

Revisiting redox-active antioxidant defenses in response to hypoxic challenge in both hypoxia-tolerant and hypoxia-sensitive fish species

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Abstract It is not known whether changes in antioxidant levels always occur in fish in response to the oxidative stress that usually accompanies a hypoxic challenge. The studies of antioxidant responses to hypoxia in fish have mostly focused on very anoxia-tolerant species and indicate that there is an enhancement of antioxidant defenses. Here we present new data on redox-active antioxidants from three species, which range in their tolerance to hypoxia: the epaulette shark, threespine stickleback, and rainbow trout, together with a compilation of results from other studies that have measured oxidative stress parameters in hypoxia-exposed fish. The results suggest that in general, fish do not show an increase in redox-active antioxidant defense in response to oxidative stress associated with hypoxia. Rather, the changes in antioxidant defenses during hypoxia are very much

species- and tissue-specific and are not linked to the level of hypoxia tolerance of the fish species.

Keywords ROS · Hypoxia · Oxidative stress · Threespine stickleback · Rainbow trout · Epaulette shark

Introduction

For all animals, tightly regulated oxygen metabolism is a central feature of cellular homeostasis. While oxygen shortage (hypoxia) challenges oxygen-dependent energy production, excess oxygen is damaging. A low level of reactive oxygen species (ROS) produced in excess of constitutive antioxidant defense is important in adaptive cellular signaling (Hamanaka and Chandel 2009) while their overabundance can be cytotoxic. During hypoxia, oxidative phosphorylation is compromised so less ROS is produced in the mitochondria of hypoxia-intolerant mammals (Hoffman et al. 2007). Interestingly, in some species, hypoxia is known to increase ROS production in a tissue-specific manner (Chandel and Budinger 2007), representing a physiological paradox in which more ROS is produced in hypoxia than either in normoxia or in anoxia, the latter having almost zero ROS production.

In most species when oxygen is reintroduced after hypoxia/anoxia, a burst of ROS overproduction

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exceeds antioxidant defense resulting in oxidative stress, which if severe causes reperfusion/reoxygenation injury. This elevation in ROS causes both reversible and irreversible damage to DNA, lipids, and proteins as well as dysfunction in cellular organelles such as the endoplasmic reticulum and mitochondria and can initiate cell death pathways (Scandalios 2005). Anoxia-tolerant species, which are well adapted to fluctuating environmental oxygen, show increased amounts of antioxidants and redox enzyme activities during anoxia in aquatic environments (Lushchak 2011). This response could protect tissues from harmful ROS production upon reoxygenation following an anoxic insult (Hermes-Lima et al. 1998). The paradoxical increase in ROS production and subsequent development of oxidative stress during anoxia/hypoxia were first demonstrated in hypoxia-exposed Chinese sleeper (*Percottus glenii*) where ROS production increased rather than decreased in hypoxia and caused oxidative stress (Lushchak and Bagnyukova 2007). Additional support for the hypoxic induction of oxidative stress was demonstrated in the hypoxia-tolerant medaka (*Oryzias latipes*) (Oehlers et al. 2007). These observations regarding hypoxia-induced oxidative stress even in hypoxia-tolerant species have caused the topic to remain one of intensive discourse.

The potential of the organism to be protected from oxidative damage can be evaluated by measuring a number of redox parameters. These comprise of, first, a group of low-weight molecules, such as vitamins, carotenoids, glutathione (GSH), and thioredoxin, that react with the oxidizing compounds and secondly, by measuring the activities of redox-activated enzymes with antioxidant capacity, such as superoxide dismutase (SOD), catalase (CAT), and GSH peroxidase (GP), which detoxify ROS in simple redox reactions. The function of these enzymes is strongly associated with increased levels of glutathione reductase (GR), glutathione *S*-transferase (GST), and glucose 6-phosphate dehydrogenase (G6PDH) (Scandalios 2005). Since the activities of both this set of redox enzymes as well as the activities of the enzymes associated with them have been shown to increase in some fish species encountering severe hypoxia (Lushchak and Bagnyukova 2006), we measured the level of redox-activated enzymes in response to a hypoxic challenge in three separate fish lineages spanning a range of tolerances to hypoxia in order to determine whether

there is a uniform increase in redox-activated antioxidant defense.

Currently, hypoxia-induced oxidative stress has been referred to as a generalized response in fish (Gorr et al. 2010) although it has been evidenced only in a few species with exceptionally good hypoxia tolerance. The approximately 32,500 fish species known, at present (www.fishbase.org 2013), are a very heterogeneous group with a wide array of physiological adaptations, and therefore, evidence from a few species does not justify any generalizations. We hypothesize that hypoxia does not disturb the balance between ROS production and the constitutive level of antioxidant defense, and hence, a hypoxia-induced upregulation of redox-active antioxidants that is indicative of oxidative stress cannot be observed in most fish species. Here we report data from three species which occupy very different ecological niches and represent a range of levels of hypoxia tolerance: the epaulette shark (*Hemiscyllium ocellatum*) is hypoxia tolerant (Wise et al. 1998), the threespine stickleback (*Gasterosteus aculeatus*) can only tolerate moderate hypoxia (Leveelahti et al. 2011; O'Connor et al. 2011), and the rainbow trout (*Oncorhynchus mykiss*) is hypoxia sensitive (Kutty 1968). We measured the activities of CAT, SOD, and GP and associated enzymes GR, GST and G6PDH from each species after exposure to hypoxia. The duration and dose in each hypoxia treatment regime was selected so that data could be collected after a severe hypoxic challenge rather than allowing acclimation to hypoxia. These data were compared with available literature on hypoxia-exposed fish. The collected data suggests that the alterations in antioxidant–redox enzyme levels in response to hypoxic insults among fish species are not alike.

Materials and methods

Epaulette shark collection, hypoxia exposure regime, and tissue collection

Epaulette sharks (mean length 57 ± 4.9 cm) were collected from the reef platform surrounding Heron Island Research Station, ($23^{\circ}27'S$, $151^{\circ}55'E$) during the summer when water temperatures were 25 ± 2 °C. Collection permits (G25214.1 and G04/12777.1) were obtained from the Great Barrier Reef Protection Authority. The sharks were held in a 1,000 L flow-

through holding pool at the ambient reef conditions of 25 ± 2 °C and 36 ‰ salinity, for 24–48 h prior to the start of the experiments during which they were not fed. Sharks were pair-matched for sex, weight, and length before being randomly assigned to either hypoxic ($n = 10$) or normoxia conditions as a control ($n = 10$). The hypoxia exposure of each individual animal consisted of 2 h of hypoxia at 0.34 mg O₂/L (5 % of air saturation) using nitrogen gas to displace dissolved O₂, which represented the upper limit of hypoxia tolerance for this species (Wise et al. 1998). The information gained on epaulette shark with the previously used 2-h time point encouraged to keep the regime unchanged (Rytkönen et al. 2012). Immediately after the hypoxic exposure, sharks were euthanized by anesthesia (7.5 mL of 5 % benzocaine in 1 L of fresh seawater at 0.34 mg O₂/L for hypoxia-treated animals or at normoxia for control animals). In order to sample two highly metabolic tissues, the brains were rapidly removed and dissected to isolate the cerebellum, followed by the removal of gills. The brain has previously been shown to be susceptible to lipid peroxidation, a marker of oxidative stress, following the protocol outlined below (Renshaw et al. 2012). The tissues were immediately frozen in liquid nitrogen and stored at -80 °C.

Teleost maintenance and hypoxia exposure regime and tissue collection

Adult threespine sticklebacks (*Gasterosteus aculeatus*, L) were originally caught in brackish water in the Turku Archipelago (Finland) and rainbow trout (*Oncorhynchus mykiss*, Walbaum) obtained from the Finnish Institute for Fisheries and Environment (Parainen, Finland). Fish were maintained for at least 1 week before the experiments at 15 and 11 °C, respectively, at a photoperiod of 12 h/12 h light/dark in activated-charcoal-filtered tap water (Turku, Finland). Sticklebacks were kept in 40 L glass aquaria and two-thirds of the water was changed every 3–5 days. Fish density in aquaria was maximally one fish per 1.6 L water, and fish were fed 6 days a week with commercial frozen chironomid larvae at a ratio of approximately 2 % of total fish weight. Rainbow trout were kept in 400 L aquaria with recirculating water at a fish density below 1 kg m⁻³ and were fed commercial trout pellet twice a week.

For hypoxia exposure of threespine sticklebacks and rainbow trout, the fish were placed into separate aquaria. The partial pressure of oxygen was adjusted by replacing oxygen by nitrogen supplied via air stones to the aquarium water. The oxygen level was controlled by an O₂ analyzer and regulator system (D202, Qubit Systems, Kingston, Canada). Oxygen content and temperature were continuously recorded throughout the hypoxia exposure with an effective sampling rate of 0.5 Hz. The signal of the oxygen electrode was calibrated according to the electrode's manual. During the 3-h hypoxia exposure of sticklebacks, the average oxygen level was moderate at 24 ± 0.68 % of air saturation. This protocol was adopted because sticklebacks acclimate to hypoxia within 48 h at which time they do not show signs of oxidative stress (Levelahti et al. 2011). Rainbow trout are not known to acclimate to hypoxia and do not tolerate even moderate hypoxia, so the oxygen was increased to 33 % of maximum saturation which is very close to the P_{crit} of rainbow trout (Kutty 1968). The longer treatment time (48 h) was chosen because earlier pilot measurements of samples from rainbow trout exposed to hypoxia did not show alterations in redox enzyme activities. Following hypoxia exposure, fish were individually removed, alternately, from the hypoxia treatment and control aquaria and killed by cutting the spine directly behind the head (sticklebacks) or a sharp blow to the head (trout). Length and weight were measured and livers were dissected, snap-frozen in liquid nitrogen, and stored at -84 °C. The liver is a highly metabolic tissue and unlike brains and gills provided enough tissue for all of the assays and could be removed quickly enough to ensure accurate protein assays.

Sample preparation and measurement of redox enzyme activities and EROD activity

Protein homogenates were prepared as described previously by Vuori et al. (2008) with some modifications. In brief, frozen pieces of tissue were crushed in liquid nitrogen and homogenized in 0.1 M K₂HPO₄ + 0.15 M KCl-buffer (pH 7.4) using TissueLyser II (Qiagen, Hilden, Germany). The homogenate was centrifuged (15 min at 10,000 g and +4 °C) and the supernatant was divided into aliquots, frozen in liquid nitrogen and stored at -84 °C.

Enzyme activity measurements were performed as described previously by Vuori et al. (2008). In brief, the enzyme activities were measured in triplicate using 96-well or 384-well microplates according to the manufacturer's instructions with some modifications to take into account the reduced reaction volumes. The catalase (CAT), glutathione peroxidase (GP), reductase (GR), and (GST) activities were measured with Sigma kits (Sigma–Aldrich, St. Louis, Missouri, USA). In the measurement of the GP activity, 2 mM H₂O₂ was used as a substrate. The inhibition rate of (SOD) was measured using a Fluka (Fluka, Buchs, Germany) kit. Glucose-6-phosphate dehydrogenase (G6PDH) activity was measured according to Noltmann et al. (1961), and ethoxyresorufin-O-deethylase (EROD) activity was measured according to Burke and Mayer (1974). The protein content was determined with Bradford method using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California, USA) with bovine serum albumin (Sigma–Aldrich, St. Louis, MI, USA) as the standard. All measurements were done with EnVision™ (PerkinElmer, Wallac Oy, Turku, Finland) microplate reader. Variance analysis with covariates (ANCOVA) was performed with SAS software.

Results

Superoxide dismutase (SOD) and catalase (CAT) activities increase or decrease in response to oxidative stress depending on its intensity and form (together with glutathione peroxidase (GP)) the first line of defense against oxidative damage. CAT and SOD activities in hypoxia-treated samples did not differ from control levels in any of the samples measured from any of the species sampled (Fig. 1). Enzymes supporting glutathione metabolism (GP, GR, and GST) or associated with it (G6PDH) can also function as indicators of overwhelmed reductive capacity and were hence measured. No differences were found in any of the samples (Table 1).

Since cytochrome P450 1A (CYP1A) enzyme is well characterized in fish and a fairly good indicator of stress in general and can be measured as ethoxyresorufin-O-deethylase (EROD) activity, this was measured to determine the level of physiological stress induced by hypoxic exposure. Samples from hypoxia-treated fish did not differ from control-treated fish (Fig. 2). In

rainbow trout, EROD activity in hypoxia tended to decrease but this difference was not significant.

Discussion

The connection between elevated antioxidant defense and hypoxia/anoxia tolerance was first made by Reischl (1986). Later Hermes-Lima et al. (1998) formed a hypothesis based on their experiments with land snails proposing that activation of the antioxidant defenses during oxygen shortage was a preparative mechanism that protected the organism from oxidative damage upon reoxygenation. This mechanism was found to operate in snakes (Hermes-Lima and Storey 1993), frogs (Hermes-Lima and Zenteno-Savin 2002), and turtles (Reischl 1986). In some fish, the goldfish, the common carp, and the Chinese sleeper, hypoxia and anoxia alone were shown to cause elevation of the activities of enzymes involved in antioxidant defense suggesting that the specimens experienced oxidative stress in response to diminished oxygen (Lushchak et al. 2001, 2005; Lushchak and Bagnyukova 2007). Based on these experiments, performed mostly on highly hypoxia- or anoxia-tolerant species, a generalized model suggesting that oxidative stress occurs during hypoxia in fish (and other animal groups) (Lushchak 2011; Gorr et al. 2010). The collection of data from several studies in Table 2 suggests, however, that the hypoxic induction of oxidative stress is a species- and tissue-specific phenomenon, which does not occur in many fish and correlates only partly with hypoxia tolerance.

The paradoxical increase in ROS production during oxygen shortage is usually discussed in connection with changes in redox-activated antioxidant levels. According to the model of ROS overproduction during hypoxia (Chandel and Budinger 2007), an elevation in the activity of most enzymes (CAT, SOD, GP, GR, GST, GSH, G6PDH) is expected, as shown in Table 2. Although some increases in redox-activated antioxidant levels have been observed, the majority of observed changes have been decreased. Probably, the most commonly measured parameters are CAT, SOD, and GP activities. The roles of GP and CAT in oxidative stress overlap and their activities appear to be tissue-specific with CAT operating especially in liver tissue (Storey 1996). In the studies depicted in Table 2, the activities of SOD and CAT were never

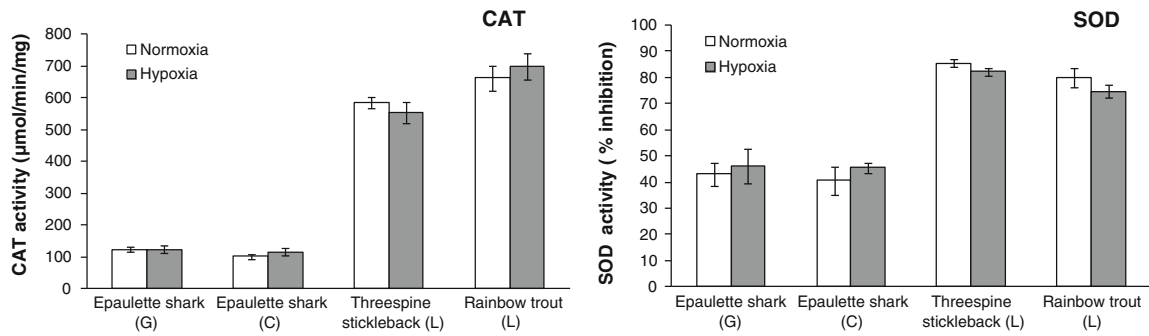


Fig. 1 Catalase (CAT) and superoxide dismutase (SOD) activities did not alter in response to hypoxia. Activities were measured from metabolically highly active tissues (*G* gill, *C* cerebellum, *L* liver) and are given as mean ± SEM

Table 1 Glutathione peroxidase (GP), glutathione reductase (GR), glutathione *S*-transferase (GST), and glucose 6-phosphate dehydrogenase (G6PDH) activities did not alter in epaulette shark, threespine stickleback, and rainbow trout tissues in response to hypoxia

O ₂ saturation treatment		GP nmol/min/mg	GR nmol/min/mg	GST nmol/min/mg	G6PDH nmol/min/mg
Epaulette shark (G)					
100 %	Control	68 ± 7	10 ± 0.6	18 ± 3	15 ± 3
5 %	Hypoxia	67 ± 7	9.2 ± 0.9	18 ± 2	12 ± 2
Epaulette shark (C)					
100 %	Control	46 ± 5	9.5 ± 0.9	30 ± 5	21 ± 2
5 %	Hypoxia	50 ± 12	8.2 ± 1.4	28 ± 4	18 ± 1
Threespine stickleback (L)					
100 %	Control	37 ± 6	12 ± 1.8	87 ± 15	25 ± 4
24 %	Hypoxia	32 ± 5	14 ± 1.3	77 ± 9	18 ± 2
Rainbow trout (L)					
100 %	Control			24 ± 4	78 ± 11
33 %	Hypoxia			24 ± 3	68 ± 13

Activities were measured from metabolically highly active tissues (*G* gill, *C* cerebellum, *L* liver) and are given as mean ± SEM

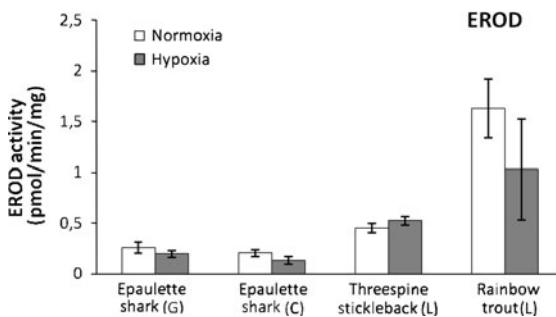


Fig. 2 Ethoxyresorufin-O-deethylase (EROD) activity did not alter in response to hypoxia. Activities were measured from metabolically highly active tissues (*G* gill, *C* cerebellum, *L* liver) and are given as mean ± SEM did not alter in response to given treatments

found to be upregulated simultaneously. Instead, their activity was either downregulated, altered in opposite direction, or the activities did not change at all in

response to the hypoxic insult. Some of the variations in response may be explained by the environment that the fish originate from or it could depend on the activity level of the species, and physically active species were proposed to have higher concentrations of SOD and CAT than less active ones (Wilhelm-Filho 1996). This is illustrated by a study performed on eels; exercise training is often associated with increased ROS and oxidative stress; however, exercise training of eels lowered the levels of lipid peroxidation as well as those of SOD and CAT which may be due to more efficient oxidative phosphorylation and/or more effective ROS scavenging (Mortelette et al. 2010). This finding indicates that physically active species may be better equipped for handling oxidative stress (Lopez-Cruz et al. 2010).

In Atlantic cod and Nile tilapia exposed to hypoxia, the glutathione redox state was unchanged (Olsvik

Table 2 Collection of studies with fish exposed to hypoxia/anoxia and sampled for measurement of redox parameters

Order, Family	Latin name	Name	Treatment	Duration hours (h), days (d), weeks (w)	Tissue	Measured parameter(s):		Publication
						up regulated	down regulated	
Belontiiformes, Adrianichthyidae	<i>Oryzias latipes</i>	Medaka	H	8 d	brain	GST	-	Oehlers et al. 2007
Characiformes, Anostomidae	<i>Leporinus macrocephalus</i>	Plavucu	H	96 h	liver	-	CAT, SOD, GST, LP, TBARS	Riffel et al. 2012
Characiformes, Characidae	<i>Piaractus mesopotamicus</i>	Pacu	H	48 h	muscle	-	CAT, GP, CAT, SOD, GP	Garcia Sampalo et al. 2008
Cypriniformes, Cyprinidae	<i>Carassius auratus</i>	Goldfish	A	8 h	brain liver muscle	G6PDH, GP CAT LP	- CAT, SOD, GR, GST, LP SOD, GP, GR, GST, G6PDH, LP CAT, SOD, GP, GR, GST, G6PDH	Lushchak et al. 2001
Cypriniformes, Cyprinidae	<i>Cyprinus carpio</i>	Common carp	H	5.5 h	brain liver muscle	CAT, GP TBARS GP, CP	- - -	Lushchak et al. 2005
Cypriniformes, Cyprinidae	<i>Danio rerio</i>	Zebrafish	H	30 d	liver & gills	COMET	GP	Mustafa et al. 2011
Gadiformes, Gadidae	<i>Gadus morhua</i>	Atlantic cod	H	24 h	embryo	-	transcriptome	Ton et al. 2003
Gasterosteiformes, Gasterosteidae	<i>Gasterosteus aculeatus</i>	Threespine stickleback	H	6 w	liver	-	SOD, GP and CYP1A mRNA	Olsvik et al. 2006
Perciformes, Cichlidae	<i>Oreochromis niloticus</i>	Nile tilapia	H	3 h	liver	-	SOD, GP, GR, GST, G6PDH, CYP1A transcriptome	see. Results
Perciformes, Gobiidae	<i>Gillichthys mirabilis</i>	Longjaw mudsucker	H	48 h	liver	CYP1A CAT	-	Levelehti et al. 2011
Perciformes, Odontobutidae	<i>Percottus glenii</i>	Chinese sleeper	H	3 h	liver	-	SOD, GP, GR, GSH, GST, G6PDH	Levelehti et al. unpublished data
Perciformes, Sciaenidae	<i>Leiostomus Xanthurus</i>	Spot (croaker)	H	8 h, 24 h, 72 h, 144 h	various	-	CAT, SOD, GP, GSH, LP, CP, TBARS transcriptome	Welker et al. 2012
Perciformes, Salmonidae	<i>Oncorhynchus mykiss</i>	Rainbow trout	H	2 h, 6 h, 10 h	brain liver muscle	CAT, CP, TBARS, LP SOD, CP, LP GST, CP, LP	LP, SOD, GST, GR GP, G6PDH GP, GST, G6PDH CAT, SOD, GP, GR, TBARS, CAT, SOD, GP, GR, TBARS,	Gracey et al. 2001 Lushchak and Bagryukova 2007
Perciformes, Salmonidae	<i>Hemiscyllum ocellatum</i>	Epaulette shark	H	12 h	gills & muscle	SOD	CAT	Cooper et al. 2002
Perciformes, Hemiscyillidae	<i>Hemiscyllum ocellatum</i>	Epaulette shark	H	48 h	liver	-	CAT, SOD, GST, G6PDH,	see. Results
Measured redox parameters in hypoxia-treated (H) shark tissues.								
Perciformes, Hemiscyillidae	<i>Hemiscyllum ocellatum</i>	Epaulette shark	H	2 h	gills & brain	-	CAT, SOD, GP, GR, GST, G6PDH	see. Results

COMET Comet assay for oxidative damage of DNA. CP carbonylation of proteins. LP lipid peroxidation, TBARS thiobarbituric acid-reactive substance (lipid injury)

et al. 2006; Welker et al. 2012). This may be due to an intensified maintenance of its reductive capacity by the auxiliary proteins such as GST, GR, and G6PDH. In cases of ROS overproduction in response to hypoxia and subsequent reoxygenation, both GP and GST activities should have been elevated. This was not, however, the case in the hypoxia studies performed on medaka and common carp (Lushchak et al. 2005; Oehlers et al. 2007). GR facilitates the reduction in oxidized glutathione, and G6PDH supplies the NADPH required in GSSH reduction. Consequently, an increase in the activity of these proteins must occur if glutathione is to be reduced to its active form to counteract increased ROS production. However, on the basis of the studies depicted in Table 2, it appears that the activities of the majority of enzymes are either decreased or unresponsive to lowered oxygen tension which supports our conclusion that redox-activated antioxidant levels are not always increased in response to physiologically relevant hypoxia across all fish species.

While elasmobranchs differ from the more recently evolved teleost phyla, most animals examined to date, including fish, share the same molecules involved in redox-activated antioxidant defense. The epaulette shark used in our investigation represents an ancestral vertebrate with a higher hypoxia tolerance than most fish or mammals (Nilsson and Renshaw 2004). The responses of this species to anoxia and reoxygenation, including changes in antioxidants and markers of oxidative stress, were recently reviewed by Renshaw et al. (2012). Our results concur with previous findings that CAT and SOD activities and GSH: GSSG ratio remained unchanged after anoxic or anoxia-reperfusion challenges (Renshaw et al. 2012). Comparative studies on the antioxidant defenses in fish have indicated that while the antioxidant enzyme level in elasmobranchs appeared to be lower than that of teleosts (Wilhelm-Filho and Boveris 1993; Gorbi et al. 2004), the activity level of pelagic marine species was higher than bottom dwellers, as reflected by their higher levels of SOD and CAT (Wilhelm-Filho 1996). This “activity-based hypothesis” was recently tested in 3 species of elasmobranchs by Lopez-Cruz et al. (2010), one of the sharks with a high activity level, the silky shark, also had higher levels of Cu, Zn-SOD, and GP, while the other, the mako shark, did not, and the shark with the lowest activity level of the three, the hammerhead shark, had lower antioxidant levels than

the other species. Taken together with the data from our study, the divergent findings may indicate that there are at least three possible interpretations of the findings in fish species studied so far: (1) there is not a systematic relationship between activity levels and antioxidant defense; (2) fish use other endogenous antioxidants, not usually determined, to defend themselves from oxidative stress; and (3) that some fish species, like the epaulette shark (Hickey et al. 2012), may have other adaptations, such as altered mitochondrial function, which could lower ROS production during hypoxia and reoxygenation.

While low molecular mass antioxidants (described by Livingstone 2001) such as vitamins and carotenoids are equally important for the antioxidant defense as are the high molecular weight antioxidant enzymes, changes in their levels are not included in the discussions of the relationship between hypoxia and oxidative stresses, largely because the measured changes in them do not allow unequivocal conclusions to be made. However, there is evidence that a marker of lipid peroxidation (malondialdehyde, MDA) was present at lower levels when the carotenoid astaxanthin was added to salmon feed (Brambilla et al. 2009).

When oxygen supply is limited, organisms down-regulate their metabolic activity. As ATP production becomes oxygen limited, this can be accompanied by increased ROS production during hypoxia because the flow of electrons to complex IV ceases when oxidative phosphorylation shuts down. Consequently, the back flow of electrons to complex III/I can result in the formation of ROS. Alternatively, if oxidative phosphorylation continues at a lower rate with a decrease in general energy metabolism or if mitochondria respire in a quiescent state, a decreased formation of ROS occurs (Hoffman et al. 2007). The current results together with our previous findings on unchanged ROS levels in hypoxia-exposed HeLa cells and rainbow trout gill cell (RTGW1) (Leveelahti et al. unpublished data) agree with the general downshift in energy expenditure.

While the focus of this study was to examine the redox-activated antioxidants following ROS production, there is strong evidence that mitochondria may reflect ecophysiological adaptations and partly explain the absence of oxidative stress. In two species from the *elasmobranchii* mitochondria in permeabilized heart fibers from the epaulette shark (*Hemiscyllium ocellatum*) produced 50–80 % less reactive species in

normoxia than did those from the shovel nose ray (*Rhinobatus typus*) (Hickey et al. 2012), which is more sensitive to hypoxia than the epaulette shark as indicated by their critical oxygen tension (P_{crit}) of 5.10 ± 0.37 kPa for the epaulette and 7.23 ± 0.40 kPa for the shovel nose ray (Speers-Roesch et al. 2012). In response to hypoxia, mitochondria from the epaulette shark maintained the normoxia mitochondrial efficiency without increasing the level of reactive species production while the ray mitochondria significantly increased the level of reactive species produced. It seems likely that if mitochondria are resistant to hypoxic stress and/or produce reduced levels of ROS, the need for antioxidant defense in hypoxia would be diminished, and the data support the finding that the epaulette shark did not increase redox-activated antioxidant defense in response to hypoxia.

EROD activity, which is usually associated with xenobiotic exposure and biotransformation reactions, was measured because earlier experiments demonstrated that EROD activity could be elevated even though there were unchanged *CYP1A* mRNA levels in liver tissue of 48-h hypoxia-exposed stickleback (Leveelahti et al. 2011). Similarly, EROD activity was unchanged after 3-h hypoxia in sticklebacks or after 2-h hypoxia in epaulette sharks. In rainbow trout subjected to 48-h hypoxia, the EROD activity appeared to decrease but the change was not statistically significant. *CYP1A* mRNA transcription differs spatially and hence the activity of the protein product may vary in different parts of the fish liver (Olsvik et al. 2007).

In conclusion, the results of the present study and compilation of data from earlier studies suggest that (1) hypoxia does not always result in unchecked oxidative stress that culminates in tissue damage and (2) not all fish show a general increase in key markers of redox-activated antioxidant defense that is usually found in response to elevated hypoxia-induced oxidative stress. Furthermore, the results suggest that there is no clear association between the oxidative stress response and hypoxia tolerance of the species. Rather, in several of the species studied, the observed redox-activated antioxidant defenses were tissue-specific and may be more aligned to phylogenetic relationships than to the species-specific oxygen requirements.

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