Ionoregulatory Aspects of the Osmorespiratory Compromise during Acute Environmental Hypoxia in 12 Tropical and Temperate Teleosts

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ABSTRACT

In the traditional osmorespiratory compromise, as seen in the hypoxia-intolerant freshwater rainbow trout (*Oncorhynchus mykiss*), the branchial modifications that occur to improve O_2 uptake during hypoxia result in unfavorable increases in the fluxes of ions and water. However, at least one hypoxia-tolerant freshwater species, the Amazonian oscar (*Astronotus ocellatus*), shows exactly the opposite: decreased branchial flux rates of ions, water, and nitrogenous wastes during acute hypoxia. In order to find out whether the two strategies were widespread, we used a standard 2-h normoxia, 2-h hypoxia (20%–30% saturation), 2-h normoxic recovery protocol to survey 10 other phylogenetically diverse tropical and temperate species. Unidirectional influx and efflux rates of Na^+ and net flux rates of K^+, ammonia, and urea-N were measured. The flux reduction strategy was seen only in one additional species, the Amazonian tambaqui (*Colossoma macropomum*), which is similarly hypoxia tolerant and lives in the same ion-poor waters as the oscar. However, five other species exhibited evidence of the increased flux rates typical of the traditional osmorespiratory compromise in the trout: the rosaceu tetra (*Hyphessobrycon bentosi rosaceus*), the moenkhausia tetra (*Moenkhausia diktyota*), the bluegill sunfish (*Lepomis macrochirus*), the zebra fish (*Danio rerio*), and the goldfish (*Carassius auratus*). Four other species exhibited no marked flux changes during hypoxia: the cardinal tetra (*Paracheirodon axelrodi*), the hemigrammus tetra (*Hemigrammus rhodostomus*), the pumpkinseed sunfish (*Lepomis gibbosus*), and the Atlantic killifish (*Fundulus heteroclitus*). Overall, a diversity of strategies exist; we speculate that these may be linked to differences in habitat and/or lifestyle.

Keywords: trout, oscars, sodium fluxes, ammonia fluxes, P_{50}.

Introduction

In circumstances where O_2 uptake from the aquatic environment becomes difficult or restricted (e.g., aquatic hypoxia) or where metabolic O_2 demand increases (e.g., exercise), teleost fish can implement complex branchial alterations that increase the functional surface area of the gill and/or decrease the mean blood-to-water diffusion distance. These changes have been well studied in the freshwater rainbow trout (*Oncorhynchus mykiss*), a highly aerobic, hypoxia-intolerant salmonid. They include increases in the rates and alterations in the patterns of branchial blood and water flow (Holeton and Randall 1967; Stevens and Randall 1967; Booth 1979; Soivio and Tuurula 1981; Sundin and Nilsson 1997) that elevate gill O_2 transfer factor during both hypoxia and exercise (Randall et al. 1967). However, as a consequence of these changes, branchial ion losses from the fish to the hypotonic environment (as well as osmotic water uptake) also increase—a phenomenon that has been termed the "osmorespiratory compromise" (Randall et al. 1972; Nilsson 1986). In salmonids, this phenomenon has been well documented during exercise, with some studies showing increases in both unidirectional Na^+ influx and efflux rates, as well as greater net Na^+ loss rates and water fluxes (Stevens 1972; Wood and Randall 1973*a*, 1973*b*; Hofmann and Butler 1979; Gonzalez and McDonald 1992, 1994; Postlethwaite and McDonald 1995). The phenomenon has been less extensively studied during hypoxia in salmonids but with evidence again for increased Na^+ and K^+ loss rates (Thomas et al. 1986; Iftikar et al. 2010).

In contrast, recent studies on the oscar (*Astronotus ocellatus*), an extremely hypoxia-tolerant cichlid native to the Brazilian Amazon, suggest that at least one other species may show a very different response (Richards et al. 2007; Wood et al. 2007, 2009; Scott et al. 2008; DeBoeck et al. 2013)—that is, the osmorespiratory compromise seen in trout may not be representative of all teleosts. Indeed, during acute environmental hypoxia, the oscar is able to increase its branchial O_2 transfer factor (Scott et al. 2008) like the trout (Randall et al. 1967) yet at the same time reduces both passive Na^+ efflux and active Na^+ influx rates at the gills. Ammonia, urea-N, and K^+ loss rates and water fluxes

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are also decreased, suggesting that a general reduction in gill permeability occurs (Wood et al. 2007, 2009; DeBoeck et al. 2013). This is presumably adaptive to the frequent hypoxia that occurs in its ion-poor environment.

Based on these two very different species, there appear to be two different patterns to the osmorespiratory compromise during acute hypoxia. While both species can promote O_2 uptake during hypoxia, the oscar controls ionoregulation and reduces gill ion fluxes, while the trout sacrifices ionoregulation, allowing gill ion fluxes to increase. Given the paucity of species studied to date, this raises the question as to whether these two strategies are widespread in different species of fish and whether intermediate patterns also occur.

With this background in mind, a normoxia-hypoxia-normoxia recovery screening protocol was devised to help rapidly identify the two strategies, using the rainbow trout (order Salmoniformes) and oscar (order Perciformes) as validation organisms. The approach involved radioisotopic measurements of the unidirectional flux rates of Na^+, as well as the net flux rates of Na^+, K^+, ammonia, and urea-N. The latter three have been used as indicators of gill transcellular permeability in recent studies on these two species (Wood et al. 2007, 2009; Iftikar et al. 2010; Deboeck et al. 2013). The protocol was then applied to five other Brazilian species (all members of the order Characiformes) that live in habitats similar to that of the oscar and that were available opportunistically on two research trips to the Rio Negro region of the Amazon basin. The protocol was also applied to five other tropical- and temperate-zone species, covering a wide range of natural habitats, hypoxia tolerances, and phylogenies (members of the orders Perciformes, Cyprinodontiformes, and Cypriniformes). To help interpret these results, we compiled additional available information on respiratory parameters (partial pressure of oxygen [P_{O_2}] at which the O_2 uptake rate becomes dependent on environmental P_{O_2} [P_{crit}] and P_{O_2} at which the blood is 50% saturated with O_2 [blood P_{50}]) in these 12 species.

Material and Methods

Experimental Animals in Canada

In Canada, all fish were held at McMaster University at ~18°C in dechlorinated Hamilton tap water (moderately hard: $[Na^+] = 0.6$ mM, $[Cl^-] = 0.8$ mM, $[Ca^{2+}] = 0.9$ mM, $[Mg^{2+}] = 0.15$ mM, $[K^+] = 0.05$ mM; pH ~8.0) under a 12L : 12D daily photoperiod and fasted at least 24 h before experimentation. All experiments were performed under the same water quality conditions. All procedures were approved by the McMaster University Animal Research Ethics Board and were in accordance with the Guidelines of the Canadian Council on Animal Care.

Rainbow Trout

Juvenile rainbow trout (*Oncorhynchus mykiss*; 20–30 g, ~12 cm) were obtained from Humber Springs trout farm (Orangeville, Ontario) and held for a minimum of 1 wk before use, at ~18°C in 500-L flow-through tanks containing Hamilton dechlorinated tap water. Fish were fed every other day with a 10% body mass ration of five-point trout pellets (Martin Mills, Elmira, Ontario).

Pumpkinseed and Bluegill Sunfish

Mature pumpkinseed (*Lepomis gibbosus*) and bluegill sunfish (*Lepomis macrochirus*; 70–90 g, 15–20 cm) were caught by angling from Lake Opinicon, Ontario, and held under the same lab conditions as trout. They were fed a combination of blackworms (*Lumbriculus variegatus*), beef liver, and squid daily at a 2%–3% body mass ration.

Goldfish

Mature *Carassius auratus* (15–25 g, 15–20 cm) were obtained from AQUAlity Tropical Fish Wholesale (Mississauga, Ontario) and held under the same lab conditions as the previous species. Goldfish were fed ad lib. daily with nutrient flakes manufactured by Big Al's Aquarium Supercentres (Woodbridge, Ontario).

Killifish

Mature *Fundulus heteroclitus* (2.5–3.5 g) were collected from tidal flats in New Hampshire by Aquatic Research Organisms (Hampton, New Hampshire) and initially held in 30% seawater. At McMaster University, they were acclimated to freshwater (dechlorinated Hamilton tap water) for 3 wk in aerated 40-L buckets. Water was changed daily, and fish were fed ad lib. daily with nutrient flakes (Big Al's Aquarium Supercentres).

Zebra Fish

Danio rerio (0.3–0.4 g) were obtained from AQUAlity Tropical Fish Wholesale and held in 38-L aquaria with recirculating charcoal-filtered Hamilton tap water at ~18°C. Fish were fed ad lib. daily with nutrient flakes (Big Al's Aquarium Supercentres).

Experimental Animals in Brazil

In Brazil, all fish were held under a natural 12L : 12D daily photoperiod in typical Amazonian soft water either at the Ecophysiology and Molecular Evolution Laboratory of the Instituto Nacional de Pesquisas da Amazônia (INPA) in Manaus or on board a research vessel (the *Ana Clara*, from Manaus) moored in the Rio Negro approximately 50 km northeast of Barcelos. Experiments were performed at either INPA or on the *Ana Clara* in the same respective water qualities (which differed between the two sites). All procedures were in compliance with Brazilian national and INPA animal care regulations.

Amazonian Oscar, Tambaqui, and Rosaceu Tetra

Juvenile Amazonian oscars (*Astronotus ocellatus*; 20–30 g, ~12 cm) and tambaqui (*Colossoma macropomum*; 10–25 g, ~10 cm) were

obtained from Sítio dos Rodrigues (Km 35, Rod.AM-010, Brazil) and moved to INPA for at least 2 wk before use. Mature rosaceu tetra (*Hyphessobrycon bentosi rosaceus*; 0.5–0.8 g) were wild caught from the Rio Negro and maintained under lab conditions at INPA for at least 1 mo before use. Oscar and tambaqui were held in 500-L flow-through tanks and rosaceu in 50-L non-flow-through tanks, at ~28°C. The holding and experimental water was typical Amazonian soft water obtained from a well on INPA property ($[Na^+] = 35\ \mu M$, $[Cl^-] = 36\ \mu M$, $[Ca^{2+}] = 18\ \mu M$, $[Mg^{2+}] = 4\ \mu M$, $[K^+] = 16\ \mu M$; pH 6.5). Fish were fed daily with commercial pellets (Nutripeixe Tr 36, Purina, São Paulo, SP, Brazil) and fasted at least 24 h before experimentation.

Cardinal, Hemigrammus, and Moenkhausia Tetra

Experiments were performed during a research expedition on board the *Ana Clara* moored in the Rio Negro approximately 50 km northeast of Barcelos, Amazonas, Brazil. Cardinal tetra (*Paracheirodon axelrodi*), hemigrammus (*Hemigrammus rhodostomus*), and moenkhausia (*Moenkhausia diktyota*) of approximately 0.3–0.5 g were collected locally using baited minnow traps and held for 3–7 d in large aerated tanks. The holding and experimental water was pumped directly from the Rio Negro ($[Na^+] = 20\ \mu M$, $[Cl^-] = 20\ \mu M$, $[Ca^{2+}] = 10\ \mu M$, $Mg = 5\ \mu M$, $[K^+] = 10\ \mu M$; pH 4.5). After collection, the fish were not fed, so the fasting period was 3–7 d before experimentation. In other experiments directed at Na^+ and ammonia transport mechanisms (Wood et al. 2014), all parameters remained stable after 3 d of fasting. On the research vessel, fish were exposed to ambient conditions—a natural photoperiod of 12L:12D and water temperature ~30°–35°C.

Experimental Design

A 2-h normoxia, 2-h acute hypoxia, 2-h normoxic recovery protocol was employed, in which the unidirectional Na^+ influx rate was measured radioisotopically with ^{22}Na and Na^+ net flux rates were also measured, allowing calculation of unidirectional Na^+ efflux rates by difference. The simultaneous net flux rates of K^+, ammonia, and urea-N were also determined. In Brazil, ^{22}Na (as NaCl) manufactured by New England Nuclear (Dupont, Boston, MA) was obtained from REM (São Paulo) and in Canada from Eckert and Ziegler Isotope Products (Valencia, CA).

All fish were transferred to plastic containers served with capillary aeration devices and allowed to settle for 30 min before experimentation. Wild-caught tetra (*Paracheirodon axelrodi*, *Hemigrammus rhodostomus*, and *Moenkhausia diktyota*) were held at ~30°–35°C in 60 mL of Rio Negro water; rosaceu tetra (*Hyphessobrycon bentosi rosaceus*), Amazonian oscar (*Astronotus ocellatus*), and tambaqui (*Colossoma macropomum*) were held at ~28°C in 60 mL (rosaceu) and 500 mL (oscar and tambaqui) of INPA well water. Rainbow trout (*Oncorhynchus mykiss*), sunfish (*Lepomis gibbosus* and *Lepomis macrochirus*), and goldfish (*Carassius auratus*) were held at ~18°C in 1,000 mL of dechlorinated Hamilton tap water. Killifish (*Fundulus heteroclitus*) were held at ~18°C in 400 mL of dechlorinated Hamilton tap water, and zebra fish (*Danio rerio*) were held at ~18°C in 50 mL of the same water.

The waters were dosed with ^{22}Na (typically 1 μCi of ^{22}Na per 100 mL) and allowed to mix for 15 min, after which a 2-h normoxic flux period (O_2 saturation >85%, ~135 torr) was started and hourly water samples (5 mL) were taken. The holding water was then changed with minimal disturbance to the fish and dosed with ^{22}Na again, and nitrogen was bubbled through to deoxygenate it to the required level. After a 15-min mixing period, the 2-h hypoxic flux period started, again with hourly water sampling. The hypoxia level was 20%–30% O_2 saturation (32–48 torr) for all species except the rainbow trout, and this level could be easily maintained simply by turning the N_2 gassing on and off periodically. Some trout died in initial trials at 30% O_2, so instead they were brought to 45% O_2 saturation (~72 torr) for the hypoxic period. However, trout consumed oxygen so rapidly that intermittent aeration was necessary every half hour to raise saturation back to 45%, so average saturation over the entire 2-h period was actually ~35% (~56 torr). Finally, after the hypoxic period, a 2-h normoxic recovery period was instituted; water was again changed with minimal disturbance, dosed with ^{22}Na, and then maintained at normoxic levels (O_2 saturation >85%, ~135 torr) with continual aeration. After a 15-min mixing period, the 2-h normoxic recovery flux period was begun, with hourly water sampling.

Water Analyses

In Canada, water oxygen partial pressure (P_{O_2}) was measured using a Clarke-type oxygen electrode (Cameron Instruments, Port Aransas, TX) connected to a 1900 Polarographic amplifier (A-M Systems, Carlsborg, WA) and in Brazil using a WTW Oxi325 Oximeter (WTW, Weilheim, Germany). Water samples taken in every trial were measured for ^{22}Na. In Brazil, this was done by scintillation counting of subsamples using Ultima Gold scintillation fluid (Perkin-Elmer, Waltham, MA; 5 mL fluor:5 mL water) on either a Triathler portable counter (Hidex, Helsinki, Finland) for the experiments on the research vessel *Ana Clara* or a Beckman LS6500 counter (Beckman Coulter, Fullerton, CA) for experiments at the INPA laboratory. Tests showed that quenching was constant so correction was unnecessary. In Canada, analysis was done by gamma counting using a Perkin-Elmer Wizard 1480 3-inch Auto Gamma Counter (Perkin-Elmer, Shelton, CT). Separate subsamples were also measured for total ion concentrations (Na^+, K^+), as well as ammonia and urea-N as detailed below.

In Brazil, Na^+ and K^+ concentrations were measured by atomic absorption spectrophotometry using an AAnalyst 800 (PerkinElmer, Singapore) for experiments performed at the INPA laboratory, whereas on the research vessel *Ana Clara*, Na^+ and K^+ concentrations were measured by flame photometry, using a 910 Digital Flame Photometer (Instrumentação Analítica, São Paulo). In Canada, Na^+ and K^+ concentrations were measured via atomic absorption spectrophotometry using

a SpectrAA 220FS AAS (Varian Canada, Missisauga, Ontario). Water total ammonia and urea-N concentrations were measured colorimetrically by the salicylate hypochlorite assay (Verdouw et al. 1978) and the diacetyl monoxime assay (Rahmatullah and Boyde 1980), respectively.

Calculations

As hourly water samples were taken throughout the three experimental periods (control, hypoxia, and recovery), flux rates were available for the first hour as well as the second. There were no systematic differences; therefore, values displayed in "Results" are the average of these two 1-h flux rates, for each period.

Na influx was calculated based on ^{22}Na disappearance from water as

$$J_{\text{Na in}} = \frac{\Delta\text{cpm} \times V}{\text{average SA} \times M \times \Delta t}, \quad (1)$$

where V is the effective volume of the container (mL), M is the weight of the fish (g), and Δt is the duration of the flux period (h). Average specific activity (SA) was determined by averaging initial and final specific activities, the mean ratio of ^{22}Na cpm concentration to total water Na^+ concentration over that time period.

Net Na^+ flux was calculated from the change in the total concentration of Na^+ in the water as measured by atomic absorption or flame photometry:

$$J_{\text{Na net}} = \frac{\Delta\text{Na} \times V}{M \times \Delta t}, \quad (2)$$

and Na^+ efflux was calculated by difference as

$$J_{\text{Na out}} = J_{\text{Na net}} - J_{\text{Na in}}. \quad (3)$$

Net fluxes for K, ammonia, and urea-N were all calculated by analogy to equation (2).

Statistics

Data have been expressed as the means ± 1 SEM (N). All statistical tests were performed using the program Sigma Plot 10.1 with Sigma Stat Integration 3.0. Figure legends denote the specific statistical test performed in each case, but in general, one-way repeated-measures ANOVA, followed by a Holm-Sidak post hoc test, was used for multiple comparisons since the same fish were monitored continuously throughout all three experimental periods. If the normality test failed, a Kruskal-Wallis ANOVA on ranks analysis was executed before a Tukey post hoc test. In all cases, differences were considered statistically significant at $P \leq 0.05$.

Results

In figures 1–4, the responses for each flux parameter are shown separately for each species, with the oscar responses on the left and the trout responses on the right (or middle in fig. 1) as points of reference. In figure 1, note that it was necessary to use a different Y-axis to encompass the data for the three species on the right. The hypoxia level used in the final trout tests was ~35% (~56 torr), as the fish were intolerant of a more severe level.

Na^+ Influx Rates (Fig. 1A)

Oscars exhibited a significant decrease in Na^+ influx rate of about 30% during hypoxia, with recovery on return to normoxia. In contrast, the trout showed the opposite, a significant increase by more than twofold during hypoxia, with partial recovery in the following normoxic period. The tambaqui was the only species to show a significant decrease in Na^+ influx rate similar to the oscar, and this decrease persisted during the normoxic recovery period. However, several species exhibited a significant increase similar to the trout: rosaceu, bluegill, and zebra fish. The zebra fish demonstrated a particularly large threefold increase. In all cases, there was partial or complete restoration of the control rate during the normoxic recovery period. Cardinal tetra, hemigrammus, moenkhausia, pumpkinseed, killifish, and goldfish showed no significant changes in Na^+ influx rate during hypoxia.

Na^+ Efflux Rates (Fig. 1B)

Oscars displayed a significant drop in Na^+ efflux rate during hypoxia, with recovery during normoxia, mirroring the changes in their Na^+ influx rate (see fig. 1*A*). As with the latter, this contrasted with the trout responses, which were exactly the opposite. Again, the tambaqui was the only species to show a response similar to that of the oscar, a significant decrease in Na^+ efflux rate during hypoxia. The effect persisted during the normoxic recovery period. In general, the same species that had shown the trout pattern of increased Na^+ influx rate during hypoxia and/or decreased Na^+ influx rate during normoxic recovery also exhibited similar significant responses in Na^+ efflux rates. These included rosaceu, bluegill, zebra fish, and moenkhausia. There were no significant changes in cardinal tetra, hemigrammus, pumpkinseed, killifish, and goldfish.

Na^+ Net Flux Rates (Fig. 1C)

Oscars maintained a constant net Na^+ flux rate during hypoxia, as changes in Na^+ influx and efflux rates were identical (see fig. 1*A*). In trout, the negative Na^+ flux rate during hypoxia was significantly attenuated during normoxic recovery. Like the oscar, the tambaqui maintained an unchanged Na^+ net flux rate during hypoxia and normoxic recovery, despite the significant changes in unidirectional influx and efflux rates (see fig. 1*A*, 1*B*). Na^+ net loss rates followed the trout pattern only in rosaceu and moenkhausia, though similar nonsignificant changes were apparent in zebra fish and goldfish. Na^+ net flux remained unchanged during hypoxia in cardinal tetra, hemigrammus,

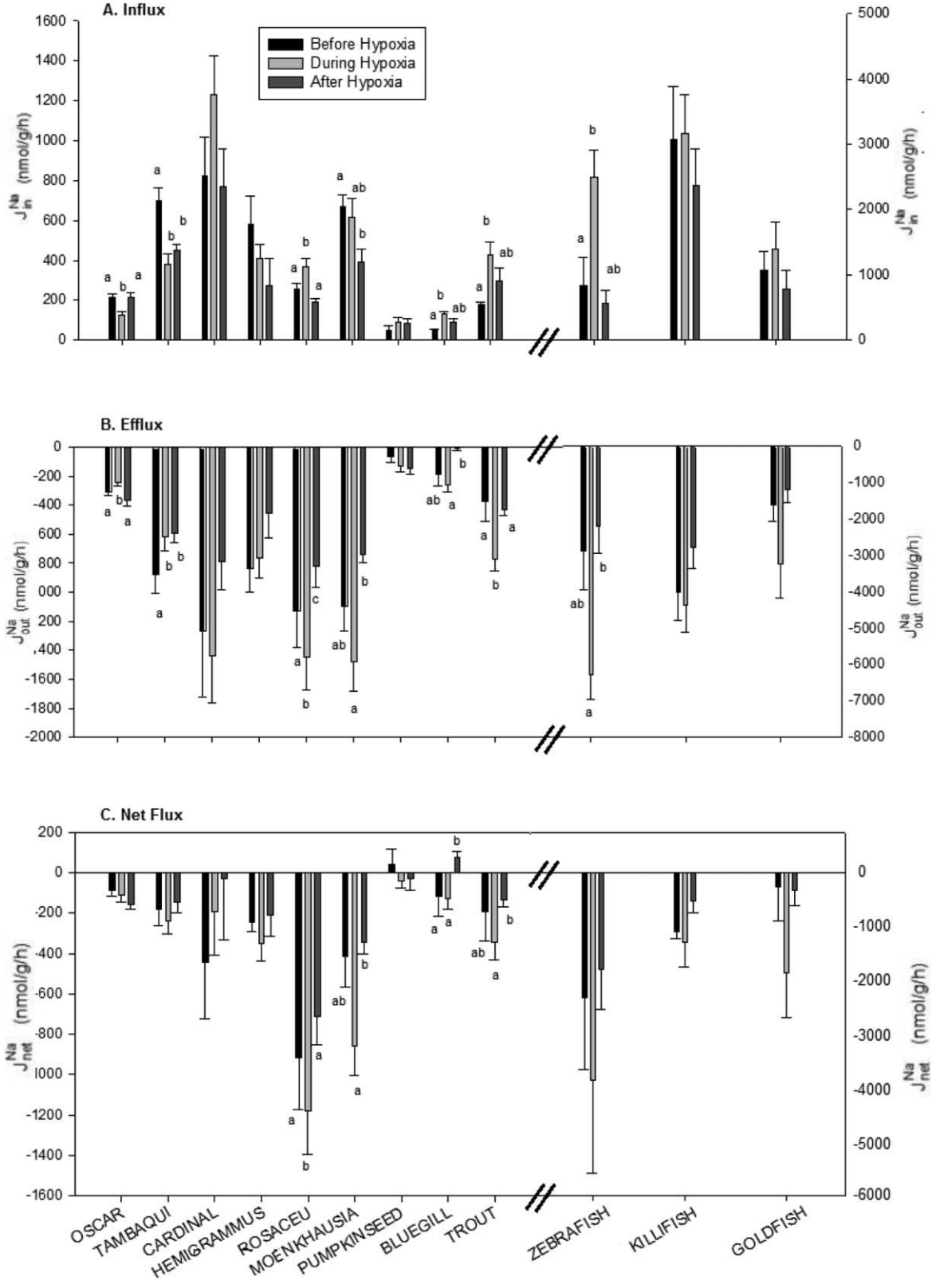

Figure 1. Changes in unidirectional Na^+ influx rates (*A*), unidirectional Na^+ efflux rates (*B*), and net Na^+ flux rates (*C*) during a 2-h normoxia, 2-h hypoxia, 2-h normoxia recovery protocol in 12 species of teleost fish. Means + SEM ($N = 5–12$) are shown. Within a species, means sharing the same letter are not significantly different ($P > 0.05$).

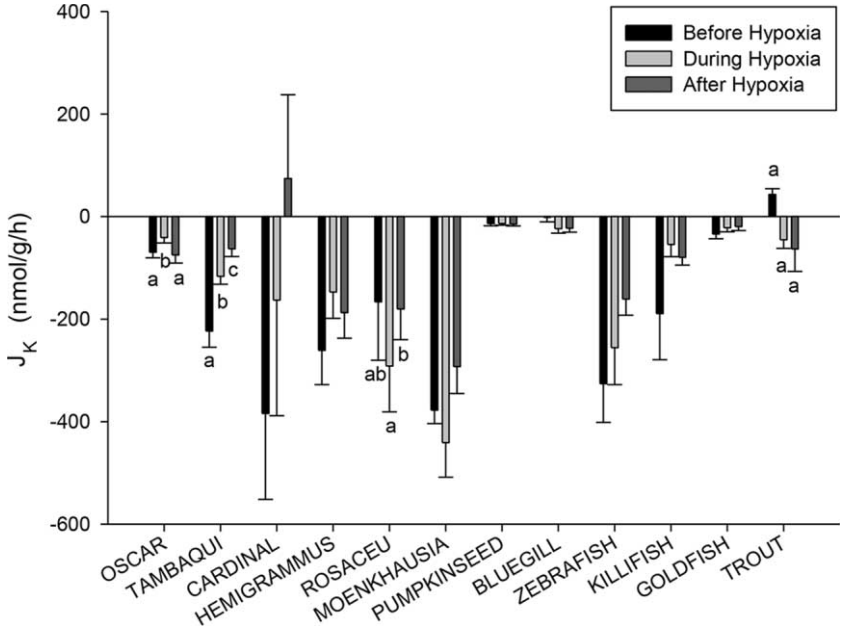

Figure 2. Changes in K^+ net flux rates during a 2-h normoxia, 2-h hypoxia, 2-h normoxia recovery protocol in 12 species of teleost fish. Positive values represent uptake by the fish; negative values represent loss by the fish. Means + SEM ($N = 5–12$) are shown. Within a species, means sharing the same letter are not significantly different ($P > 0.05$).

pumpkinseed, killifish, and bluegill, though it did become positive in the latter during the normoxic recovery period.

K^+ Net Flux Rates (Fig. 2)

In oscars, net loss rates of K^+ decreased significantly during hypoxia and returned to control levels on return to normoxia. In tambaqui, the net loss rate of K^+ decreased significantly during hypoxia and even further during normoxic recovery. The decrease during hypoxia was similar to the response of the oscar. The persistence of this effect during recovery was similar to that seen for Na^+ influx and efflux rates (see fig. 1*A*, 1*B*). The only other significant changes were seen in the rosaceu, with a pattern of increased loss during hypoxia and recovery there-

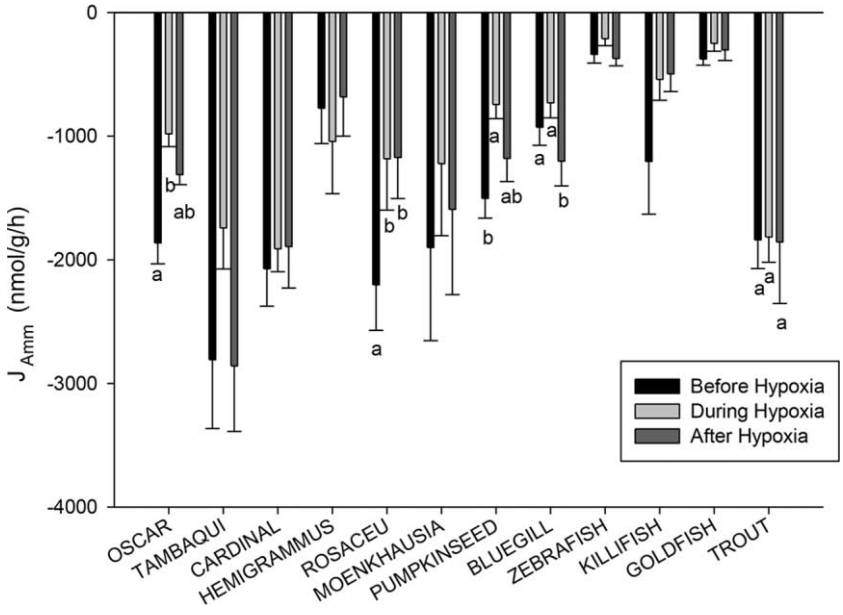

Figure 3. Changes in total ammonia excretion rates during a 2-h normoxia, 2-h hypoxia, 2-h normoxia recovery protocol in 12 species of teleost fish. Means + SEM ($N = 5–12$) are shown. Within a species, means sharing the same letter are not significantly different ($P > 0.05$).

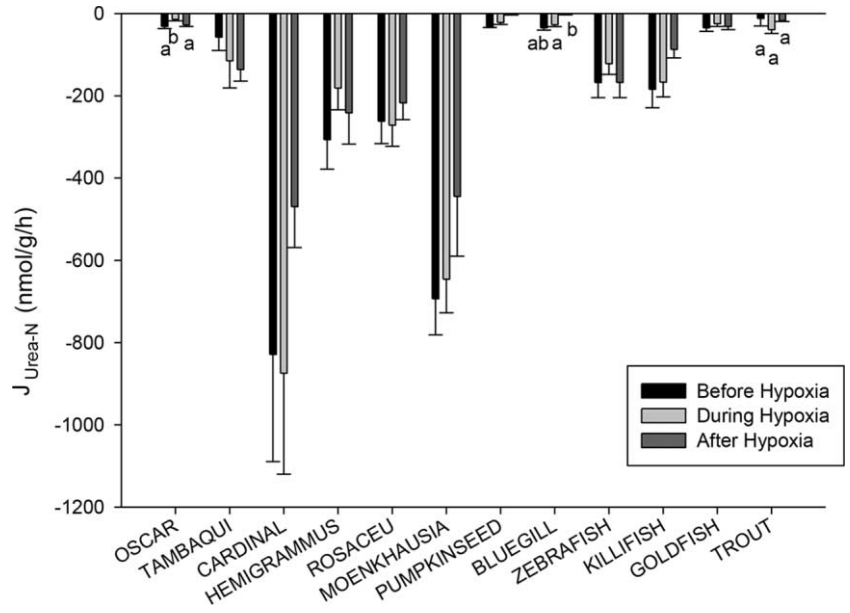

Figure 4. Changes in urea-N excretion rates during a 2-h normoxia, 2-h hypoxia, 2-h normoxia recovery protocol in 12 species of teleost fish. Means + SEM ($N = 5–12$) are shown. Within a species, means sharing the same letter are not significantly different ($P > 0.05$).

after. There were no clear K^+ net flux responses in the other species.

Ammonia Net Flux Rates (Fig. 3)

Oscars exhibited a significant 50% decrease in ammonia excretion rate during hypoxia, with partial recovery thereafter, whereas the trout maintained a constant ammonia excretion rate throughout all three periods. Ammonia excretion decreased significantly during hypoxia in rosaceu and pumpkinseed, with a similar recovery response in bluegill. There were no significant changes in the other species.

Urea-N Efflux Rates (Fig. 4)

Oscars significantly reduced net excretion rates of urea-N by about 50% during hypoxia, with recovery thereafter. In the other species, urea-N efflux rates were generally unresponsive to hypoxia, with none exhibiting the significant decrease during hypoxia seen in the oscar. Indeed, the only other significant change was a reduction in urea-N excretion rate during normoxic recovery in the bluegill.

Discussion

Validation of the Protocol

Clearly, the 2-h normoxia, 2-h hypoxia, 2-h normoxia recovery screening protocol was successful in identifying the very different osmorespiratory response patterns of oscars versus trout, as outlined below, thereby validating this as a rapid screening protocol for different patterns of osmorespiratory compromise. However, virtually all of the species exhibited negative net Na^+ flux rates under control conditions (fig. 1*C*), and for most, these net loss rates were higher than in trout or oscars. This may have been due to experimental stress. The trout and oscars were obtained from aquaculture, and this domestication may have rendered them less susceptible to handling stress. Most of the other species were collected from the wild. While net ion balance as an indicator of a nonstressed condition may be seen in long-term laboratory acclimation studies with fasted fish, this may not be a valid criterion for wild fish collected from their natural environment. These circumstances are artificial and not representative of the real world, where the fish are eating and swimming in natural water. There is an increasing body of evidence that in the wild, fish normally obtain a substantial amount of their ions from food (reviewed in Wood and Bucking 2011). When food is not present during flux measurements, it is not surprising that net balance is negative. This is probably especially true in the Rio Negro species such as the various tetra species studied here that live in such ion-poor, acidic waters and that are voracious eaters.

Different Mechanisms of Osmorespiratory Compromise

The two reference species showed characteristically different responses to hypoxia. Oscars exhibited all of the decreased flux responses during hypoxia that have been reported in previous studies using a variety of hypoxia exposure regimes (Wood et al. 2007, 2009; De Boeck et al. 2013). These include decreased Na^+ influx (fig. 1*A*), Na^+ efflux (fig. 1*B*), and net loss rates of K^+ (fig. 2), ammonia (fig. 3), and urea-N (fig. 4) but unchanged Na^+ net flux rate (fig. 1*C*), with recovery thereafter. The trout exhibited the same increased flux responses dur-

ing hypoxia that have been reported previously (Thomas et al. 1986; Iftikar et al. 2010). These include increases in Na^+ influx (fig. 1*A*), Na^+ efflux (fig. 1*B*), and Na^+ net loss rates (fig. 1*C*), as well as a tendency for greater net K^+ loss rates (fig. 2). Notably, neither Iftikar et al. (2010) nor this study reported any change in ammonia excretion rates during hypoxia (fig. 3), whereas we are aware of no previous trout studies against which to compare the nonsignificant rise in urea-N excretion during hypoxia seen in this study (fig. 4).

Until now, the reduction in gill fluxes during hypoxia was unique to the oscar, but this study shows that it occurs in at least one additional species, the tambaqui. Indeed, for Na^+ influx (fig. 1*A*), Na^+ efflux (fig. 1*B*), and K^+ net flux (fig. 2) rates, the response persisted during recovery in tambaqui, an effect that has been seen in previous studies on the oscar (Wood et al. 2007, 2009), indicating that it is a regulated phenomenon. On the other hand, several other species clearly exhibited the trout pattern of increased fluxes during hypoxia; these include the rosaceu, bluegill, and zebra fish, while moenkhausia and goldfish tended nonsignificantly toward this pattern.

Four species (pumpkinseed, killifish, cardinal tetra, and hemigrammus tetra) exhibited negligible ionoregulatory responses to hypoxia (figs. 1, 2). There would appear to be two possible explanations: either that this is truly a third pattern with animals able to maintain unchanged flux rates during hypoxia or that the level of hypoxia used in this study (20%–30% saturation) was not severe enough to elicit a distinctive response pattern. These possibilities are amenable to future experimental test.

How is it possible that both oscars and trout increase gill O_2 transfer factor (Randall et al. 1967; Scott et al. 2008) during acute hypoxia yet show opposite ionoregulatory and osmoregulatory flux changes during acute hypoxia? Morphometric studies have indicated that these very different osmorespiratory compromises in oscar versus trout can be explained by differences in the morphological responses of gill pavement cells (PVCs) and mitochondria-rich cells (MRCs). While MRCs are generally recognized as the sites of active ion uptake, the major sites of diffusive effluxes are often thought to be the PVCs because of their much larger area, but this idea is an assumption only, without support from specific unidirectional flux data. However, in the oscar, diffusive unidirectional Na^+ effluxes, measured radioisotopically, decrease during hypoxia (Wood et al. 2007, 2009; DeBoeck et al. 2013), yet PVC surface area gets larger and MRC surface area gets smaller (Wood et al. 2009; Matey et al. 2011). The obvious conclusion is that the MRCs are the major site of diffusive loss. Conversely, in the rainbow trout, diffusive unidirectional Na^+ effluxes, measured radioisotopically, increase during hypoxia (Iftikar et al. 2010), yet PVC surface area gets smaller and MRC surface area gets larger (Matey et al. 2011). Again, the obvious conclusion is that the MRCs are the major site of diffusive loss. Therefore, in this model, the MRCs are the putative sites of both active uptake and passive diffusive losses. In trout, the PVCs retract during hypoxia, increasing the apical exposure of the MRCs that bulge outward so that unidirectional and net ion fluxes increase. In contrast, in oscars, the PVCs extend in area, thereby covering the MRCs and reducing their apical exposure so that unidirectional and net ion fluxes decrease. Thus, by this interpretation, transcellular permeability changes in opposite directions due to opposite effects on channel and transporter exposure in the MRCs of the two species (Matey et al. 2011), while parallel adjustments in effective exchange area, diffusion distance, and blood flow pattern can still promote O_2 uptake in both. In oscar, branchial Na^+,K^+ ATPase activity is also downregulated during hypoxia (Richards et al. 2007; Wood et al. 2007), though this effect occurs more slowly than the rapid morphological and flux changes. With respect to other species exhibiting oscar-type responses (tambaqui) or trout-type responses (rosaceu, bluegill, zebra fish, moenkhausia, goldfish) during hypoxia, morphometric and enzymatic studies will be required to determine whether the cellular changes are the same as in oscars and trout, respectively. An additional cautionary note is that overall PVC area is much larger than MRC area, so it remains possible that the majority of flux changes could occur via the PVCs or through specific permeability changes unrelated to morphological responses.

Findings using the scanning ion-selective electrode technique (SIET) applied to larval fish skin have identified keratinocytes, which are often considered analogous in morphology and function to gill PVCs in adult fish, as the sites of net ion losses (e.g., Horng et al. 2009; Wu et al. 2010). However, these electrophysiological data on skin do not conflict with our model whereby MRCs are simultaneously the major sites of active ion uptake and passive diffusive ion losses in fish gills. SIET measures only net fluxes, whereas radioisotopes are needed to separate the unidirectional fluxes of active uptake and passive diffusive efflux.

In oscars, this conclusion that the responses are transcellular is reinforced by the fact that gill permeability to polyethylene glycol-4000 (PEG-4000, a classic paracellular permeability marker) did not change during acute hypoxia (Wood et al. 2009). In trout, gill permeability to PEG-4000 has not been measured during hypoxia, but it did increase during sustained exercise (Robertson and Wood 2014). Thus, it is possible that increases in both paracellular and transcellular permeability occur during hypoxia and exercise in this species. Branchial PEG-4000 permeability has not been assessed during exercise or hypoxia in any of the other species studied.

With respect to other moieties, K^+ flux was originally attributed to a simple diffusive loss from the gill cells via the transcellular pathway (McDonald et al. 1986; Wood et al. 2009; Iftikar et al. 2010; Deboeck et al. 2013), though recent evidence suggests that active efflux may occur through the MRCs, at least in seawater teleosts (Furukawa et al. 2012). Regardless, the covering (in oscars) or uncovering (in trout) of MRCs by PVCs during hypoxia would explain the observed differences, and the same may be true of the other species that exhibited these differing response patterns (fig. 2). Urea-N and ammonia excretion were also reduced in the oscars during hypoxia, but in the tambaqui, this pattern occurred only for ammonia and not for urea-N (figs. 3, 4). Indeed, urea-N fluxes were unresponsive to hypoxia in most of the species (fig. 4). Furthermore, several species that exhibited trout-like patterns for Na^+ and K^+ fluxes

(rosaceu, bluegill) actually exhibited oscar-like patterns for ammonia excretion, whereas in trout, ammonia flux remained unchanged (fig. 3).

Interpretation of ammonia and urea-N fluxes is confounded by the fact that metabolic production rates of these waste products may change during hypoxia, so simultaneous measurements of plasma levels would be informative (e.g., Wood et al. 2007). Recent evidence suggests that a substantial portion, though not necessarily all, of these N-waste fluxes occur via Rh (Wright and Wood 2012) and UT (McDonald et al. 2012) proteins, which facilitate diffusion through the transcellular pathway in the gills. A further complication is that this pathway may involve the MRCs, the PVCs, or both, depending on species; for example, in trout, Rh proteins dominate in the PVCs rather than in the MRCs (Nawata et al. 2007), which may explain the lack of change in ammonia excretion during hypoxia in this species.

Variation in Response Patterns among Species

At the outset, we acknowledge that our primary goal in this study was simply to survey whether the two prominent response patterns to acute hypoxia exemplified by the oscar (decreased flux rates) and the rainbow trout (increased flux rates) occurred in other species or whether there were alternative patterns. Our selection of species was to a large extent opportunistic; the various species, most of which were wild, came from very different backgrounds, and no attempt was made to "common garden" them (see Garland and Adolph 1991). In addition to background, the species varied in body mass, phylogeny, habitat/lifestyle, hypoxia tolerance, and respiratory physiology, and at least some of these are independent variables. The difficulty of disentangling the role of these various factors in interspecies investigations, particularly in the absence of phylogenetically based statistical analyses, has been discussed in several reviews (e.g., Garland and Adolph 1994; Blomberg et al. 2003; Garland et al. 2005; Rezende and Diniz-Filho 2012). We therefore acknowledge that any post hoc explanations of the present data in terms of adaptive significance are highly speculative, because no formal tests were performed.

The data of figures 1–4 were examined to see whether there was any correlation between the response patterns to hypoxia and body mass among the 12 species (mean species values tabulated in table 1). While smaller fish generally had higher flux rates as expected from standard allometric theory, there were no significant relationships between body mass and the percentage changes in any of these fluxes during hypoxia challenge (all $P > 0.10$) using the various analyses suggested by White and Kearney (2014).

The oscar is a member of the Perciformes, whereas the tambaqui is a member of the Characiformes. Yet rosaceu and moenkhausia, with opposite responses to the tambaqui, are also both members of the Characiformes, while the goldfish, also showing an opposite response pattern, is a member of the Cypriniformes. Conversely, trout (Salmoniformes) and zebra fish (Cypriniformes) are phylogenetically distant yet share similar response patterns. A third group tolerates acute hypoxia with negligible changes in gill fluxes; this consists of largely unrelated species: pumpkinseed (Perciformes), killifish (Cyprinodontiformes), and cardinal and hemigrammus tetra (both Characiformes). Thus, there is no obvious phylogenetic signal in the response pattern data.

Oscars and tambaqui live in Amazonian blackwaters, which are not only frequently hypoxic but also very low in ions (Val and Almeida-Val 1995; Duncan and Fernandes 2010). Both have remarkable tolerance to hypoxia without resorting to air breathing, though the tambaqui does grow a lip for surface skimming if hypoxia is prolonged and oscars decrease their metabolic rates (Saint-Paul 1984; Rantin and Kalinin 1996; Araujo-Lima et al. 1997; Muusze et al. 1998; Almeida-Val et al. 2000; Chippari-Gomes et al. 2005; Florindo et al. 2006; Sloman et al. 2006; Lewis et al. 2007; Richards et al. 2007). However, the four other Brazilian species are all small tetras that are also endemic to the same ion-poor Amazonian blackwaters as inhabited by oscars and tambaqui. Rosaceu and moenkhausia exhibited the trout-like increased Na^+ flux pattern, while cardinals and hemigrammus exhibited unchanged fluxes during hypoxia. In contrast to oscars and tambaqui, which are known to invade hypoxic floodplains, tetras appear to prefer flowing waters; all were caught close to the shore in the main Rio Negro river. Their hypoxia tolerance has not been documented in the literature, but in our hands they appeared less tolerant of hypoxia than oscars or tambaqui.

The zebra fish is also a tropical species but endemic to streams in the Indian subcontinent, including ion-poor waters (Krisnaswami and Sarin 1984; Talwar and Jhingran 1991). They are both extremely active (e.g., McClelland et al. 2006) and extremely hypoxia tolerant (e.g., Rees et al. 2001), surviving at 10% O_2 saturation for at least 6 mo (van der Meer et al. 2005). The goldfish is a eurythermal species of East Asian origin that can thrive under tropical conditions; like the zebra fish, it is extremely tolerant of hypoxia (Fry et al. 1947). The killifish is endemic to estuaries of eastern North America and is both eurythermal and euryhaline; it, too, is generally considered to be extremely tolerant of hypoxia (Kneib 1986). Zebra fish and goldfish exhibited the same increased flux patterns as rainbow trout, whereas the killifish exhibited no flux response when all four species were tested under the same conditions. Trout are a highly aerobic species originating from cold-water streams in western North America and have some euryhaline capacity. They are one of the most poorly hypoxia-tolerant species (Davis 1975), generally succumbing within 24 h at about 30% O_2 saturation, based on data summarized in Doudoroff and Shumway (1970), in agreement with our preliminary trial (see "Material and Methods").

The conspecific perciforms (centrarchids) bluegill and pumpkinseed sunfish, endemic to lakes in eastern North America, are eurythermal and exhibit intermediate hypoxia tolerance according to the summary of Doudoroff and Shumway (1970). However, in preliminary experiments we found that bluegills were clearly less tolerant than pumpkinseeds, a finding supported by research comparing loss of equilibrium in declining O_2 concentrations (Farwell et al. 2007), as well as differences in activity and gene expression patterns of glycolytic and aerobic

Table 1: Values of P_{crit} and whole-blood P_{50} of the species investigated in this study, grouped according to flux response during hypoxia

Response	Species	P_{crit} (torr)	P_{50} (torr)	Species body mass (g)
Decreased	Oscar (*Astronotus ocellatus*)	27,[a] 70,[c] 74[d]	14[b]	20–30
Decreased	Tambaqui (*Colossoma macropomum*)	29,[e] 40[g]	13,[f] 8[h]	10–25
Increased	Rainbow trout (*Oncorhyncus mykiss*)	27,[i] 63,[k] 118[m]	31,[j] 25[l]	20–30
Increased	Goldfish (*Carassus auratus*)	29–34[n]	3,[o] 23[p]	15–25
Increased	Zebra fish (*Danio rerio*)	20[q]	20[r]	.3–.4
Increased	Bluegill sunfish (*Lepomis macrochirus*)	27,[s] 95,[t] 102,[u] 115[v]	…	70–90
Increased	Moenkhausia tetra (*Moenkhausia diktyota*)	…	22[w]	.3–.5
Increased	Rosaceu tetra (*Hyphessobrycon bentosi rosaceus*)	…	…	.5–.8
No change	Cardinal tetra (*Paracheirodon axelrodi*)	…	28[x]	.3–.5
No change	Hemigrammus tetra (*Hemigrammus rhodostomus*)	…	23[y]	.3–.5
No change	Atlantic killifish (*Fundulus heteroclitus*)	35,[z] 64,[B] oxyconformer[C]	4[A]	2.5–3.5
No change	Pumpkinseed sunfish (*Lepomis gibbosus*)	30,[D] 42[E]	…	70–90

Sources. a = De Boeck et al. 2013, 28°C; b = A. L. Val, unpublished data, 25°C; c = Sloman et al. 2006, 28°C; d = Scott et al. 2008, 28°C; e = L. M. Robertson, A. L. Val, V. F. Almeida-Val, and C. M. Wood, unpublished data, 28°C; f = A. L. Val, unpublished data, 32°C; g = Saint-Paul 1984, 30°C; h = Gonzalez et al. 2005, 28°C; i = Ott et al. 1980, 20°C; j = Eddy 1971, 20°C; k = McIntyre 2011, 15°C; l = Cameron 1971, 20°C; m = Marvin and Heath 1968, 12°C; n = Fry and Hart 1948, 15°–20°C; o = Burggren 1982, 26°C; p = A. L. Val, unpublished data, 25°C; q = Barrionuevo and Burggren 1999, 25°C; r = A. L. Val, unpublished data, 25°C; s = K. Crans and G. Scott, personal communication, unpublished data, 15°C; t = Spitzer et al. 1969, 25°C; u = L. M. Robertson, A. L. Val, V. F. Almeida-Val, and C. M. Wood, unpublished data, 18°C; v = Marvin and Heath 1968, 25°C; w = A. L. Val, unpublished data, 25°C; x = A. L. Val, unpublished data, 25°C; y = A. L. Val, unpublished data, 25°C; z = Cochran and Burnett 1996, 30°C; A = DiMichelle and Powers 1982, 10°C; B = Richards et al. 2008, 20°C; C = Blewett et al. 2013, 18°C; D = K. Crans and G. Scott, personal communication, unpublished data, 15°C; E = L. M. Robertson, A. L. Val, V. F. Almeida-Val, and C. M. Wood, unpublished data, 18°C.

enzymes among sunfish species (Davies et al. 2011). This lower tolerance of the bluegill is in accord with its trout-like increased Na^+ flux response to acute hypoxia and the general lack of flux response in the pumpkinseed.

Thus, the nature of the flux response during hypoxia exhibits no clear relationship with the hypoxia tolerance of the species. However, we speculate that what may be more important than the absolute hypoxia tolerance is the lifestyle of the species—for example, how often it must deal with hypoxia in its natural habitat or how often it encounters ion-poor water.

While the strategy of reducing gill transcellular permeability in response to hypoxia seems particularly useful for the lifestyle of oscar and tambaqui in ion-poor water, why do other freshwater species not do the same? Indeed, all freshwaters are extremely ion poor relative to blood plasma. It may be that the strategy to some degree limits the extent to which O_2 uptake can be promoted. McDonald and Gonzalez (1994) addressed this same question with respect to the osmoregulatory compromise during exercise in a range of fish species from diverse habitats. They concluded that while decreasing permeability to ions during exercise would be energetically beneficial, it could well compromise other gill functions such as acid-base regulation and N-waste excretion. The same may also apply to the osmorespiratory compromise during acute hypoxia, and, indeed, the observed decreases in ammonia excretion in oscar and tambaqui could be interpreted as evidence supporting this explanation.

Gonzalez and McDonald (1994) concluded that the ability to limit ion loss during exercise was related to the ability to raise O_2 consumption ($\dot{M}_{O_2}$) during exercise, such that species with high swimming $\dot{M}_{O_2}$ lost fewer ions per mole of O_2 uptake. In order to explore whether the nature of the osmorespiratory compromise during hypoxia is linked to the respiratory physiology of the species, available data for two commonly used indexes of respiratory performance under hypoxia (P_{crit}, blood P_{50}) have been tabulated for the 12 species in table 1. These include some of our own previously unpublished measurements on these species. An effort has been made to choose measurements made at temperatures as close as possible to those of this study. P_{crit} is the partial pressure of O_2 (P_{O_2}) at which O_2 uptake rate becomes dependent on environmental P_{O_2}, and P_{50} is the P_{O_2} at which the blood is 50% saturated with O_2; lower values of both are often thought to be associated with hypoxia tolerance. There were no significant relationships between either P_{crit} or P_{50} and body mass ($P > 0.10$). Overall, it is apparent that there is no clear relationship between the ionoregulatory response during hypoxia and P_{crit}, and this was confirmed by simple regression analyses of percentage changes in any of the fluxes against P_{crit} (all $P > 0.10$). However, for several species there are multiple reports of P_{crit}, and these are notable for their variability, indicating that the value obtained may be very dependent on physiological conditions such as feeding (e.g., DeBoeck et al. 2013) or on the methodology used and therefore not a useful comparator among different studies. Indeed, Scott et al. (2008, p. 114) concluded, "While the P_{crit} of oscar is at or above that of many

other fish, their hypoxia/anoxia tolerance is exceptional . . . which suggests that P_{crit} is not a reliable sole indicator of hypoxia tolerance in fishes."

For blood P_{50}, the two very low values in table 1 (3 torr for goldfish [Burggren 1982], 4 torr for killifish [DiMichelle and Powers 1982]) are included for completeness but should perhaps be disregarded, as they were obtained with very different methodology from the other data. Both the tambaqui and oscar have relatively low P_{50} and the trout a relatively high P_{50} (table 1), in accord with their different ionoregulatory responses to hypoxia. However, three other species that tended to increase ionic fluxes during hypoxia (goldfish, zebra fish, and moenkhausia) exhibit blood P_{50} in the same range as those that show no flux changes (table 1), and overall, there were no significant relationships ($P > 0.10$) between the percentage flux changes during hypoxia and P_{50}. Of course, other factors may come into play that further confound the comparisons such as the harder, more ion-rich freshwater and the lower temperature used for the Canadian species versus the Brazilian species.

Conclusions

Clearly, a diversity of strategies exists among the ionoregulatory responses of teleost fish to hypoxia. We speculate that these may be linked to differences in habitat and/or lifestyle. We further speculate that from an energetic point of view, reducing ionoregulatory costs at a time of severe energetic limitation may be adaptive for fish such as tambaqui and oscar that encounter frequent hypoxia in ion-poor waters. In contrast, for very active fish such as rainbow trout and zebra fish it may be more important to maximize O_2 uptake rather than to conserve ions. In future studies, it will be of interest to test whether the same interspecific differences seen during hypoxia also occur during exercise, the other condition that invokes the osmorespiratory compromise.

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