazza and Catalá, 2006; Tanito et al., 2008; Huang et al., 1984). The results from feeding studies are less clear; however, several reports demonstrate that mammals consuming feeds fortified with fish oil (e.g. ω-3 PUFA including 20:5 and 22:6) over a prolonged period have increased hepatic GPx activity (likely to represent total GPx activity) (Brigelius-Flohé, 1999) and also increased tissue levels of ω-3 PUFA (Kaasgaard et al., 1992; Ruiz-Gutiérrez et al., 1999; Gutiérrez et al., 2001; Venkatraman et al., 1994). More specifically, increases of 1.4 times in hepatic GPx activity have been reported for both monkeys and mice fed diets enriched in marine oil, relative to animals consuming corn oil diets (Kaasgaard et al., 1992; Venkatraman et al., 1994). In contrast, consumption of marine oil has been shown to reduce the activity of hepatic GPx (Luostarinen et al., 2001; Pulla Reddy and Lokesh, 1994; Venkatraman and Pinnavaia, 1998; Venkatraman et al., 1998), and there is no clear explanation for the conflicting reports of GPx activity in feeding studies, even by the same authors (Venkatraman et al., 1994; Venkatraman and Pinnavaia, 1998; Venkatraman et al., 1998). As GPx1 and GPx4 can both detoxify soluble hydroperoxides (e.g. H_2O_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. Data from the current study on marine fish link membrane unsaturation to the activity of both GPx4 and GPx1, and therefore the reported increases in glutathione peroxidase activity following marine oil consumption are likely to reflect increased activity of both GPx isozymes.

A closer look at GPx4 and GPx1 activities across a taxonomic range of marine fish may also shed light on how antioxidant defenses are modified in response to species-specific oxidative stress. Ratios of GPx4/GPx1 activity are generally similar among species (Fig. 5A), regardless of differences in CS activity (Fig. 3) or membrane unsaturation (Table 3), indicating that marine fish partition the abundant selenium available in marine habitats (Wrench and Measures, 1982) between the biosynthesis of GPx4 and GPx1 in order to maintain an array of glutathione-dependent antioxidant defenses. In contrast, membranes from the livers of mice contain 1.3 times fewer double bonds (UI=357), and a significantly lower GPx4/GPx1 activity, when compared with marine fish (J.M.G. and E.L.C., unpublished). Under selenium-limited conditions, GPx4 (and to a lesser extent GPx1) is (are) preferentially retained, relative to other GPx isosymes, in both mammalian brain and testis when selenium is limiting (Brigelius-Flohé, 1999), and therefore the disparate strategies for maintaining GPx antioxidant defenses (i.e. GPx4/GPx1 activity) in marine fish and mouse are unlikely to be a function of selenium limitation derived from either diet or habitat. Selective partitioning of selenium amongst GPx isozymes exists in

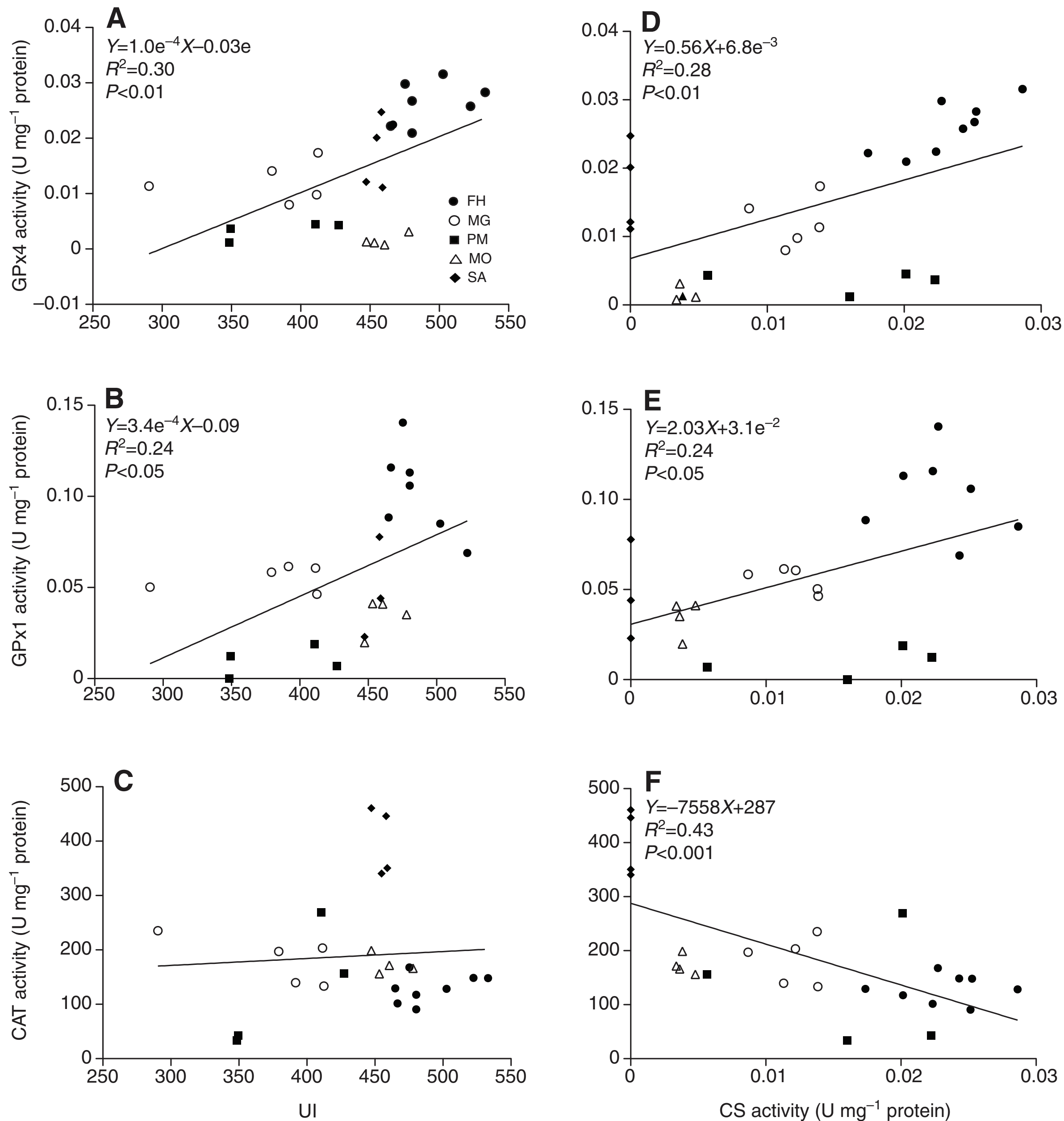

Fig. 4. Linear relationships between GPx4 activity and either unsaturation index (UI, A–C) or CS activity (D–F) among marine fish: killifish (FH), dogfish shark (SA), Atlantic hagfish (MG), sea lamprey (PM) and longhorn sculpin (MO); minimum *N*=4 per species.

animals (Brigelius-Flohé, 1999), and species may benefit from prioritizing the maintenance of different GPx isozymes, especially given the redundancy in selenium requirement and the overlap in substrate compatibility between GPx1 and GPx4. It is likely, therefore, that the elevated PUFA content in the tissues of marine fish (Table 3) warrants a more uniform partitioning of resources between GPx4 and GPx1 (shown as elevated GPx4/GPx1) than in terrestrial vertebrates or other animals with lower PUFA levels. By deploying a more balanced complement of GPx isozymes than in terrestrial mammals, marine fish would be provided with comparable protection against H_2O_2, while simultaneously mitigating the potential for increased lipid peroxidation associated with phospholipid hydroperoxides.

In contrast to the fundamental similarities between the GPx isozymes GPx4 and GPx1 (e.g. selenium incorporation and substrate overlap), GPx4 and CAT have little in common aside from the ability to detoxify H_2O_2. The low GPx4/CAT values (Fig. 5B) for all marine fish tested are therefore unlikely to represent a metric for potential resource partitioning among species, but rather reflect the primary localization of CAT to peroxisomes, which are particularly abundant in liver (de Duve and Baudhuin, 1966). It has been hypothesized, however, that GPx1 and CAT work cooperatively *in vivo* to detoxify endogenous H_2O_2, with GPx1 (by virtue of its relatively small K_m for H_2O_2) responding to eliminate lower concentrations of H_2O_2, while CAT (high K_m) is recruited to assist at higher concentrations of H_2O_2 (Halliwell and Gutteridge, 2007). Baud and colleagues proposed that GPx1 protects CAT in oligodendrocytes from irreversible inactivation by maintaining H_2O_2 concentration at $<100\,\mu mol\,l^{-1}$ (Baud et al., 2004). It is unclear what role GPx4 plays relative to CAT, *in vivo* in other cell types, or how a more balanced

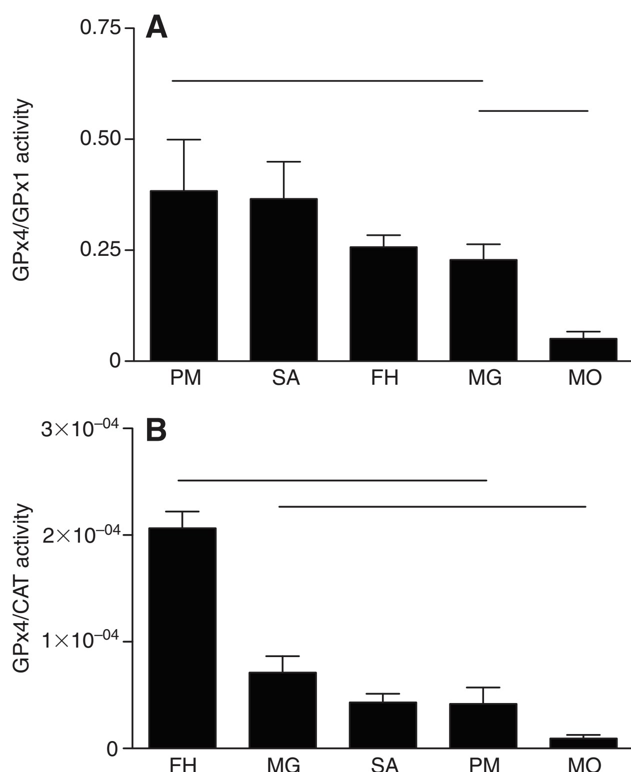

Fig. 5. Ratios of GPx4/GPx1 (A) and GPx4/CAT (B) enzyme activity among marine fish: killifish (FH), dogfish shark (SA), Atlantic hagfish (MG), sea lamprey (PM) and longhorn sculpin (MO). Means and s.e.m. are plotted (minimum *N*=4 per species). Bars not connected by horizontal lines are significantly different.

complement of GPx isozymes in marine fish may potentially affect the catalytic cooperativity between GPx1 and CAT.

While there is no immediate explanation for why the marine teleost longhorn sculpin possesses such low levels of GPx4 protein and activity in contrast with other marine fish, several points should be considered. 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) are extremely low in sculpin compared with other teleosts examined (E.L.C., unpublished), in spite of relatively high membrane unsaturation (Table 3). 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 low molecular weight antioxidants like α-tocopherol or other antioxidant enzymes) or PLA_2. Because longhorn sculpin are a relatively derived marine teleost (Kontula and Väinölä, 2003), and duplicate GPx4 genes have been reported in teleosts (Thisse et al., 2003; Hermesz and Ferencz, 2009), there also exists the possibility that a novel GPx isozyme (undetected by our methods) may be occupying the role typically associated with GPx4. In light of the relationship between membrane unsaturation and GPx4, future studies may consider how the relatively high levels of highly oxidizable phospholipids of sculpin are protected against oxidation.

Additional GPx4 function(s)

In addition to protecting membranes from oxidative stresses, GPx4 has been implicated in a wide-variety of biological functions. Although the earliest reports of GPx4 focused on its ability to detoxify complex lipid hydroperoxides (Ursini et al., 1982), several recent reports have recognized additional functions for this protein in mammalian tissues and cells, including roles as a structural protein during spermatogenesis, and as a regulator of apoptosis, eicosanoid production and redox signaling (Brigelius-Flohé, 1999; Ursini et al., 1999; Imai and Nakagawa, 2003; Conrad et al., 2007; Flohé, 2007; Seiler et al., 2008; Liang et al., 2009). 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 likely paramount to the suite of complex processes now ascribed to the protein. At the same time, not much is known about the fundamental mechanism(s) that drive(s) GPx4 expression within tissues of an organism and/or between organisms within a taxonomic group.

CONCLUSIONS

The broad substrate specificity of GPx4, including complex lipid hydroperoxides, functions, at least in part, to protect the high PUFA content in marine fish against excess lipid peroxidation. The proposed link between GPx4 and PUFA content is supported by the positive relationship between GPx4 activity and UI among marine fish in the current study. Furthermore, relative activities of glutathione peroxidases (i.e. GPx4/GPx1 ratios) in marine fish reveal partitioning of resources among GPx isozymes, which indicates that abundant PUFA levels in these organisms may require increased protection from initial oxidative insults (*via* GPx1 activity), as well as an increased ability to repair oxidized phospholipids (*via* GPx4). Preliminary data from our laboratory suggest that marine fish have increased GPx4 protein and activity, relative to terrestrial vertebrates (newt and mouse), indicating that antioxidant defenses are likely prioritized to meet species-specific oxidative stresses. Finally, the positive relationship between GPx4 and membrane unsaturation in the current study suggests that other functions attributed to GPx4 may have evolved secondarily in response to the elevated need for antioxidant protection in PUFA-rich tissues.

LIST OF SYMBOLS AND ABBREVIATIONS

CAT	catalase
CS	citrate synthase
GPx	glutathione peroxidase (general)
GPx1	glutathione peroxidase 1
GPx4	glutathione peroxidase 4
LPO	lipid peroxidation
PLA_2	phospholipase A_2
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
UI	unsaturation index

ACKNOWLEDGEMENTS

Special thanks to Drs Susan Edwards and Steve Reilly for access to livers from their experimental animals. Additionally the authors are grateful to Ms Hae Lim Yook (MDIBL High School Fellow funded by the Kathryn A. Davis Foundation) and Ms Liz Simonik (Ohio University) for assisting with the enzymatic analyses. We would also like to recognize Drs Christopher Pomory and Sharlene Santana for providing their statistical expertise.

FUNDING

This work was supported by an Ohio University SEA [SEA-08-39], Mount Desert Island Biological Laboratory's (MDIBL) Stan and Judy Fund Graduate Fellowship, and Ohio Center for Ecology and Evolutionary Studies (OCEES) Fellowship (all to J.M.G.); by MDIBL's National Institute for Environmental Health Sciences Center for Membrane Toxicity Studies [P30 ES003828-20], American Physiological Society Research Career Enhancement Award, and National Science Foundation [IOS no. 0842624] (all to E.L.C.); by National Institutes of Health [CA72630] (to A.W.G.); and National Science Foundation grants [MCB 0455318 and DBI 0521587 to the Kansas State University Lipidomics Research Center (KLRC)],

and National Science Foundation EPSCoR [grant EPS-0236913] with matching support from the State of Kansas through Kansas Technology Enterprise Corporation and Kansas State University. The KLRC is also supported by a K-INBRE National Institutes of Health grant [P20 RR16475] from the INBRE program of the National Center for Research Resources. Deposited in PMC for release after 12 months.

REFERENCES

Aniagu, S., Day, N., Chipman, J. K., Taylor, E. W., Butler, P. J. and Winter, M. J. (2006). Does exhaustive exercise result in oxidative stress and associated DNA damage in the chub (*Leuciscus cephalus*). *Environ. Molecular Muta.* **47**, 616-623.

Antunes, F., Salvador, A. and Pinto, R. E. (1995). PHGPx and phospholipase A2/GPx: comparative importance on the reduction of hydroperoxides in rat liver mitochondria. *Free Rad. Biol. Med.* **19**, 669-677.

Baud, O., Greene, A. E., Li, J., Wang, H., Volpe, J. J. and Rosenberg, P. A. (2004). Gluathione peroxidase-catalase cooperativity is required for resistance to hydrogen peroxide by mature rat oligodendrocytes. *J. Neurosci.* **24**, 1531-1540.

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

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

Brigelius-Flohé, R. (1999). Tissue-specific functions of individual glutathione peroxidases. *Free Rad. Bio. Med.* **27**, 951-965.

Choudhary, S., Zhang, W., Zhou, F., Campbell, G. A., Chan, L. L., Thompson, E. B. and Ansari, N. H. (2002). Cellular lipid peroxidation end-products induce apoptosis in human lens epithelial. *Free Rad. Bio. Med.* **32**, 360-369.

Conrad, M., Schneider, M., Seiler, A. and Bornkamm, G. (2007). Physiological role of phospholipid hydroperoxide glutathione peroxidase in mammals. *Biol. Chem.* **388**, 1019-1025.

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

de Duve, C. and Baudhuin, P. (1966). Peroxisomes (microbodies and related particles). *Physiol. Rev.* **46**, 323-357.

Edwards, S. L., Claiborne, J. B., Morrison-Shetlar, A. I. and Toop, T. (2001). Expression of Na^+/H^+ exchanger mRNA in the gills of the Atlantic hagfish (*Myxine glutinosa*) in response to metabolic acidosis. *Comp. Biochem. Physiol.* **130A**, 81-91.

Flohé, L. (2007). Selenium in mammalian spermiogenesis. *Biol. Chem.* **388**, 987-995.

Flohé, L. and Günzler, W. F. (1984). Assays of glutathione peroxidase. *Meth. Enzym.* **105**, 114-121.

Floyd, R. A. (1999). Antioxidants, oxidative stress, and degenerative neurological disorders. *Proc. Soc. Exp. Biol. Med.* **222**, 236-245.

Gavazza, M. B. and Catalá, A. (2006). The effect of α-tocopherol on lipid peroxidation of microsome and mitochondria from rat testis. *Prostag. Leukotr. Ess.* **74**, 247-254.

Grim, J. M., Miles, D. R. B. and Crockett, E. L. (2010). Temperature acclimation alters oxidative capacities and composition of membrane lipids without influencing activities of enzymatic antioxidants or susceptibility to lipid peroxidation in fish muscle. *J. Exp. Biol.* **213**, 445-452.

Halliwell, B. and Gutteridge, J. M. C. (2007). *Free Radicals in Biology and Medicine*. New York, NY: Oxford University Press.

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

Hermesz, E. and Ferencz, Á. (2009). Identification of two phospholipid hydroperoxide glutathione peroxidase (*gpx4*) genes in common carp. *Comp. Biochem. Phys.* **150C**, 101-106.

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

Huang, Y. S., Horrobin, D. F., Manku, M. S., Mitchell, J. and Ryan, M. A. (1984). Tissue phospholipid fatty acid composition in the diabetic rat. *Lipids.* **19**, 367-370.

Hulbert, A. J., Rana, T. and Couture, P. (2002). The acyl composition of mammalian phospholipids: an allometric analysis. *Comp. Biochem. Phys.* **132B**, 515-527.

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

Hurst, R., Korytowski, W., Kriska, T., Esworthy, R. S., Chu, F.-F. and Girotti, A. W. (2001). Hyperresistanve to cholesterol hydroperoxide-induced peroxidative injury and apoptotic death in a tumor cell line that overexpresses glutathione peroxidase isotype-4. *Free Rad. Biol. Med.* **31**, 1051-1065.

Hyndman, K. A. and Evans, D. H. (2009). Effects of environmental salinity on gill endothelin receptor expression in the killifish, *Fundulus heteroclitus*. *Comp. Biochem. Physiol. A.* **152**, 58-65.

Hyndman, K. A., Choe, K. P., Havird, J. C., Rose, R. E., Piermarini, P. M. and Evans, D. H. (2006). Neuronal nitric oxide synthase in the gill of the killifish, *Fundulus heteroclitus*. *Comp. Biochem. Physiol. B.* **144**, 510-519.

Imai, H. and Nakagawa, Y. (2003). Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. *Free Rad. Biol. Med.* **34**, 145-169.

Kaasgaard, S. G., Hølmer, G., Høy, C.-E., Behrens, W. A. and Beare-Rogers, J. L. (1992). Effects of linseed oil and marine oil on lipid peroxidation in monkey liver *in vivo* and *in vitro*. *Lipids* **27**, 740-745.

Kernstock, R. M. and Girotti, A. W. (2008). New strategies for the isolation and determination of naturally occurring type-4 gluathione peroxidase. *Protein Expres. Purif.* **62**, 216-222.

Kontula, T. and Väinölä, R. (2003). Relationships of Paleartic and Nearctic 'glacial relict' *Myoxocephalus* sculpins from mitochondrial DNA data. *Mol. Ecol.* **12**, 3179-3184.

Kriska, T. and Girotti, A. W. (2005). A thin layer chromatographic method for determining the enzymatic activity of peroxidases catalyzing the two-electron reduction of lipid hydroperoxides. *J. Chromatography.* **827**, 58-84.

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

Lambert, A. J., Boysen, H. M., Buckingham, J. A., Yang, T., Podlutsky, A., Austad, S. N., Kunz, T. H., Buffenstein, R. and Brand, M. D. (2007). Low rates of hydrogen peroxide production by isolated heart mitochondria associate with long maximum lifespan in vertebrate homeotherms. *Aging Cell.* **6**, 607-618.

Liang, H., Van Remmen, H., Frohlich, V., Lechleiter, J., Richardson, A. and Ran, Q. (2007). GPx4 protects mitochondrial ATP generation against oxidative damage. *Biochem. Biophys. Res. Comm.* **356**, 893-898.

Liang, H., Ran, Q., Jang, Y. C., Holstein, D., Lechleiter, J., McDonald-Marsh, T., Musatov, A., Song, W., Van Remmen, H. and Richardson, A. (2009). Glutathione peroxidase 4 differentially regulates the release of apoptogenic proteins from mitochondria. *Free Rad. Biol. Med.* **47**, 312-320.

Luostarinen, R. L., Laasonen, K. and Calder, P. C. (2001). α-Tocopherol concentrations, lipid peroxidation and superoxide dismutase and glutathione peroxidase activities in rat heart and liver after feeding stabilized and unstabilized fish oil. *Nut. Res.* **21**, 1529-1544.

Pulla Reddy, A. C. and Lokesh, B. R. (1994). Alterations in lipid peroxides in rat liver by dietary n-3 faty acids: modulation of antioxidant enzymes by curcumin, eugenol, and vitamin E. *J. Nutr. Biochem* **5**, 181-188.

R Development Core Team (2008). *A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing, Vienna. Available at: http://www.R-project.org

Roveri, A., Casasco, A., Maiorino, M., Dalan, P., Calligaro, A. and Ursini, F. (1992). Phospholipid hydroperoxide glutathione peroxidase of rat testis: gonadotropin dependency and immunocytochemical indentification. *J. Biol. Chem* **267**, 6142-6146.

Roveri, A., Maiorino, M. and Ursini, F. (1994). Enzymatic and immunological measurements of soluble and membrane-bound phospholipid-hydroperoxide glutathione peroxidase. *Meth. Enzymol.* **223**, 202-212.

Ruiz-Gutiérrez, V., Pérez-Espinosa, A., Vásquez, C. M. and Santa-María, C. (1999). Effects of dietary fats (fish, olive, and high-oleic-acid sunflower oils) on lipid composition and antioxidant enzymes in rat liver. *Brit. J. Nutri.* **82**, 233-241.

Ruiz-Gutiérrez, V., Vásquez, C. M. and Santa-María, C. (2001). Liver lipid composition and antioxidant enzyme activities of spontaneously hypertensive rats after ingestion of dietary fats (fish, olive, and high-oleic sunflower oils). *Biosci. Reports.* **21**, 271-285.

Sargent, J., McEvoy, L., Estevez, A., Bell, G., Bell, M., Henderson, J. and Tocher, D. (1999). Lipid nutrition of marine fish during early development: current status and future directions. *Aquaculture.* **179**, 217-229.

Seiler, A., Schneider, M., Förster, H., Roth, S., Wirth, E. K., Culmsee, C., Plesnila, N., Kremmer, E., Rådmark, O., Wurst, W. et al. (2008). Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. *Cell Metabolism* **8**, 237-248.

Srere, P. A., Brazil, H. and Gonen, L. (1963). The citrate condensing enzyme of pigeon breast muscle and moth flight muscle. *Acta. Chem. Scand.* **17**, S219-S234.

Takezaki, N., Figueroa, F., Zaleska-Rutczynska, Z. and Klein, J. (2003). Molecular phylogeny of early vertebrates: monophyly of the agnathans as revealed by sequences of 35 genes. *Mol. Biol. Evol.* **20**, 287-292.

Tanito, M., Brush, R. S., Elliot, M. H., Wicker, L. D., Henry, K. R. and Anderson, R. E. (2008). High levels of retinal membrane docoshexaenoic acid increase susceptibility to stress-induced degeneration. *J. Lipid Res.* **50**, 807-819.

Thisse, C., Degrave, A., Kryukov, G. V., Gladyshev, V. N., Obrecht-Pflumio, S., Krol, A., Thisse, B. and Lescure, A. (2003). Spatial and temporal expression patterns of selenoprotein genes during embryogenesis in zebrafish. *Gene Expr. Patterns.* **3**, 525-532.

Thomas, J. P., Maiorino, M., Ursini, F. and Girotti, A. W. (1990). Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. *J. Biol. Chem.* **265**, 454-461.

Thompson, J. L., See, V. H. L., Thomas, P. M. and Schuller, K. A. (2010). Cloning and characterization of two glutathione peroxidase cDNAs from southern bluefin tuna (*Thunnus maccoyii*). *Comp. Biochem. Physiol.* **156B**, 287-297.

Ursini, F., Maiorino, M., Valente, M., Ferri, L. and Gregolin, C. (1982). Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides. *Biochim. Biophys. Acta.* **710**, 197-211.

Ursini, F., Heim, S., Kiess, M., Maiorino, M., Roveri, A., Wissing, J. and Flohé, L. (1999). Dual function of the selenoprotein PHGPx during sperm maturation. *Science.* **285**, 1393-1396.

Venkatraman, J. T. and Pinnavaia, L. (1988). Effects of saturated, ω-6 and ω-3 lipids on activities of enzymes involved in antioxidant defense in normal rats. *Nut. Res.* **18**, 341-350.

Venkatraman, J. T., Angkeow, P., Satsangi, N. and Fernandes, G. (1988). Effects of n-6 and n-3 lipids on antioxidant defense system in livers of exercised rats. *J. Am. College Nut.* **17**, 586-594.

Venkatraman, J. T., Chandrasekar, B., Kim, J. D. and Fernandes, G. (1994). Effects of n-3 and n-6 fatty acids on the activities and expression of hepatic antioxidant enzymes in autoimmune-prone NZBxNZW F_1 mice. *Lipids* **29**, 561-568.

Wang, L., Lam, T. T., Lam, K.-W. and Tso, M. O. M. (1994). Correlation of phospholipid hydroperoxide glutathione peroxidase activity to the sensitivity of rat retinas to photic injury. *Ophthalmic Res.* **26**, 60-64.

Wilhelm Filho, D., Giulivi, C. and Boveris, A. (1993). Antioxidant defenses in marine fish – I. Teleosts. *Comp. Biochem. Physiol. C.* **106**, 409-413.

Wrench, J. J. and Measures, C. I. (1982). Temporal variations in dissolved selenium in a coastal ecosystem. *Nature* **30**, 431-433.

Yang, T., Forrest, S. J., Stine, N., Endo, Y., Pasumarthy, A., Castrop, H., Aller, A., Forrest, J. N., Jr, Schnermann, J. and Briggs, J. (2002). Cyclooxygenase cloning in dogfish shark, *Squalus acanthias*, and its role in rectal gland Cl secretion. *Am. J. Physiol.* **283**, R631-R637.