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Critical oxygen tensions as predictors of hypoxia tolerance and tissue metabolic responses during hypoxia exposure in fishes

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ABSTRACT

An organism's critical oxygen tension (P_{crit}) reflects its ability to extract environmental O_2. Consequently, P_{crit} has been used as an indicator of hypoxia tolerance in aquatic animals. The relationship between P_{crit} and hypoxia tolerance and hypoxic metabolic responses, however, remains incompletely understood. Among several species of sculpin fishes (superfamily Cottoidea), we previously demonstrated a correlation between P_{crit} and hypoxia tolerance, as measured as the time required for 50% of a group of fish to lose equilibrium (LOE_{50}) at a water PO_2 of 6.4 Torr. In the present study, we further investigated the relationship between P_{crit}, hypoxia tolerance, and hypoxic metabolic responses by examining the effects of hypoxia exposure at a fixed percentage of P_{crit} (30%; termed relative hypoxia exposure) on LOE_{50} and metabolic responses in brain, liver, and white muscle in three sculpin species that differ in P_{crit} and hypoxia tolerance at 6.4 Torr. We also assessed the tissue metabolic responses underlying hypoxic loss of equilibrium (LOE). The species, from most to least hypoxia-tolerant at 6.4 Torr and from lowest to highest P_{crit} values, were the tidepool sculpin (*Oligocottus maculosus*), staghorn sculpin (*Leptocottus armatus*), and silverspotted sculpin (*Blepsias cirrhosus*). If P_{crit} predicts hypoxia tolerance, then we expected similar LOE_{50} values and similar tissue metabolic responses across all species during relative hypoxia exposure. LOE_{50} values were similar in staghorn sculpins and tidepool sculpins, but not in silverspotted sculpins, which had a comparatively lower relative hypoxia LOE_{50} value. Thus, P_{crit}, and consequently the ability to extract environmental O_2, cannot predict hypoxia tolerance in all species, at least at a water PO_2 of 30% of P_{crit}. During relative hypoxia exposure, tissue lactate accumulation and ATP levels were similar between species, suggesting that the ability to extract environmental O_2 is an important determinant of cellular energy status and reliance on anaerobic glycolysis in hypoxic sculpins. However, whereas tissue glycogen content and utilization were similar between tidepool sculpins and staghorn sculpins, there were lower normoxic levels and greater hypoxic depletion in silverspotted sculpins, potentially explaining their poorer relative hypoxia tolerance. In all species, LOE was associated with depletion of brain [ATP]. Overall, caution is warranted when P_{crit} is used as an indicator of hypoxia tolerance, especially when considering temporal aspects of hypoxia tolerance and related metabolic characteristics (e.g. glycogen availability). Ideally, comparative studies of hypoxia tolerance should feature multiple measures (e.g. P_{crit} and LOE_{50}) in order to assess the overall responses of fishes to hypoxia.

1. Introduction

Environmental hypoxia is a common abiotic stressor in aquatic environments (Diaz and Breitburg, 2009). Unsurprisingly, many fishes have evolved the ability to survive periods of hypoxia, although there is large interspecific variation in the severity and duration of hypoxia that can be tolerated. An important factor thought to underlie the variation in hypoxia tolerance is the critical O_2 tension of whole-animal O_2 consumption rate ($\dot{M}_{O_2}$), or P_{crit}, which is the water PO_2 (P_wO_2) at which the $\dot{M}_{O_2}$ of an organism transitions from being independent of, to being dependent upon, environmental O_2. Differences in P_{crit} are primarily due to variation in the physiological variables that influence the diffusive and convective movement of O_2 from the water to the mitochondrion (Richards, 2011). A lower P_{crit}, in general, has been thought to be associated with greater hypoxia tolerance because it indicates improved O_2 uptake and transport to tissues at low P_wO_2, which would decrease the requirement for O_2-independent ATP production, a relatively inefficient process that is accompanied by accumulation of deleterious by-products (e.g., H^+) (Nilsson and Östlund-Nilsson, 2008; Mandic et al., 2009; Speers-Roesch et al., 2012a). In fact, we recently demonstrated that P_{crit} was negatively

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correlated with hypoxia tolerance, measured as the time required for 50% of a group of fish to show loss of equilibrium (LOE_{50}) at a P_wO_2 of 6.4 Torr, across 11 species of sculpins (superfamily Cottoidea) (Mandic et al., 2013). These findings corroborate the widespread use of P_{crit} as a useful measure of hypoxia tolerance that may facilitate cross-species comparisons of the metabolic responses of aquatic organisms to environmental hypoxia (Pörtner and Grieshaber, 1993; Chapman et al., 2002; McKenzie et al., 2008; Nilsson and Östlund-Nilsson, 2008; Mandic et al., 2009; Speers-Roesch et al., 2012a). However, with the exception of Mandic et al. (2013), few studies have explored the relationship between P_{crit} and hypoxia tolerance or responses, including determining if hypoxia exposures scaled to P_{crit} result in similar metabolic disturbances across multiple species.

At P_wO_2 below P_{crit}, where O_2 availability is constrained, hypoxic survival is thought to be dependent upon the ability of a fish to maintain cellular energy balance (i.e., stable ATP levels) in tissues despite diminished aerobic energy supply (Richards, 2009). This may be achieved by an increased activation of O_2-independent ATP production, in particular anaerobic glycolysis, as well as a reversible metabolic rate depression (Richards, 2009). Provision of exclusively aerobic fuels such as non-esterified fatty acids appears to be downregulated during hypoxia exposure in some hypoxia-tolerant fishes (Van den Thillart et al., 2002; Speers-Roesch et al., 2010). In contrast, mobilization of stored fermentable fuels (e.g., glycogen) is crucial to sustain anaerobic glycolysis and hypoxia-tolerant fishes are generally considered to have greater glycogen stores than hypoxia-sensitive fishes (Richards, 2009).

Species that can maintain cellular energy balance to lower P_wO_2, particularly in vital organs such as the brain, should have greater hypoxia tolerance (Richards, 2009) because they can prevent catastrophic drops in tissue [ATP] that lead to cellular dysfunction and necrosis, and, consequently, loss of equilibrium (LOE) and organismal death (Boutilier, 2001). However, direct support for the existence of a link between hypoxia-induced disruption of cellular energy balance and hypoxic survival is equivocal, with some species showing substantial decreases in [ATP] in brain and other tissues at LOE or moribundity (DiAngelo and Heath, 1987; Van Raaij et al., 1994; Van Ginneken et al., 1996; Ishibashi et al., 2002), while in other species such changes are not evident (DiAngelo and Heath, 1987; Van Ginneken et al., 1996). Even if a fish maintains cellular energy balance during hypoxia exposure, hypoxic survival time may be limited by glycogen availability as well as by metabolic acidosis associated with anaerobic glycolysis (Nilsson and Östlund-Nilsson, 2008; Richards, 2009). If glycogen stores are exhausted, loss of anaerobic glycolytic ATP production will result in the perturbed cellular energy balance described previously. Nonetheless, despite one study showing that moribund hypoxia-exposed fishes show depletion of brain [glycogen] (DiAngelo and Heath, 1987), it is unclear whether glycogen exhaustion is a major limiting factor affecting hypoxic survival. Even if glycogen stores remain available, metabolic acidosis can constrain hypoxic survival and more hypoxia-tolerant species are thought to minimize acidosis by decreasing energy demands via metabolic rate depression or by improved acid–base regulation (Jackson, 2004). Tissue lactate levels can be a simple indicator of the degree of acidosis because of the equimolar production of lactate and H^+ by anaerobic glycolysis (Nilsson and Östlund-Nilsson, 2008). There is some evidence that excessive lactate loads in brain and other tissues are associated with LOE during hypoxia exposure in fishes (DiAngelo and Heath, 1987; Van Raaij et al., 1994).

In the present study, we examined the relationship between P_{crit} and hypoxia tolerance and assessed the biochemical limitations on hypoxia tolerance in three species of sculpins that vary in P_{crit} and hypoxia tolerance as measured by LOE_{50} at 6.4 Torr. Specifically, we addressed three questions: 1) Does exposure to hypoxia at a fixed percentage of P_{crit} result in similar LOE_{50} in these species?, 2) Does exposure to hypoxia at a fixed percentage of P_{crit} result in metabolic responses in brain, liver, and muscle that are similar in all three species?, and 3) What metabolic responses in brain, liver, and muscle are associated with hypoxic LOE in these species? Our study species were the silverspotted sculpin (*Blepsias cirrhosus*), the staghorn sculpin (*Leptocottus armatus*), and the tidepool sculpin (*Oligocottus maculosus*), which are known to have different P_{crit} values (44.4, 37.4, and 25.9 Torr, respectively) that are correlated negatively with the interspecific variation in LOE_{50} values determined at 6.4 Torr (25, 281, and 538 min, respectively) (Mandic et al., 2009; Mandic et al., 2013). To address question 1, we exposed each species to a level of hypoxia equal to 30% of their P_{crit} (henceforth termed relative hypoxia) and measured the time to LOE_{50} (henceforth termed relative hypoxia LOE_{50}). If variation in P_{crit}, and consequently the ability to extract environmental O_2, explains interspecific variation in whole-animal hypoxia tolerance, as suggested by Mandic et al. (2013), then relative hypoxia LOE_{50} values would be similar between species. To address question 2, we measured the metabolic profile (i.e. levels of ATP, glycogen, glucose, and lactate) in brain, liver, and white muscle of each species exposed to 6 h of relative hypoxia. If variation in P_{crit} explains interspecific variation in hypoxia tolerance, then relative hypoxia exposure in the three species should result in similar tissue metabolic profiles. On the other hand, if factors other than P_{crit} play a role in explaining the variation in hypoxia tolerance among these species, then tissue metabolic profiles would vary between species. To address question 3 and ascertain the proximate cause of LOE in fishes, we measured metabolic profiles in brain, liver, and white muscle of individuals of each species at the point they displayed LOE during hypoxia exposure.

2. Materials and methods

2.1. Animals

Silverspotted sculpins (7.0 ± 0.4 g, $n = 58$), staghorn sculpins (34.3 ± 2.0 g, $n = 54$), and tidepool sculpins (5.2 ± 0.4 g, $n = 45$) were collected using handheld nets or beach seines during the lowest tidal cycle of July 2008 at Ross Islets (48°52.4′ N, 125°9.7′ W) and Wizard's Rock (48°51.5′ N, 125°9.4′ W), near the Bamfield Marine Sciences Centre (BMSC), Bamfield, British Columbia, Canada. All three species of sculpins were transported to BMSC and held in fiberglass aquaria supplied with aerated flow-through filtered seawater (12 °C, 33 ppt). Fishes were allowed to recover from capture for at least one week before experimentation during which they were fed daily with mussels, bloodworms and frozen baitfish, except for 24 h before experimentation when food was withheld. All experiments were conducted according to guidelines set out by the Canadian Council for Animal Care and approved institutional protocols.

2.2. Experimental protocols

2.2.1. Determination of relative hypoxia LOE_{50} values

A total of three trials were carried out on each species using the following protocol. For each trial, eight individuals from a randomly selected species were transferred from holding tanks to a 40 L glass aquarium supplied with aerated flow-through filtered seawater and partially submerged in a wet table supplied with the same seawater (12 °C) for temperature regulation. The fishes were allowed to acclimate overnight under well-aerated conditions. Two small submersible pumps were placed in the aquarium to ensure adequate mixing of the water. Following the acclimation period, the flow-through seawater supply and aeration was stopped and hypoxia was induced by bubbling N_2 into the aquarium, which was covered with plastic bubble wrap to prevent O_2 ingress. Each species was exposed to a level of hypoxia corresponding to 30% of their P_{crit} (relative hypoxia): $P_wO_2 = 13.2$ Torr for silverspotted sculpins, 11.1 Torr for staghorn sculpins, and 7.7 Torr for tidepool sculpins. The rate of decrease of P_wO_2 was similar across all trials and hypoxic levels were reached after approximately 30 min of N_2

bubbling. Water PO_2 was monitored continuously using handheld O_2 meters and maintained at the desired level by adjusting the N_2 bubbling. The water PO_2 never deviated more than 4% from the target value. Fish behavior was monitored for evidence of LOE every 10 min. For these benthic species, LOE was defined as the inability of an individual fish to right itself after gentle prodding with a plastic rod. LOE was associated with a cessation of opercular movements. The time at LOE was noted for each individual fish and at the point of LOE each fish was removed from the aquarium and euthanized in seawater containing benzocaine ($0.2\,g\,L^{-1}$, initially dissolved in 95% ethanol). Each trial ended when the last fish in the aquarium displayed LOE.

2.2.2. Relative hypoxia exposures (6 h)

The following procedure for relative hypoxia exposures was carried out on one of the three species (randomly selected) on consecutive days. Twenty fish of the same species were divided equally between two 40 L glass aquaria under the same conditions as described in Section 2.2.1. Within each aquarium, 2 to 3 fish were held within one of four, submerged, 4 L plastic tubs with large mesh windows that allowed water movement. Following overnight acclimation under well-aerated, flow-through conditions, two tubs were gently removed from each aquarium (eight fish total; normoxia group) and benzocaine ($0.2\,g\,L^{-1}$, initially dissolved in 95% ethanol) was added to the water in the tub to euthanize the fish. Animals remained calm during this procedure. The brain, liver, and a 1 cm segment of posterior trunk white muscle were quickly excised and frozen in liquid N_2. The entire liver was sampled in all cases and for the normoxic groups the liver was weighed for calculation of hepatosomatic index ([liver mass/body mass] $*$ 100). Concomitant with tissue sampling, blood was sampled from the severed caudal artery/vein using hematocrit (Hct) capillary tubes. Plasma was collected from the Hct capillary tube following centrifugation at 5000 *g* and frozen in liquid N_2. All samples were stored at −80 °C until analysis.

Following the sampling of the normoxic fish, relative hypoxia was induced as described in Section 2.2.1. Final exposure P_wO_2 for each species was the same as described in Section 2.2.1. At 6 h of relative hypoxia exposure, four fish from each aquarium (total of 8 fish) were sampled as previously described for the normoxia group.

2.2.3. LOE exposures

2.2.3.1. LOE exposures at 6.4 Torr. During a previous study (Mandic et al., 2013), we measured the LOE_{50} of the three study species at a single level of hypoxia (6.4 Torr), using the same procedure as described in Section 2.2.1 (except for the use of a single rather than a relative level of hypoxia exposure). We sampled blood and tissues as described in Section 2.2.2 from ten randomly selected individuals of all three study species immediately upon LOE (as defined in Section 2.2.1) during the LOE_{50} trials at 6.4 Torr.

2.2.3.2. Relative hypoxia LOE exposure for silverspotted sculpins. During the LOE exposures at 6.4 Torr (Section 2.2.3.1), silverspotted sculpins lost equilibrium in <1 h ($LOE_{50} = 25 \pm 2$ min) whereas staghorn sculpins and tidepool sculpins both tolerated several hours of hypoxia exposure (staghorn sculpin $LOE_{50} = 281 \pm 25$ min; tidepool sculpin $LOE_{50} = 518 \pm 12$ min) (Mandic et al., 2013). To address the question of whether tissue-level responses at LOE are affected by the duration to LOE, we also sampled tissues from silverspotted sculpins during the relative hypoxia LOE_{50} exposure described in Section 2.2.1 ($P_wO_2 = 13.2$ Torr), where this species tolerated several hours of exposure. Sampling procedures are the same as described in Section 2.2.2.

2.3. Analytical protocols

Frozen tissue was broken into small pieces under liquid N_2 using an insulated mortar and pestle. For extraction of metabolites, 25–75 mg of tissue was combined with 10 volumes of ice-cold 1 M $HClO_4$ in a microcentrifuge tube and immediately sonicated on ice with three bursts of 10 s using a Kontes sonicator on its highest setting. An aliquot was frozen at −80 °C for measurement of [glycogen] according to the protocol outlined in Bergmeyer (1983). The remaining homogenate was centrifuged (10,000 *g*; 10 min; 4 °C) and the supernatant neutralized with 3 M K_2CO_3. Neutralized extracts were assayed spectrophotometrically for [ATP], [lactate], and [glucose] following methods described in Bergmeyer (1983). [Glycogen] was corrected for measured endogenous glucose levels. Plasma [NEFA] was measured spectrophotometrically using a commercially available kit (NEFA-HR(2); Wako, Osaka, Japan). Hematocrit was determined by centrifugation (5000 *g*) of whole blood in a sealed Hct capillary tube.

2.4. Statistics

For the 6 h relative hypoxia exposure, the effects of species and hypoxia exposure on Hct and metabolite levels were tested using a two-way ANOVA followed by Holm–Sidak post hoc (H–S) tests. The hepatosomatic indices of the normoxic fishes were compared between species with a one-way ANOVA with H–S test.

The relative hypoxia LOE_{50} median value of each species was determined using the Kaplan-Meier survival curve analysis in SigmaStat 3.0. There was no significant difference in LOE_{50} among trials within a species so data from individual trials were combined for each species to establish the LOE_{50}. Relative hypoxia LOE_{50} values were compared between species using a one-way ANOVA with H–S tests.

For data from the LOE exposures, the effects of species and LOE at 6.4 Torr (see Section 2.2.3.1) on Hct and metabolite levels were tested using a two-way ANOVA followed by H–S tests. Data for silverspotted sculpins exposed to relative hypoxia until LOE (see Section 2.2.3.2) were separately compared with the values for silverspotted sculpins exposed to normoxia and the values for silverspotted sculpins exposed to LOE at 6.4 Torr using a one-way ANOVA with H–S tests.

Statistical significance was accepted when $p < 0.05$ and analyses were carried out using SigmaStat 3.0. Data were log or square root transformed prior to statistical analyses if assumptions of equal variance or normality were not met. All data are presented as means ± SE.

3. Results

3.1. Relative hypoxia LOE_{50} values

There were no signs of LOE in any species until ~200 min (~3.3 h) of relative hypoxia exposure (Fig. 1), at which point the occurrence of LOE increased in all species at similar rates until ~400 min (~6.7 h) of relative hypoxia exposure. After 400 min, the rate at which silverspotted sculpins showed LOE increased dramatically compared with staghorn sculpins and tidepool sculpins, which showed similar survivorship curves (Fig. 1). As a result, the calculated LOE_{50} values were similar for staghorn sculpins and tidepool sculpins while the silverspotted sculpins had a significantly shorter LOE_{50} (Fig. 1).

3.2. Metabolic responses to 6 h of relative hypoxia exposure

Under normoxic conditions, Hct was significantly different between species, with the highest level in tidepool sculpins and the lowest level in staghorn sculpins (Table 1). Exposure to relative hypoxia caused Hct to increase significantly in silverspotted sculpins and staghorn sculpins but it remained unchanged in tidepool sculpins (Table 1). Tidepool sculpins and silverspotted sculpins had a similar hypoxic level of Hct that was significantly greater than that in staghorn sculpins (Table 1).

Levels of plasma non-esterified fatty acids during normoxia were significantly different between species, being greatest in tidepool sculpins and lowest in staghorn sculpins (Table 1). Relative hypoxia exposure caused no change in plasma [NEFA] in silverspotted sculpins

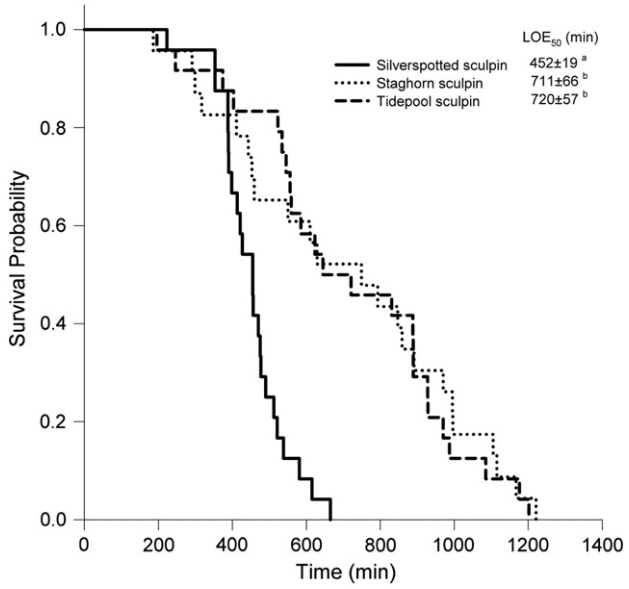

Fig. 1. Relative hypoxia LOE_{50} survivorship curves and LOE_{50} values of silverspotted sculpins, staghorn sculpins, and tidepool sculpins exposed to relative hypoxia (water PO_2 = 30% of each species' P_{crit}; 13.2 Torr for silverspotted sculpins, 11.1 Torr for staghorn sculpins, and 7.7 Torr for tidepool sculpins) until loss of equilibrium (LOE) (see Section 2.2.1 of text). Values are means ± s.e.m. (n = 24). LOE_{50} values sharing the same letter are not significantly different from one another (one-way ANOVA with Holm–Sidak tests, $p < 0.05$).

and staghorn sculpins, whereas levels dropped significantly in tidepool sculpins (Table 1).

Under normoxic conditions, brain [glycogen] was similar between silverspotted sculpins and staghorn sculpins whereas tidepool sculpins had higher levels (Fig. 2A). Liver [glycogen] was not significantly different between species (Fig. 2B). The hepatosomatic index was not different between species (silverspotted sculpin, 1.44 ± 0.11%; staghorn sculpin, 1.70 ± 0.35%; tidepool sculpin, 1.26 ± 0.12%; n = 8 for all species). White muscle [glycogen] was significantly greater in staghorn and tidepool sculpins compared with silverspotted sculpins (Fig. 2C). After 6 h of relative hypoxia exposure, brain [glycogen] decreased significantly compared with normoxia in tidepool sculpins only (Fig. 2A). Liver and white muscle [glycogen] were unchanged in staghorn sculpins and tidepool sculpins during relative hypoxia exposure but was greatly decreased in silverspotted sculpins (Fig. 2B and C).

Glucose levels in brain, liver, and white muscle during normoxia were similar in all three species (Table 1). In brain and white muscle, relative hypoxia exposure caused increases in [glucose] in all species (Table 1). Higher [glucose] were observed in brain of hypoxic staghorn sculpins and tidepool sculpins compared with silverspotted sculpins but in white muscle levels were similar between species (Table 1). In liver, only staghorn sculpins showed a significant increase in [glucose] during relative hypoxia exposure and silverspotted sculpins had the lowest level of [glucose] compared with the other two species (Table 1).

Normoxic levels of lactate in brain, liver, and white muscle were low and similar in all three species (Fig. 3A, B, and C). In all species and all tissues examined, lactate increased significantly after relative hypoxia exposure with the exception of liver in staghorn sculpins (Fig. 3A, B, and C). The level of [lactate] during relative hypoxia exposure was similar in all species for all tissues, except [lactate] in tidepool sculpin brain and liver was higher compared with silverspotted sculpins or staghorn sculpins (Fig. 3A, B, and C).

Levels of ATP under normoxic conditions were generally similar between species in brain, liver, and white muscle, although staghorn sculpins had the highest ATP levels in white muscle and brain (Fig. 4A, B, and C). In all species, brain and white muscle [ATP] was unaffected by relative hypoxia exposure (Fig. 4A and C) whereas liver [ATP] decreased significantly by 2- to 5-fold to a level not different between species (Fig. 4B).

3.3. Metabolic profiles at LOE

Normoxic Hct was significantly different between species, with the highest level in tidepool sculpins and the lowest level in staghorn sculpins (Table 2). At LOE, Hct was significantly increased in all species (Table 2).

Under normoxic conditions in brain, liver, and white muscle [glycogen] levels were lowest in silverspotted sculpins, highest in tidepool sculpins, and intermediate in staghorn sculpins (Fig. 5A, B, and C). At LOE, [glycogen] levels were significantly lower compared with normoxic levels in all species and tissues with the exception of brain and white muscle of silverspotted sculpins exposed to 6.4 Torr until LOE (Fig. 5A, B, and C). In all tissues, silverspotted sculpins exposed to relative hypoxia until LOE showed a significant decrease from normoxic values and a greater depletion of [glycogen] compared with the value for LOE at 6.4 Torr (Fig. 5A, B, and C). At LOE, levels of glycogen in brain were similar between species, whereas in liver and

Table 1
Hematocrit of whole blood, plasma [non-esterified fatty acids], and [glucose] in brain, liver, and white muscle of silverspotted sculpins, staghorn sculpins, and tidepool sculpins exposed to normoxia and 6 h of relative hypoxia (30% of each species' P_{crit}; 13.2 Torr for silverspotted sculpins, 11.1 Torr for staghorn sculpins, and 7.7 Torr for tidepool sculpins).

	Silverspotted sculpin		Staghorn sculpin		Tidepool sculpin	
	Normoxia	6 h relative hypoxia	Normoxia	6 h relative hypoxia	Normoxia	6 h relative hypoxia
Blood						
Hct	26.6 ± 2.0^{a}	$37.3 \pm 1.8^{z,*}$	18.8 ± 1.8^{b}	$26.6 \pm 1.9^{y,*}$	34.3 ± 1.9^{c}	37.2 ± 2.6^{z}
Plasma						
[NEFA]	186.1 ± 9.7^{a}	186.2 ± 8.5^{z}	67.7 ± 7.8^{b}	66.7 ± 21.7^{y}	401.8 ± 30.5^{c}	$255.4 \pm 38.9^{x,*}$
Brain						
[Glucose]	0.36 ± 0.04^{a}	$2.03 \pm 0.67^{z,*}$	0.47 ± 0.05^{a}	$3.55 \pm 1.02^{y,*}$	0.38 ± 0.01^{a}	$4.68 \pm 0.37^{y,*}$
Liver						
[Glucose]	4.93 ± 1.21^{a}	3.41 ± 1.78^{z}	3.90 ± 0.77^{a}	$8.91 \pm 1.55^{y,*}$	5.37 ± 0.89^{a}	10.0 ± 1.6^{y}
White muscle						
[Glucose]	0.23 ± 0.07^{a}	$0.86 \pm 0.13^{z,*}$	0.10 ± 0.05^{a}	$1.40 \pm 0.45^{z,*}$	0.12 ± 0.08^{a}	$1.13 \pm 0.12^{z,*}$

Values are means ± s.e.m. (n = 8 for Hct; n = 5–8 for [NEFA]; n = 4–8 for brain [glucose]; n = 5–8 for liver [glucose]; n = 6–8 for white muscle [glucose]). [Glucose] is expressed as μmol · g ww^{-1}, Hct as %, and [NEFA] as μmol · L^{-1}. Hct, hematocrit; NEFA, non-esterified fatty acids. * denotes that the value for hypoxia exposure is significantly different from the normoxia value within species (two-way ANOVA with Holm–Sidak post-hoc test, $p < 0.05$). Comparing normoxia values across species, values with the same letter are not significantly different from one another (two-way ANOVA with Holm–Sidak post-hoc test, $p < 0.05$). Comparing hypoxia values across species, values with the same letter are not significantly different from one another (two-way ANOVA with Holm–Sidak post-hoc test, $p < 0.05$).

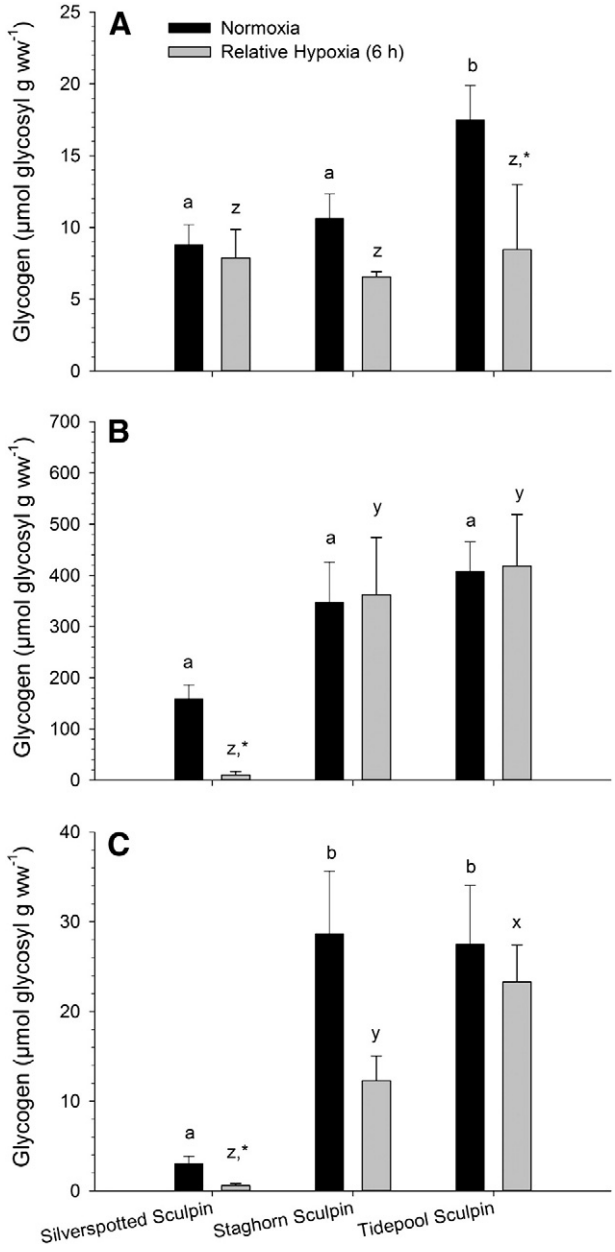

Fig. 2. [Glycogen] in brain (A), liver (B), and white muscle (C) of silverspotted sculpins, staghorn sculpins, and tidepool sculpins exposed to normoxia or 6 h of relative hypoxia (water PO_2 = 30% of each species' P_{crit}; 13.2 Torr for silverspotted sculpins, 11.1 Torr for staghorn sculpins, and 7.7 Torr for tidepool sculpins). Values are means ± s.e.m. (n = 5–6 for brain; n = 5–7 for liver; n = 6–8 for white muscle). * denotes that the value for relative hypoxia exposure is significantly different from the normoxia value within species (two-way ANOVA with Holm–Sidak post-hoc test, $p < 0.05$). Comparing normoxia values across species, values with the same letter are not significantly different from one another (two-way ANOVA with Holm–Sidak post-hoc test, $p < 0.05$). Comparing relative hypoxia values across species, values with the same letter are not significantly different from one another (two-way ANOVA with Holm–Sidak post-hoc test, $p < 0.05$).

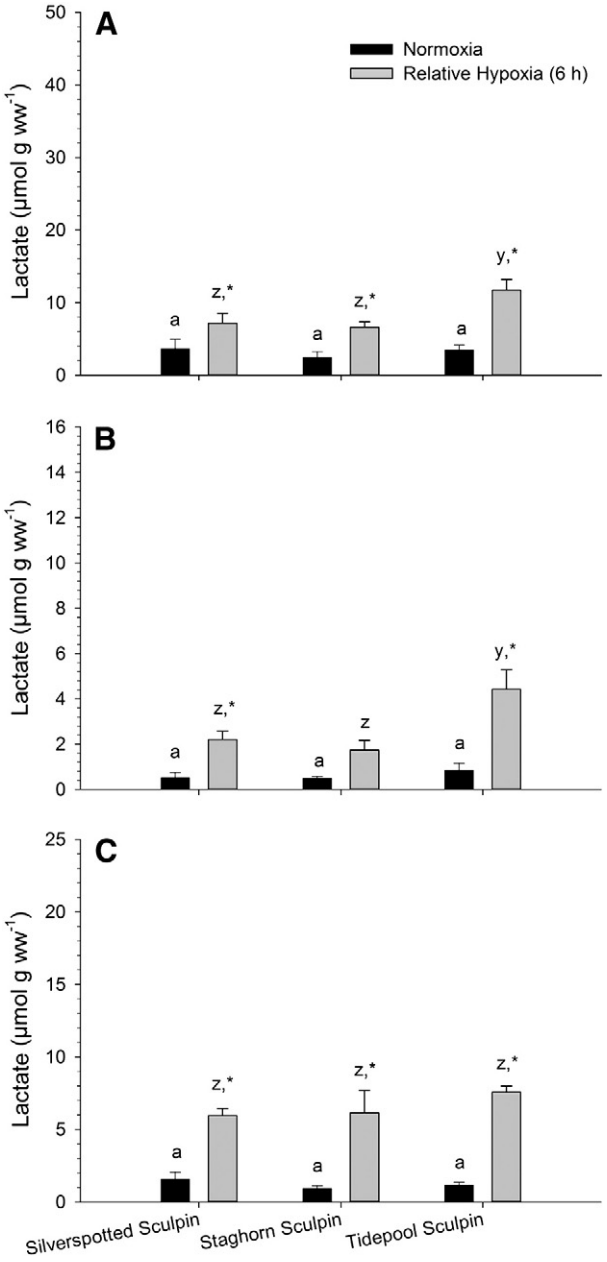

Fig. 3. [Lactate] in brain (A), liver (B), and white muscle (C) of silverspotted sculpins, staghorn sculpins, and tidepool sculpins exposed to normoxia or 6 h of relative hypoxia (water PO_2 = 30% of each species' P_{crit}; 13.2 Torr for silverspotted sculpins, 11.1 Torr for staghorn sculpins, and 7.7 Torr for tidepool sculpins). Values are means ± s.e.m. (n = 6–8). See Fig. 2 caption for more details.

white muscle, tidepool sculpins had higher levels compared with the other species (Fig. 5A, B, and C).

Glucose levels in all tissues during normoxia were similar between species (Table 2). At LOE in all tissues, silverspotted sculpins and staghorn sculpins showed no significant change in [glucose] compared with normoxic levels, whereas in tidepool sculpins levels were elevated and reached levels higher than in the other species (Table 2). Levels of glucose in liver of silverspotted sculpins at LOE in relative hypoxia were below the level of detection but in brain and white muscle, glucose levels were not lower compared with normoxia (Table 2).

Lactate levels were generally similar between all species under normoxia in each tissue (Fig. 6A, B, and C). At LOE (including silverspotted sculpins exposed to relative hypoxia until LOE), [lactate] was greatly increased in all tissues and species, except for silverspotted sculpin liver during LOE at 6.4 Torr (Fig. 6A, B, and C). Brain showed particularly high levels of lactate at LOE, ranging from ~12 to 40 μmol g ww^{-1} depending on the species (Fig. 6A). At LOE, tidepool sculpins had higher [lactate] in all tissues compared with the other species (Fig. 6A, B, and C).

Under normoxic conditions, [ATP] in each tissue was generally similar between species although levels were higher in staghorn sculpins in white muscle and lower in silverspotted sculpin liver compared with the other species (Fig. 7A, B, and C). At LOE (including silverspotted sculpins exposed to relative hypoxia until LOE), brain

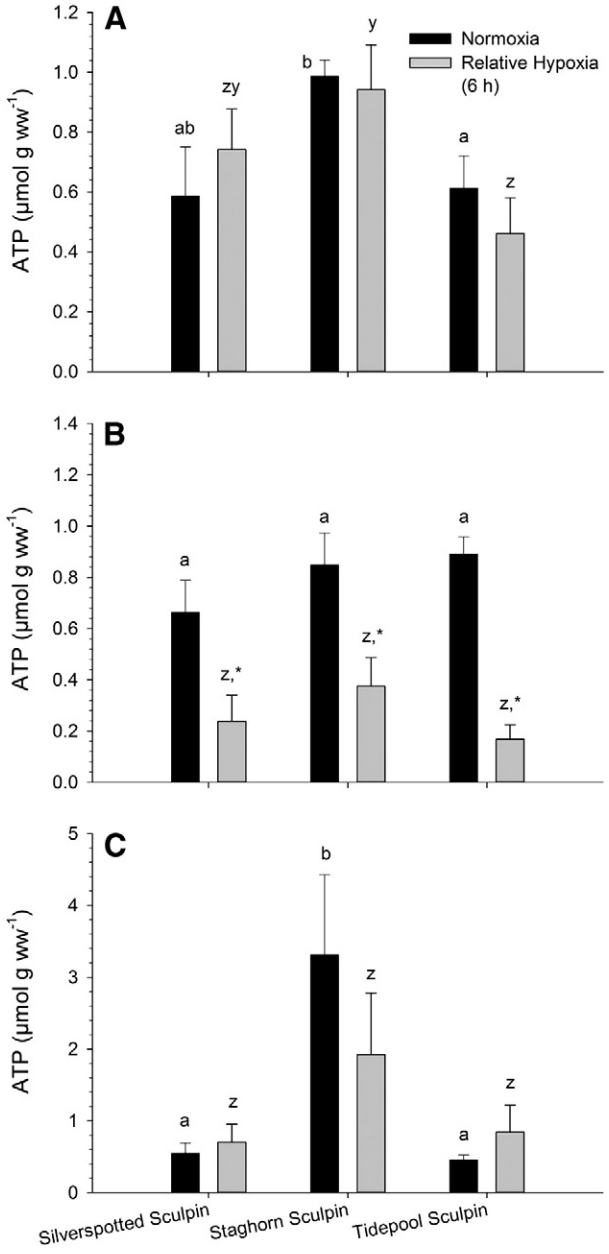

Fig. 4. [ATP] in brain (A), liver (B), and white muscle (C) of silverspotted sculpins, staghorn sculpins, and tidepool sculpins exposed to normoxia or 6 h of relative hypoxia (water $PO_2 = 30\%$ of each species' P_{crit}; 13.2 Torr for silverspotted sculpins, 11.1 Torr for staghorn sculpins, and 7.7 Torr for tidepool sculpins). Values are means ± s.e.m. ($n = 6–8$). See Fig. 2 caption for more details.

[ATP] was lower by >2-fold in all species (Fig. 7A). In all cases in liver at LOE, [ATP] was decreased by up to 14-fold compared with normoxia and levels were similar between species (Fig. 7B). In white muscle at LOE, ATP levels decreased significantly only in staghorn sculpins but the lower levels present in the other species remained unchanged from normoxic levels (Fig. 7C).

4. Discussion

4.1. P_{crit} as an indicator of whole-animal hypoxia tolerance

Critical oxygen tensions are widely considered to be good, ecologically-relevant measures of hypoxia tolerance in fishes because they attempt to quantify the P_wO_2 below which fishes will be physiologically compromised by a limitation of routine aerobic metabolism (Nilsson and Östlund-Nilsson, 2008; Mandic et al., 2009; Speers-Roesch et al., 2012a). We have previously shown a significant correlation between P_{crit} and hypoxia tolerance, as measured by LOE_{50} at a P_wO_2 of 6.4 Torr, among several species of sculpins (Mandic et al., 2013). Under these conditions, species with low P_{crit} values survived longer (had higher LOE_{50} values) than species with higher P_{crit} values. To investigate further whether P_{crit} is a good measure of hypoxia tolerance, we examined the effects of relative hypoxia exposure on the time to LOE (assessed as LOE_{50}), as well as tissue metabolic responses (see Section 4.2), in three species of sculpins known to differ in their P_{crit} and their LOE_{50} at a P_wO_2 of 6.4 Torr. Contrary to our prediction of similar relative hypoxia LOE_{50} values across species, we found that controlling for variation in P_{crit} equalized LOE_{50} values in staghorn sculpins and tidepool sculpins, but not in silverspotted sculpins, which had a significantly lower relative hypoxia LOE_{50} compared with the other two species (Fig. 1). These results suggest that P_{crit}, and consequently the ability to extract environmental O_2, is not an accurate indicator of whole-animal hypoxia tolerance in all species. An incongruence between P_{crit} and hypoxia tolerance also was found for the electric fish *Gnathonemus petersii*, which has a very low P_{crit} yet shows poor survival at P_wO_2 below P_{crit} (Nilsson, 1996). Thus, we suggest that caution is warranted when considering P_{crit} as a comparative measure of hypoxia tolerance among fishes (see Section 4.4). Interspecific differences in tissue glycogen content also appear to contribute greatly to explaining differences in hypoxia tolerance among sculpins (see Section 4.2).

4.2. P_{crit} as an indicator of hypoxia tolerance: tissue metabolic responses to 6 h of relative hypoxia exposure

The 6 h relative hypoxia exposure allowed us to ascertain if P_{crit} predicts tissue metabolic responses to hypoxia, which would further illuminate whether P_{crit} is a good indicator of hypoxia tolerance. This experiment also allowed us to identify potential metabolic explanations for the poorer hypoxia tolerance of silverspotted sculpins compared with their more tolerant close relatives, the tidepool sculpin and staghorn sculpin.

During relative hypoxia exposure increases in tissue [lactate] occurred in all species (Fig. 3A, B, and C), a common finding in hypoxia-exposed fishes that indicates an increased activation of anaerobic glycolysis resulting from hypoxemia (Nilsson and Östlund-Nilsson, 2008). The increases in tissue [lactate] were accompanied by increases in tissue [glucose] (Table 1), which have been observed in some hypoxia-exposed fishes although there is variability in this glucose response (MacCormack et al., 2006; MacCormack and Driedzic, 2007; Richards et al., 2007). Elevation of Hct can improve tissue O_2 delivery in hypoxia-exposed fishes (Gallaugher and Farrell, 1998), but the increased Hct in silverspotted sculpins and staghorn sculpins, and high Hct in tidepool sculpins (Table 1), evidently were unable to prevent hypoxemia considering the large accumulation of lactate. Interestingly, lactate accumulated to similar levels in all species (Fig. 3A, B, and C) suggesting that when variation in P_{crit} is controlled for, the reliance on anaerobic glycolysis to meet metabolic energy demands is similar between species. Although not directly measured in the present study, this outcome could be the result of similar interspecific levels of hypoxemia during relative hypoxia exposure, which has been shown to occur in elasmobranchs during hypoxia exposure scaled to the species-specific P_{crit} (Speers-Roesch et al., 2012a). However, the level of hypoxia-induced metabolic depression also can affect reliance on anaerobic glycolysis and could vary as a function of P_{crit}. Future studies should examine the relative importance of and interaction between whole-animal O_2 supply and metabolic rate depression in decreasing reliance on anaerobic glycolysis during hypoxia exposure in fishes.

Table 2
Hematocrit of whole blood and [glucose] in brain, liver, and white muscle of silverspotted sculpins, staghorn sculpins, and tidepool sculpins exposed to normoxia, or hypoxia exposure at a water PO_2 of 6.4 Torr until loss of equilibrium (LOE), or relative hypoxia until LOE (silverspotted sculpins only, water PO_2 = 13.2 Torr, 30% of P_{crit}).

	Silverspotted sculpin			Staghorn sculpin		Tidepool sculpin	
	Normoxia	LOE at 6.4 Torr	Relative LOE	Normoxia	LOE at 6.4 Torr	Normoxia	LOE at 6.4 Torr
Blood							
Hct	$20.5 \pm 2.3^{a,A}$	$36.0 \pm 1.4^{z,*,B}$	28.2 ± 1.4^{C}	12.4 ± 1.2^{b}	$29.4 \pm 2.2^{y,*}$	30.8 ± 1.9^{c}	$37.4 \pm 2.7^{z,*}$
Brain							
[Glucose]	$0.48 \pm 0.42^{a,AB}$	$1.33 \pm 0.20^{z,B}$	0.10 ± 0.08^{A}	0.18 ± 0.03^{a}	0.70 ± 0.25^{z}	0.64 ± 0.40^{a}	$5.19 \pm 1.72^{y,*}$
Liver							
[Glucose]	$8.32 \pm 2.54^{a,A}$	$15.6 \pm 1.2^{z,A}$	BLD^{B}	2.75 ± 0.21^{a}	4.82 ± 2.93^{y}	6.79 ± 1.28^{a}	$26.2 \pm 6.7^{x,*}$
White muscle							
[Glucose]	$0.21 \pm 0.09^{a,A}$	$0.47 \pm 0.08^{z,A}$	0.43 ± 0.16^{A}	0.14 ± 0.06^{a}	0.16 ± 0.04^{y}	0.09 ± 0.04^{a}	$1.61 \pm 0.33^{x,*}$

Values are means ± s.e.m. (n = 6–9 for Hct; n = 7–10 for brain [glucose]; n = 5–9 for liver [glucose]; n = 7–8 for white muscle [glucose]). [Glucose] is expressed as μmol · g ww^{-1} and Hct is expressed as %. Hct, hematocrit. BLD, below level of detection. * denotes that the value for absolute LOE exposure is significantly different from the normoxia value within species (two-way ANOVA with Holm–Sidak post-hoc test, $p < 0.05$). Comparing normoxia values across species, values with the same letter are not significantly different from one another (two-way ANOVA with Holm–Sidak post-hoc test, $p < 0.05$). Comparing absolute LOE values across species, values with the same letter are not significantly different from one another (two-way ANOVA with Holm–Sidak post-hoc test, $p < 0.05$). Comparing values within silverspotted sculpins, values with the same uppercase letter are not significantly different from one another (one-way ANOVA with Holm–Sidak post-hoc test, $p < 0.05$).

The ability to maintain stable tissue ATP levels during hypoxia exposure, especially in sensitive tissues such as brain, is considered to be a hallmark of hypoxia tolerance (Lutz, 1992; Hochachka et al., 1996). Indeed, the 6 h relative hypoxia exposure was associated with unchanged ATP levels in brain and white muscle in all three sculpin species (Fig 4A, B, and C). Previous studies have also shown unperturbed [ATP] in brain and white muscle of fishes during non-lethal hypoxia exposure (Jorgensen and Mustafa, 1980; Dunn and Hochachka, 1986; Van der Boon et al., 1992; Johansson and Nilsson, 1995; Van Ginneken et al., 1995; Richards et al., 2008), in contrast with the decreases seen at the time of LOE, particularly in brain (see Section 4.3). The interspecific similarity of tissue [ATP] during relative hypoxia exposure in the present study suggests that when variation in P_{crit} is controlled for via hypoxia exposures corresponding to 30% of P_{crit}, the ability to maintain cellular energy status is similar between species. This could reflect similar O_2 supply or metabolic rate depression at the level of hypoxia used in the relative exposure. The decrease in liver [ATP] seen in all three species during relative hypoxia exposure is similar to findings in other hypoxia-exposed fishes (Jorgensen and Mustafa, 1980; Dunn and Hochachka, 1986; Jibb and Richards, 2008; Speers-Roesch et al., 2012b). There is increasing evidence that in some cases, such as in the fish liver and turtle heart, normoxic ATP levels are not defended during hypoxia exposure but are reset at a lower, albeit stable, level (Richards, 2009; Stecyk et al., 2009).

Based on our metabolic analysis, the ability to extract environmental O_2, as indicated by P_{crit}, appears to be an important determinant of cellular energy status and reliance on anaerobic glycolysis during hypoxia exposure in sculpins. These data also suggest that neither excessive lactate accumulation nor an inability to maintain cellular energy balance explains the poorer relative hypoxia tolerance (i.e., lower relative hypoxia LOE_{50}) of silverspotted sculpins compared with staghorn sculpins and tidepool sculpins (Fig. 1). Instead, the explanation may involve a limitation in glycogen storage and handling. Large glycogen stores and slower depletion of glycogen have been linked to hypoxia tolerance in fishes (Nilsson and Östlund-Nilsson, 2008; Vornanen et al., 2009). Staghorn sculpins and tidepool sculpins generally had greater glycogen stores in normoxia compared with silverspotted sculpins (Fig. 2A, B, and C; see also Fig. 5A, B, and C). Additionally, both tidepool sculpins and staghorn sculpins showed only small decreases in tissue [glycogen] during relative hypoxia exposure but in silverspotted sculpins, [glycogen] was greatly decreased in white muscle and especially liver (Fig. 2), which is the major glycogen depot in most fishes (Richards, 2009). The very low glycogen levels in liver and white muscle (but sustained levels in brain) of silverspotted sculpins at 6 h of relative hypoxia exposure may explain why survival sharply declined in this species at >400 min during the relative hypoxia LOE_{50} trials (Fig. 1; also see Section 4.3). All three species had similar hepatosomatic indices (see Results) so we consider our use of mass-specific liver glycogen content for interspecific comparisons to be valid. Incidentally, there is incongruence between the levels of tissue [glycogen] depletion and lactate accumulation, which theoretically should be closely matched. This finding could be related to interspecific differences in gluconeogenesis or the ability to utilize exogenous glucose, or in the case of white muscle, potential regional heterogeneity of glycogen deposition and mobilization (this cannot be true for liver or brain, which were sampled whole). Overall, our results demonstrate that tissue glycogen content and utilization are important factors contributing to hypoxia tolerance and that they are, perhaps understandably, independent of P_{crit} (see further discussion in Section 4.4).

Plasma [NEFA] decreases during hypoxia exposure in some fishes and it has been suggested that this response contributes to hypoxia tolerance by averting lipotoxicity or aiding in metabolic rate depression (Van den Thillart et al., 2002; Speers-Roesch et al., 2010). Consistent with this idea, tidepool sculpins showed a significant decrease in plasma [NEFA] but the less hypoxia-tolerant silverspotted sculpins did not (Table 1). However, staghorn sculpins, which have similar relative hypoxia tolerance compared with tidepool sculpins (Fig. 1), showed no decrease, although their levels were low to begin with (Table 1). The relationship between hypoxia tolerance and the ability to depress plasma [NEFA] during hypoxia exposure should be examined in a wider selection of hypoxia-tolerant and -sensitive fishes.

4.3. Biochemical limitations on hypoxia tolerance: metabolic responses at LOE during hypoxia exposure

A decrease in brain [ATP] was the most striking metabolic response associated with hypoxic LOE in all species and conditions (Fig. 7A), contrasting with the maintenance of brain [ATP] seen during the relative hypoxia exposure (Fig 4A). In white muscle there was little change in [ATP] and liver [ATP] decreased in a manner similar to that seen during relative hypoxia exposure (Fig. 7B and C). These results agree with previous studies showing that loss of ATP homeostasis in brain is associated with mortality during hypoxia exposure in fishes (DiAngelo and Heath, 1987; Van Raaij et al., 1994; Van Ginneken et al., 1996;

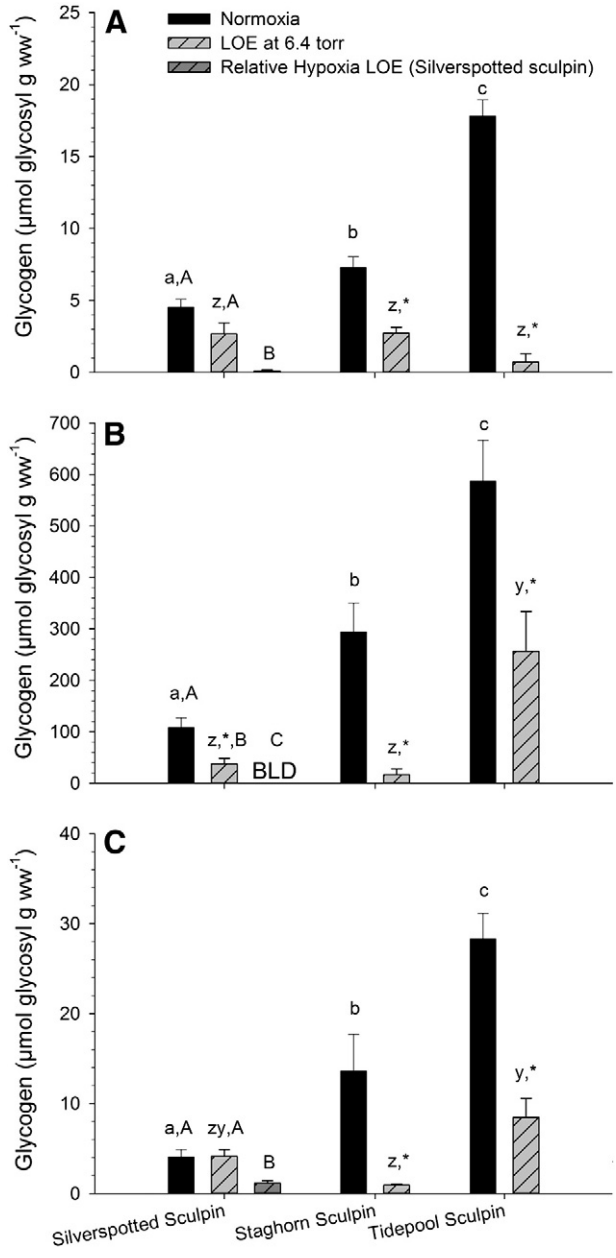

Fig. 5. [Glycogen] in brain (A), liver (B), and white muscle (C) of silverspotted sculpins, staghorn sculpins, and tidepool sculpins exposed to normoxia, or a hypoxic water PO_2 of 6.4 Torr until loss of equilibrium (LOE), or relative hypoxia until LOE (silverspotted sculpins only, water PO_2 = 13.2 Torr, 30% of P_{crit}). Values are means ± s.e.m. (n = 6–10 for brain, except tidepool sculpin LOE at 6.4 Torr where n = 4; n = 5–9 for liver; n = 8 for white muscle). * denotes that the value for LOE at 6.4 Torr is significantly different from the normoxia value within species (two-way ANOVA with Holm–Sidak post-hoc test, $p < 0.05$). Comparing normoxia values across species, values with the same letter are not significantly different from one another (two-way ANOVA with Holm–Sidak post-hoc test, $p < 0.05$). Comparing LOE at 6.4 Torr values across species, values with the same letter are not significantly different from one another (two-way ANOVA with Holm–Sidak post-hoc test, $p < 0.05$). Comparing values within silverspotted sculpins, values with the same uppercase letter are not significantly different from one another (one-way ANOVA with Holm–Sidak post-hoc test, $p < 0.05$). BLD, below level of detection.

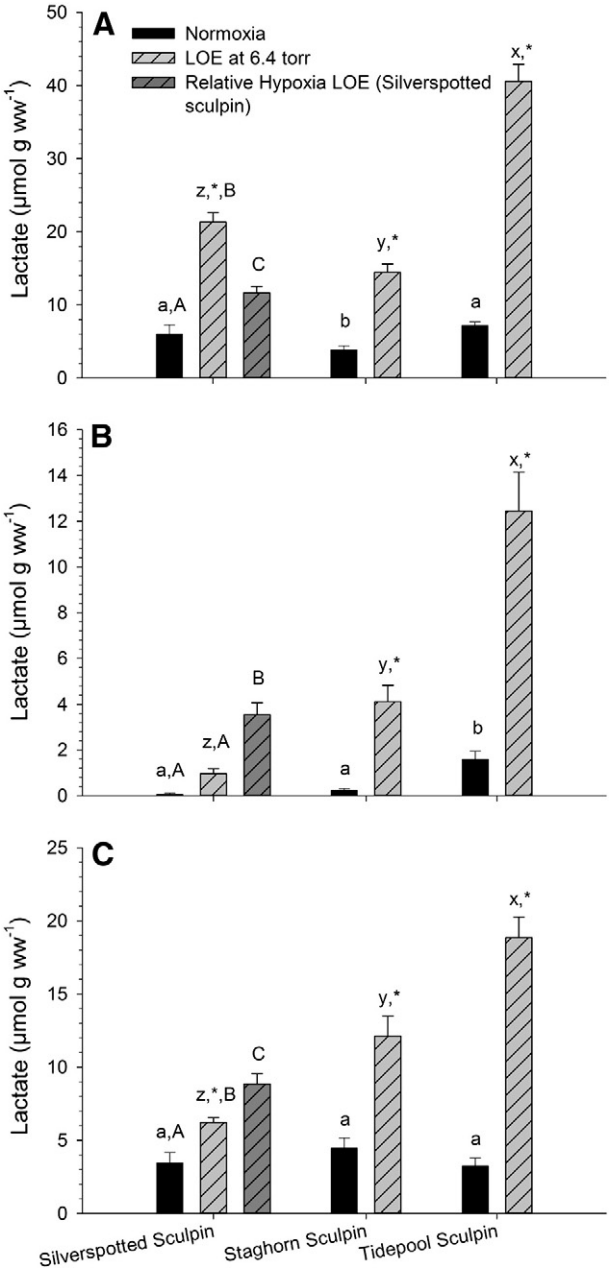

Fig. 6. [Lactate] in brain (A), liver (B), and white muscle (C) of silverspotted sculpins, staghorn sculpins, and tidepool sculpins exposed to normoxia, or a hypoxic water PO_2 of 6.4 Torr until loss of equilibrium (LOE), or relative hypoxia until LOE (silverspotted sculpins only, water PO_2 = 13.2 Torr, 30% of P_{crit}). Values are means ± s.e.m. (n = 7–10 for brain; n = 6–9 for liver; n = 8 for white muscle). See Fig. 5 caption for more details.

Ishibashi et al., 2002), which supports the notion that maintenance of ATP homeostasis, particularly in brain, is a crucial component of hypoxia survival in fishes (Lutz, 1992; Hochachka et al., 1996; Nilsson and Östlund-Nilsson, 2008). Importantly, the three sculpin species have different hypoxia tolerances (Fig. 1; Mandic et al., 2013) yet at LOE they all showed a similar pattern of depletion of brain [ATP] (Fig. 7A). Also, silverspotted sculpins lost equilibrium rapidly at 6.4 Torr compared with relative hypoxia yet showed a similar depletion of brain [ATP] in both cases (Fig. 7A). These results support the idea that loss of brain ATP homeostasis is the proximate limit to hypoxia tolerance in both hypoxia-tolerant and hypoxia-sensitive species, but that hypoxia-tolerant species are able to better maintain ATP homeostasis for a longer duration and to a lower PO_2 (Boutilier, 2001).

Depletion of glycogen stores provides one potential explanation for the loss of brain ATP homeostasis observed at LOE, with large decreases in tissue glycogen levels occurring in all species (Fig. 5A, B, and C) compared with the generally more stable levels during relative hypoxia exposure (Fig. 2A, B, and C). We suggest that glycogen depletion decreased glucose supply for anaerobic glycolysis, leading to loss of

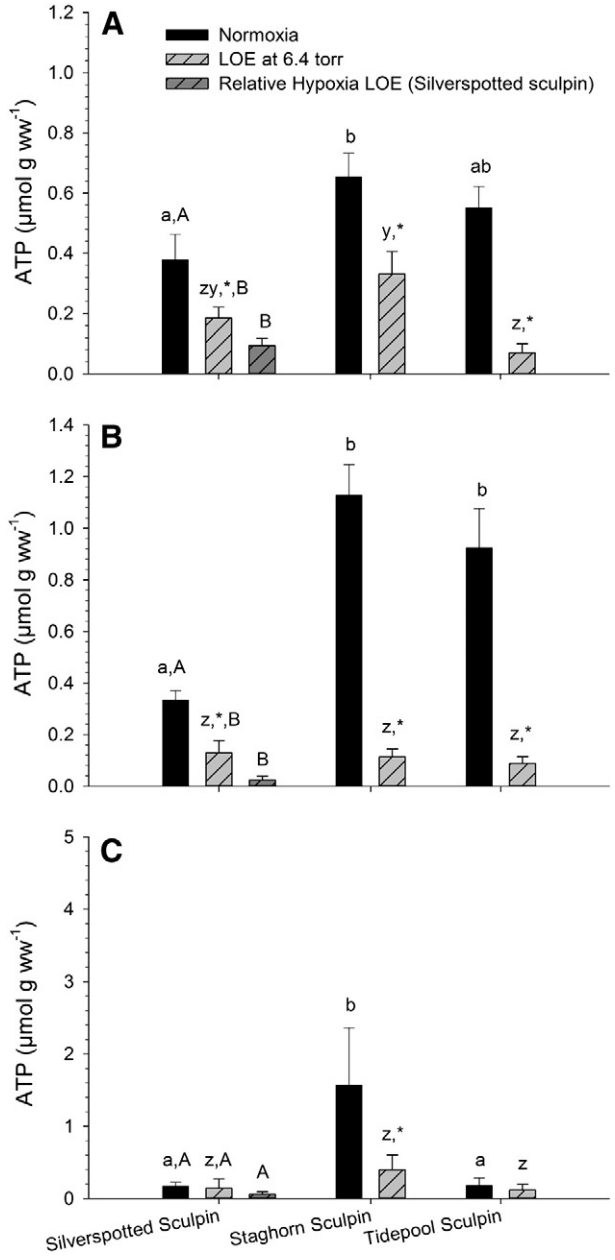

Fig. 7. [ATP] in brain (A), liver (B), and white muscle (C) of silverspotted sculpins, staghorn sculpins, and tidepool sculpins exposed to normoxia, or a hypoxic water PO_2 of 6.4 Torr until loss of equilibrium (LOE), or relative hypoxia until LOE (silverspotted sculpins only, water PO_2 = 13.2 Torr, 30% of P_{crit}). Values are means ± s.e.m. (n = 7–10 for brain; n = 7–9 for liver; n = 8 for white muscle). See Fig. 5 caption for more details.

ATP production and consequently disrupted ATP homeostasis in the brain, resulting in LOE. This scenario is most probable in staghorn sculpins and the group of silverspotted sculpins exposed to relative hypoxia until LOE, where glycogen levels fell to negligible levels, especially in the liver, and glucose levels were also low compared to the increases seen during 6 h of relative hypoxia exposure (Fig. 5A, B, and C; Table 2 cf. Table 1). These findings are consistent with previous studies demonstrating that moribund hypoxia-exposed trout and catfish show depletion of brain glycogen (DiAngelo and Heath, 1987) and that hypoxic survival in crucian carp is associated with persistence of liver glycogen stores (Vornanen et al., 2009). The present study provides significant additional evidence supporting the idea that glycogen depletion is a major ultimate limiting factor for hypoxic survival in fishes (Nilsson and Östlund-Nilsson, 2008).

Our results also support the idea that excessive lactate load is another important limitation on hypoxic survival in fishes (Nilsson and Östlund-Nilsson, 2008). At LOE, and despite a sustained elevation of Hct (Table 2), all sculpin species showed high lactate levels in brain and white muscle that were within the range that is thought to be associated with hypoxic mortality as well as total exhaustion from exercise (12–40 µmol g ww^{-1}; DiAngelo and Heath, 1987; Nilsson and Östlund-Nilsson, 2008). In particular, a high lactate load, rather than glycogen depletion, may ultimately explain hypoxic LOE in tidepool sculpins. LOE in this species was associated with very high levels of tissue lactate (likely due to their longer survival time during hypoxia exposure at 6.4 Torr compared with the other species; Mandic et al., 2013) yet a significant amount of liver glycogen stores remained and tissue glucose levels were still elevated (Fig. 5B; Fig. 6A, B, and C; Table 1). These findings appear to contradict the suggestion that the size of glycogen stores in fishes should be matched to their maximum tolerable lactate levels (Nilsson and Östlund-Nilsson, 2008). Instead, considering that glycogen is used in some fishes as a fuel during fasting (Milne et al., 1979; Mehner and Wieser, 1994), the apparent excess of glycogen stores in tidepool sculpins could be related to tolerance of the temporal fluctuations in food availability that are common in the dynamic intertidal environment (Newell, 1979).

In silverspotted sculpins exposed to a hypoxic P_wO_2 of 6.4 Torr, which was much lower compared with the relative hypoxia LOE exposure, LOE occurred rapidly and this probably explains why glycogen levels were less changed from normoxic values compared to the relative hypoxia LOE exposures (Fig. 5A, B, and C). It is possible that the rapid LOE in silverspotted sculpins exposed to 6.4 Torr was attributable to an inability to depress cellular energy demand or increase anaerobic glycolysis sufficiently to match the loss of aerobic ATP production, leading to an imbalance of energy supply and demand and a loss of ATP homeostasis. Nonetheless, very high levels of brain lactate accumulated (Fig. 6A) and this also may have contributed to the rapid LOE. Our observation that LOE was associated with loss of brain [ATP] without depletion of glycogen stores may explain why Mandic et al. (2013) found no relationship across sculpin species between liver glycogen content and hypoxia tolerance as measured by LOE_{50} at 6.4 Torr. At this severe level of hypoxia, hypoxia tolerance in some of the species (probably those with higher P_{crit}) may have been dictated primarily by the ability to match energy supply and demand, rather than by the amount of available glycogen.

4.4. Conclusions and perspectives

The present study demonstrates that three sculpin species with different P_{crit} and hypoxia tolerance at 6.4 Torr (Mandic et al., 2009; Mandic et al., 2013) showed a similar ability to minimize lactate accumulation and maintain energy status in tissues during relative hypoxia exposure, where variation in P_{crit} is controlled for. These findings suggest that the ability to extract environmental O_2 (i.e., P_{crit}) appears to be an important determinant of cellular energy status and reliance on anaerobic glycolysis during hypoxia exposure in sculpins, at least at the level of hypoxia used here (30% of P_{crit}). The present results therefore reinforce the notion that O_2 extraction from the environment and supply to tissues is important for hypoxia tolerance in fishes. Nonetheless, a similar relative hypoxia exposure in elasmobranchs showed distinct interspecific differences in tissue metabolic profiles (Speers-Roesch et al., 2012b), warning that the relationship between P_{crit} and tissue metabolic responses may vary among fishes. Furthermore, contrary to our prediction of similar LOE_{50} values between species during relative hypoxia exposure, this was only true for tidepool sculpins and staghorn sculpins, whereas silverspotted sculpins had a lower LOE_{50} value that we attribute to glycogen limitation. We conclude that while P_{crit}, in general, offers a useful measure of hypoxia tolerance in fishes, in particular the ability to maintain routine $\dot{M}_{O_2}$ at low environmental PO_2, it should be used with caution especially when considering temporal aspects of hypoxia tolerance and related metabolic characteristics such as

the size and handling of glycogen stores. Indeed, whereas lactate accumulation indicates reliance upon anaerobic glycolysis and is affected by O_2 availability, and by extension, P_{crit}, there is no obvious mechanistic link between the capacity for glycogen sequestration and O_2 availability, or P_{crit}. Furthermore, the electric fish *G. petersii*, which has a very low P_{crit} yet poor hypoxia tolerance that has been attributed to its unusually large brain and a low capacity for anaerobic glycolysis (Nilsson, 1996), provides a further warning that the physiological diversity of fishes inhabiting hypoxic waters will constrain the use of P_{crit} as an indicator of hypoxia tolerance. Ideally, comparative studies of hypoxia tolerance should use multiple measures (e.g. P_{crit} and LOE_{50}) in order to assess the overall responses of fishes to hypoxia.

Our results provide evidence to support the idea that the loss of brain ATP homeostasis is a primary and universal cause of LOE and mortality during hypoxia exposure in fishes, regardless of their hypoxia tolerance (Nilsson and Östlund-Nilsson, 2008). However, the ultimate cause of LOE may vary and we suggest that there were three major metabolic phenotypes associated with LOE during hypoxia exposure in sculpins: 1) substantial depletion of glycogen stores, particularly in the liver, leading to loss of brain ATP homeostasis due to fuel starvation of anaerobic glycolysis (e.g. staghorn sculpins, as well as silverspotted sculpins exposed to relative hypoxia until LOE); 2) excessive lactate accumulation (but incomplete depletion of glycogen stores), which was associated with loss of brain ATP homeostasis (e.g., tidepool sculpins, and silverspotted sculpins exposed to 6.4 Torr until LOE); and 3) a drastic mismatch between ATP supply and demand leading to rapid depletion of brain [ATP] (e.g., silverspotted sculpins exposed to 6.4 Torr until LOE). We propose that the relative role of each of these metabolic phenotypes in limiting hypoxia survival in fishes depends upon the severity of hypoxia exposure relative to P_{crit}. At very low relative P_wO_2 (relative to P_{crit}), phenotype 3 is most likely to occur first and the duration of hypoxia tolerance should correlate with the degree of mismatch between ATP supply and demand that sets the rate of [ATP] depletion (Knickerbocker and Lutz, 2001), which in turn depends upon the level of available O_2 as well as the capacity for anaerobic metabolism and metabolic depression. At a certain higher level of O_2 (but still below P_{crit}), there will be a P_wO_2 value at which a matching of ATP supply and demand can be achieved, enabling tissue [ATP] to be maintained. At this point, hypoxia tolerance is ultimately limited by the size of the glycogen stores (phenotype 1) and the ability to tolerate the accumulation of H^+ and lactate (phenotype 2), as previously suggested for anoxia-tolerant turtles and crucian carp (Jackson, 2004; Nilsson and Östlund-Nilsson, 2008). We suggest that, among fishes in general, phenotype 3 is more likely to limit hypoxic survival in hypoxia-sensitive species, which typically have higher P_{crit} and thus experience a greater range of environmental hypoxia at which balance of energy supply and demand is perturbed. On the other hand, glycogen limitation (phenotype 1) and lactate load (phenotype 2) are probably the more significant problems for hypoxia-tolerant species that have a superior ability to balance energy supply and demand through improved O_2 supply and metabolic depression. Overall, our findings support the notion that hypoxia tolerance in fishes is associated with the ability to maintain cellular energy balance in brain at lower P_wO_2, to sequester large glycogen stores, and to minimize lactate load resulting from anaerobic glycolysis.

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References

Bergmeyer, H.U., 1983. Methods of Enzymatic Analysis. Academic Press, New York.
Boutilier, R.G., 2001. Mechanisms of cell survival in hypoxia and hypothermia. J. Exp. Biol. 204, 3171–3181.
Chapman, L.J., Chapman, C.A., Nordlie, F.G., Rosenberger, A.E., 2002. Physiological refugia: swamps, hypoxia tolerance and maintenance of fish diversity in the Lake Victoria region. Comp. Biochem. Physiol. A 133, 421–437.
DiAngelo, C.R., Heath, A.G., 1987. Comparison of *in vivo* energy metabolism in the brain of rainbow trout, *Salmo gairdneri* and bullhead catfish, *Ictalurus nebulosus* during anoxia. Comp. Biochem. Physiol. B 88, 297–303.
Diaz, R.J., Breitburg, D.L., 2009. The hypoxic environment. In: Richards, J.G., Farrell, A.P., Brauner, C.J. (Eds.), Hypoxia, Fish Physiology, vol. 27. Elsevier Academic Press, San Diego, CA, pp. 1–23.
Dunn, J.F., Hochachka, P.W., 1986. Metabolic responses of trout (*Salmo gairdneri*) to acute environmental hypoxia. J. Exp. Biol. 123, 229–242.
Gallaugher, P., Farrell, A.P., 1998. Hematocrit and blood oxygen-carrying capacity. In: Perry, S.F., Tufts, B.L. (Eds.), Fish Respiration, Fish Physiology, 17. Academic Press, San Diego, CA, pp. 185–227.
Hochachka, P.W., Buck, L.T., Doll, C.J., Land, S.C., 1996. Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. Proc. Natl. Acad. Sci. U. S. A. 93, 9493–9498.
Ishibashi, Y., Ekawa, H., Hirata, H., Kumai, H., 2002. Stress response and energy metabolism in various tissues of Nile tilapia *Oreochromis niloticus* exposed to hypoxic conditions. Fish. Sci. 68, 1374–1383.
Jackson, D.C., 2004. Acid–base balance during hypoxic hypometabolism: selected vertebrate strategies. Respir. Physiol. Neurobiol. 141, 273–283.
Jibb, L.A., Richards, J.G., 2008. AMP-activated protein kinase activity during metabolic rate depression in the hypoxic goldfish, *Carassius auratus*. J. Exp. Biol. 211, 3111–3122.
Johansson, D., Nilsson, G., 1995. Roles of energy status, K_{ATP} channels and channel arrest in fish brain K^+ gradient dissipation during anoxia. J. Exp. Biol. 198, 2575–2580.
Jorgensen, J.B., Mustafa, T., 1980. The effect of hypoxia on carbohydrate metabolism in flounder (*Platichthys flesus* L.). II. High energy phosphate compounds and the role of glycolytic and gluconeogenic enzymes. Comp. Biochem. Physiol. B 67, 249–256.
Knickerbocker, D.L., Lutz, P.L., 2001. Slow ATP loss and the defense of ion homeostasis in the anoxic frog brain. J. Exp. Biol. 204, 3547–3551.
Lutz, P.L., 1992. Mechanisms for anoxic survival in the vertebrate brain. Annu. Rev. Physiol. 54, 601–618.
MacCormack, T.J., Driedzic, W.R., 2007. The impact of hypoxia on in vivo glucose uptake in a hypoglycemic fish, *Myoxocephalus scorpius*. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292, R1033–R1042.
MacCormack, T.J., Lewis, J.M., Almeida-Val, V.M., Val, A.L., Driedzic, W.R., 2006. Carbohydrate management, anaerobic metabolism, and adenosine levels in the armoured catfish, *Liposarcus pardalis* (Castelnau), during hypoxia. J. Exp. Zool. A 305, 363–375.
Mandic, M., Todgham, A.E., Richards, J.G., 2009. Mechanisms and evolution of hypoxia tolerance in fish. Proc. R. Soc. B Biol. Sci. 276, 735–744.
Mandic, M., Speers-Roesch, B., Richards, J.G., 2013. Hypoxia tolerance in sculpins is associated with high anaerobic enzyme activity in brain but not in liver or muscle. Physiol. Biochem. Zool. 86, 92–105.
McKenzie, D.J., Lund, I., Pederson, P.B., 2008. Essential fatty acids influence metabolic rate and tolerance of hypoxia in Dover sole (*Solea solea*) larvae and juveniles. Mar. Biol. 154, 1041–1051.
Mehner, T., Wieser, W., 1994. Energetics and metabolic correlates of starvation in juvenile perch (*Perca fluviatilis*). J. Fish Biol. 45, 325–333.
Milne, R.S., Leatherland, J.F., Holub, B.J., 1979. Changes in plasma thyroxine, triiodothyronine and cortisol associated with starvation in rainbow trout (*Salmo gairdneri*). Environ. Biol. Fishes 4, 185–190.
Newell, R.C., 1979. Biology of Intertidal Animals. Marine Ecological Surveys, Kent, UK.
Nilsson, G.E., 1996. Brain and body oxygen requirements of *Gnathonemus petersii*, a fish with an exceptionally large brain. J. Exp. Biol. 199, 603–607.
Nilsson, G.E., Östlund-Nilsson, S., 2008. Does size matter for hypoxia tolerance in fish? Biol. Rev. 83, 173–189.
Pörtner, H.O., Grieshaber, M.K., 1993. Critical PO2(s) in oxyconforming and oxyregulating animals: gas exchange, metabolic rate and the mode of energy production. In: Eduardo, J., Bicudo, P.W. (Eds.), The Vertebrate Gas Transport Cascade: Adaptations to Environment and Mode of Life. CRC Press, Boca Raton, FL, pp. 330–357.
Richards, J.G., 2009. Metabolic and molecular responses of fish to hypoxia. In: Richards, J.G., Farrell, A.P., Brauner, C.J. (Eds.), Hypoxia, Fish Physiology, vol. 27. Elsevier Academic Press, San Diego, CA, pp. 443–485.
Richards, J.G., 2011. Physiological, behavioral and biochemical adaptations of intertidal fishes to hypoxia. J. Exp. Biol. 214, 191–199.
Richards, J.G., Wang, Y.S., Brauner, C.J., Gonzalez, R.J., Patrick, M.L., Schulte, P.M., Choppari-Gomes, A.R., Almeida-Val, V.M., Val, A.L., 2007. Metabolic and ionoregulatory responses of the Amazonian cichlid, *Astronotus ocellatus*, to severe hypoxia. J. Comp. Physiol. B. 177, 361–374.
Richards, J.G., Sardella, B.A., Schulte, P.M., 2008. Regulation of pyruvate dehydrogenase in the common killifish, *Fundulus heteroclitus*, during hypoxia exposure. Am. J. Physiol. 295, R979–R990.
Speers-Roesch, B., Sandblom, E., Lau, G.Y., Farrell, A.P., Richards, J.G., 2010. Effects of environmental hypoxia on cardiac energy metabolism and performance in tilapia. Am. J. Physiol. 298, R104–R119.

Speers-Roesch, B., Richards, J.G., Brauner, C.J., Farrell, A.P., Hickey, A.J.R., Wang, Y.S., Renshaw, G.M.C., 2012a. Hypoxia tolerance in elasmobranchs. I. Critical oxygen tension as a measure of blood oxygen transport during hypoxia exposure. J. Exp. Biol. 215, 93–102.

Speers-Roesch, B., Brauner, C.J., Farrell, A.P., Hickey, A.J.R., Renshaw, G.M.C., Wang, Y.S., Richards, J.G., 2012b. Hypoxia tolerance in elasmobranchs. II. Cardiovascular function and tissue metabolic responses during progressive and relative hypoxia exposures. J. Exp. Biol. 215, 103–114.

Stecyk, J.A.W., Bock, C., Overgaard, J., Wang, T., Farrell, A.P., Pörtner, H.-O., 2009. Correlation of cardiac performance with cellular energetic components in the oxygen-deprived turtle heart. Am. J. Physiol. 297, R756–R768.

van den Thillart, G., Vianen, G., Zaagsma, J., 2002. Adrenergic regulation of lipid mobilization in fishes; a possible role in hypoxia survival. Fish Physiol. Biochem. 27, 189–204.

van der Boon, J., de Jong, R.L., van den Thillart, G.E.E.J.M., Addink, A.D.F., 1992. Reversed-phase ion-paired HPLC of purine nucleotides from skeletal muscle, heart and brain of the goldfish, *Carassius auratus* L.—II. Influence of environmental anoxia on metabolite levels. Comp. Biochem. Physiol. B 101, 583–586.

van Ginneken, V., van den Thillart, G., Addink, A., Erkelens, C., 1995. Fish muscle energy metabolism measured during hypoxia and recovery: an in vivo ^{31}P-NMR study. Am. J. Physiol. 268, R1178–R1187.

van Ginneken, V., Nieveen, M., van Eersel, R., van den Thillart, G., Addink, A., 1996. Neurotransmitter levels and energy status in brain of fish species with and without the survival strategy of metabolic depression. Comp. Biochem. Physiol. A 114, 189–196.

van Raaij, M.T.M., Bakker, E., Nieveen, M.C., Zirkzee, H., van Den Thillart, G.E.E.J.M., 1994. Energy status and free fatty acid patterns in tissues of common carp (*Cyprinus carpio*, L.) and rainbow trout (*Oncorhynchus mykiss*, L.) during severe oxygen restriction. Comp. Biochem. Physiol. A 109, 755–767.

Vornanen, M., Stecyk, J., Nilsson, G.E., 2009. The anoxia-tolerant crucian carp (*Carassius carassius* L.). In: Richards, J.G., Farrell, A.P., Brauner, C.J. (Eds.), Hypoxia, Fish Physiology, vol. 27. Elsevier Academic Press, San Diego, CA, pp. 397–441.