Am J Physiol Regul Integr Comp Physiol 292: R2048–R2058, 2007.
First published February 1, 2007; doi:10.1152/ajpregu.00640.2006.

Rapid regulation of Na^+ fluxes and ammonia excretion in response to acute environmental hypoxia in the Amazonian oscar, *Astronotus ocellatus*

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Submitted 12 September 2006; accepted in final form 28 January 2007

Wood CM, Kajimura M, Sloman KA, Scott GR, Walsh PJ, Almeida-Val VM, Val AL. Rapid regulation of Na^+ fluxes and ammonia excretion in response to acute environmental hypoxia in the Amazonian oscar, *Astronotus ocellatus. Am J Physiol Regul Integr Comp Physiol* 292: R2048–R2058, 2007. First published February 1, 2007; doi:10.1152/ajpregu.00640.2006.—The Amazonian oscar is extremely resistant to hypoxia, and tolerance scales with size. Overall, ionoregulatory responses of small (~15 g) and large oscars (~200 g) to hypoxia were qualitatively similar, but the latter were more effective. Large oscars exhibited a rapid reduction in unidirectional Na^+ uptake rate at the gills during acute hypoxia (P_{O_2} ~10 mmHg), which intensified with time (7 or 8 h); Na^+ efflux rates were also reduced, so net balance was little affected. The inhibitions were virtually immediate (1st h) and preceded a later 60% reduction (at 3 h) in gill Na^+-K^+-ATPase activity, reflected in a 60% reduction in maximum Na^+ uptake capacity without change in affinity (Km) for Na^+. Upon acute restoration of normoxia, recovery of Na^+ uptake was delayed for 1 h. These data suggest that dual mechanisms may be involved (e.g., immediate effects of O_2 availability on transporters, channels, or permeability, slower effects of Na^+-K^+-ATPase regulation). Ammonia excretion appeared to be linked indirectly to Na^+ uptake, exhibiting a Michaelis-Menten relationship with external [Na^+], but the Km was less than for Na^+ uptake. During hypoxia, ammonia excretion fell in a similar manner to Na^+ fluxes, with a delayed recovery upon normoxia restoration, but the relationship with [Na^+] was blocked. Reductions in ammonia excretion were greater than in urea excretion. Plasma ammonia rose moderately over 3 h hypoxia, suggesting that inhibition of excretion was greater than inhibition of ammonia production. Overall, the oscar maintains excellent homeostasis of ionoregulation and N-balance during severe hypoxia.

teleost fish; ionoregulation; nitrogen metabolism; sodium-potassium-ATPase; ion channels

THE PRESENT STUDY USES the hypoxia-tolerant oscar (acará-açu; *Astronotus ocellatus*), an entirely water-breathing Amazonian cichlid, to examine the effects of low environmental O_2 on two key aspects of gill function, ionoregulation and nitrogenous waste excretion. The oscar commonly encounters hypoxia in its natural environment when it enters the seasonally flooded jungle to feed and reproduce; adults are reported to survive up to 6 h of complete anoxia and can tolerate levels of 5–20% air saturation for 20–50 h (1, 2, 32). There are several reasons for believing that ionic balance and ammonia excretion may be particularly sensitive to hypoxia in freshwater fish, but to date, these areas have received little experimental attention.

First, the respiratory-osmoregulatory compromise at the gills has been well documented in exercise studies on several teleost species: the effective gill area and diffusion distance are adjusted as a trade-off between providing the permeability required for gas exchange, while minimizing diffusive ion losses and osmotic water gain (12, 13, 57, 58). During environmental hypoxia, it is probable that similar lamellar recruitment and decreased diffusion distance occurs to help sustain O_2 uptake (17, 18) because gill O_2 transfer factor, an index of effective O_2 permeability, increases markedly in both hypoxia and exercise (36). We therefore hypothesized that increases in diffusive Na^+ loss rates to the water and a possible reduction of plasma ion levels would also occur.

Secondly, ionoregulation is a costly process in freshwater fish, with estimates generally falling in the range of 2–20% of resting metabolism at the whole animal level (reviewed in Ref. 11). The very dilute nature of many Amazonian waters ("slightly contaminated distilled water;" 41) may exacerbate these costs, and a general tendency for reduced ion levels in the plasma of Amazonian teleosts has been noted (28). In other fish, the rate of O_2 utilization by artificially perfused and ventilated gill tissue amounts to about 4–12% of resting O_2 uptake by the whole animal (26, 31, 59), that is, in the same range as estimates of the costs of ionoregulation. About half of this O_2 used by gill cells comes directly from the water, and the other half comes from the perfusate or blood, which itself has just been oxygenated in the arterial-arterial pathway of the gill lamellae before reaching the ionocytes (33, 52). Thus gill ionocytes will be on the front line of hypoxia exposure and the very first cells to experience an O_2 deficit during environmental hypoxia. By way of analogy, in neural cells, the cost of ion pumping appears to be second only to that of protein synthesis, and both processes are markedly turned down during hypoxia in model species, such as the turtle and crucian carp, which are capable of severe hypometabolism (reviewed in Refs. 6, 7, and 19). Therefore, we hypothesized that active Na^+ influx rates from the water could fall during hypoxia for two reasons: *1*) as a direct response to O_2 starvation of the working ionocytes and *2*) as a result of downregulation of uptake channels and/or Na^+-K^+-ATPase activity to save metabolic costs.

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Third, if ion transport processes are affected by hypoxia, ammonia excretion might be affected as well. The gills excrete more than 80% of the metabolic ammonia production in fish, and the mechanism is thought to be linked in some way to the active uptake of Na^+ (see Refs. 50, 51, 53, 56 for a review). Original ideas about direct Na^+/NH_4^+ exchange coupling in the gill cells (e.g., 21, 27, 60), while not entirely disproven, have given way in recent years to the concept that the coupling is indirect (3, 25, 34). Thus an H^+ pump on the apical membrane provides the electrical gradient needed to drive Na^+ uptake from the water through coupled Na^+ channels, and the associated acidification of the gill boundary layer enhances the "diffusion-trapping" of NH_3 as NH_4^+, thus sustaining the PNH_3 gradient for diffusive NH_3 efflux (9, 54). We, therefore, hypothesized that if hypoxia interferes with the Na^+ uptake-H^+ pumping mechanism (or Na^+/NH_4^+ exchange), ammonia excretion would be inhibited, and/or uncoupled from Na^+ uptake. However, the situation is complicated by the fact that the rate of metabolic ammonia (and urea) production may also be reduced by down-regulation of aerobic metabolism (i.e., less deamination of amino acids for fuel; Refs. 47 and 48), though at least one study has shown an increase in ammonia production during hypoxia (22). Regardless, metabolic rate depression per se is another reason to suspect that ammonia excretion could be sensitive to hypoxia. Therefore, we also measured plasma ammonia levels as an indicator of whether the excretion processes or the production processes were altered to the greater extent, as well as changes in urea excretion as a general indicator of N-metabolism during hypoxia.

In addition to the effects of hypoxia on ion balance and ammonia excretion in general, we explored the influence of body size. Large oscars are more tolerant of hypoxia than small oscars, in contrast to the pattern in many other teleost species (e.g., 8, 38, 43). Large oscars possess lower mass-specific MO_2s and a greater aerobic ability to sustain MO_2 during hypoxia (i.e., lower P_{O_2}crit; Ref. 42), a much greater anaerobic capacity (2), and different behavioral responses (42). We therefore looked for possible qualitative or quantitative differences between large and small fish in Na^+ and ammonia flux responses to hypoxia.

MATERIALS AND METHODS

Experimental animals. Oscars (*Astronotus ocellatus*) in two weight ranges ("small": 10–25 g and "large": 130–260 g) were obtained from Sítio dos Rodrigues (Km 35, Rod. AM-010, Manaus, Brazil) in August 2004. The fish were transferred to the Ecophysiology and Molecular Evolution Laboratory at the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Amazonas, Brazil and held in 500-liter outdoor tanks at 28 ± 3°C with a natural photoperiod; 50% of the water was exchanged every 2 days. The holding and experimental water was typical Amazonian soft water taken from a well on the INPA campus (Na^+ = 19, Cl^- = 21, K^+ = 16, Ca^{2+} = 11, Mg^{2+} = 2 μmol/l, pH = 6.5, dissolved organic carbon = 0.6 mg C/l). The fish were fed daily with commercial pellets, but feeding was suspended 2 days before experimentation. All experimental procedures complied with Brazilian and INPA animal care regulations.

Experimental protocols. Experimental temperature was 28 ± 1.5°C, and the same soft water was used in all trials. Most experimental protocols consisted of exposing the fish (n = 5–9 per experiment) to various O_2 regimes, while simultaneously measuring unidirectional Na^+ fluxes with ^{22}Na (manufactured by New England Nuclear-Dupont, Boston, MA, and supplied by REM, Sâo Paulo, Brazil) and net ammonia and urea fluxes. Experimental chambers for large fish were 2.5-liter sealable Nalgene kitchen cutlery containers mounted on their sides; the horizontally flattened shape fitted the morphology of the fish. For small fish, 700-ml Nalgene beakers with sealable lids were employed. The chambers were shielded with black plastic to minimize visual disturbance and fitted with individual water lines for flushing, and air-stones for air or N_2 gassing. The chambers were 80% submerged in a flowing water bath to maintain the experimental temperature of 28 ± 1.5°C. Fish were placed in these individual containers the evening before an experiment and left overnight to settle with flow-through flushing and continuous aeration.

At the start of a standard experiment, the water flow was stopped but aeration continued, and an aliquot of ^{22}Na (typically 2 μCi/l) was added to each container and allowed to equilibrate for 1 h. Water samples (4 × 5 ml for ^{22}Na, total Na^+, ammonia, and urea measurements) were taken at the start of the experiment (0 h) and at subsequent 1-h intervals up to 7 h, when the experiment was terminated and the animal was weighed. During this period, the O_2 regime was experimentally manipulated by vigorous gassing with either air or N_2, followed by more gentle gassing for maintenance. Tests demonstrated that P_{O_2} changes were complete within 10 min, so water P_{O_2} was routinely monitored (1-ml samples) at the midpoint of each 1-h flux. The overall 7-h measurement period was chosen so as to avoid the need to perform correction for back flux of the radioisotope, with its attendant uncertainties (cf. 20, 55). Theoretical calculations, confirmed by blood samples in preliminary trials, indicated that internal specific activity reached only 2–6% of external specific activity by this time, below the threshold (10%), in which back-flux correction would become necessary.

One exception to this basic protocol occurred in an experiment with large oscars, where fluxes were measured over a 3-h period, which included 1 h of normoxia and the first 2 h of hypoxia, and then again at 7–9 h of hypoxic exposure. The external radioisotope concentration was tripled at 6 h of hypoxia so as to avoid a back-flux problem. A second exception occurred in kinetic flux experiments with large oscars, in which the concentration dependence of Na^+ uptake was measured at successively higher external Na^+ concentrations in five steps over an 11-h period (cf. 55). In these experiments, ^{22}Na and "cold" Na^+ were raised in direct proportion to one another by adding aliquots of a ^{22}Na-labeled 180 mmol/l NaCl solution in a geometrically increasing series. At each concentration, a 0.5-h mixing period was followed by a 1-h to 2-h period of flux measurement. Similar experiments were attempted with small oscars, but the higher volume-to-mass ratio used precluded accurate Na^+ uptake measurements at higher Na^+ concentrations, so only ammonia flux data from the latter experiments have been reported.

One experimental series focused on terminal blood and tissue sampling. Large oscars were set up in their individual containers exactly as during flux tests, but ^{22}Na was not added. A typical O_2 regime of normoxia, followed by 3 h of severe hypoxia and 3 h of normoxia restoration was implemented. Fish (n = 7 per sampling point) were rapidly killed with a lethal dose of benzocaine (0.5 g/l; Sigma, St. Louis, MO) during normoxia, after 1 and 3 h of severe hypoxia, and after 1 and 3 h of normoxia restoration. In this series, water P_{O_2} was measured at each sampling time and also at 10 min after the changes from normoxia-to-hypoxia and from hypoxia-to-normoxia. Blood samples (1 ml) were taken by caudal venipuncture, and then samples of gill filaments and epaxial white muscle were excised. Plasma and tissue samples were snap-frozen in liquid N_2 and stored at −80°C for later analysis.

Finally, a separate experimental series using an identical normoxia-hypoxia-normoxia regime and duplicating the exact conditions used during the flux and tissue sampling experiments was carried out with five large oscars. Water pH was monitored at 30-min intervals throughout to ensure that it was not a confounding factor in interpretation of results.

Analytical techniques and calculations. Water P_{O_2} was monitored by injecting 1-ml samples into an O_2 electrode (Radiometer-Copenhagen, Denmark) thermostatted to the experimental temperature and connected to a Cameron OM-200 oxygen meter (Cameron Instruments, Port Aransas, TX). Water pH was monitored with a Radiometer GK24O1C combination electrode suitable for use in ion-poor water. Water total ammonia (salicylate hypochlorite assay; Ref. 49) and urea concentrations (diacetyl monoxime assay; Ref. 35) were determined colorimetrically. ^{22}Na activities in water samples were measured via liquid scintillation counting (LS6500, Beckman Coulter, Fullerton, CA) on 5-ml water samples added to 5-ml of Packard Ultima Gold AB fluor (Perkin Elmer, Wellesley, MA). Tests demonstrated that quenching was constant, so no correction was necessary. Water total Na^+ concentrations were measured using flame atomic absorption spectrophotometry (AAnalyst 800, Perkin Elmer). Na^+ influx rates ($J^{Na}in$, by convention positive) were calculated from the mean external specific activity, and the disappearance of counts from the external water (factored by time, volume, and fish mass); Na^+ net flux rates ($J^{Na}net$) were calculated from the change in total Na^+ concentration in the water (similarly factored); and Na^+ unidirectional efflux rates ($J^{Na}out$, by convention negative) were calculated by difference, as outlined in detail by Wood (55). This approach makes no assumptions about steady state and allows for comparisons of different treatments over time. Net flux rates of ammonia (J^{Amm}) and urea (J^{Urea}) were calculated as for $J^{Na}net$. In kinetic analyses, the coordinates of the Michaelis-Menten equation (Km, Jmax) and their SE estimates were fitted by nonlinear regression using Sigmaplot 8.

Plasma samples were analyzed for Na^+, Ca^{2+}, Mg^{2+} (all by atomic absorption spectrophotometry using a Varian SpectrAA-220FS; Varian, Mulgrave, Australia), Cl^- by coulometric titration (CMT10, Radiometer-Copenhagen, Denmark), total ammonia by the glutamate dehydrogenase method (Raichem, Ammonia Reagent, Product No. 85446, Columbia, MD), cortisol by radioimmunoassay (ICN Pharmaceuticals, Costa Mesa, CA), and glucose and lactate by standard enzymatic assays (10, 37). Muscle samples were similarly analyzed for lactate, but some samples were lost due to accidental thawing. Gill Na^+-K^+-ATPase activity was determined by the microplate method of McCormick (29) and normalized to total protein content (measured using the bicinchoninic acid method; Sigma).

All data are reported as means ± SE (n = number of fish). Relationships were assessed by 1-way ANOVA followed by the Bonferroni multiple comparison test for independent data or Dunnett's multiple comparison test for paired data, as appropriate, to determine when values became significantly different from reference means. A significance level of $P \leq 0.05$ was used throughout.

RESULTS

Large vs. small oscars. The Na^+ and ammonia flux responses of large vs. small oscars to hypoxia were qualitatively similar and differed only in quantitative detail. Therefore, responses of large oscars have been generally presented. Interestingly, under normoxia, unidirectional Na^+ flux rates ($J^{Na}in$ and $J^{Na}out$) were of similar magnitude on a mass-specific basis in small vs. large fish (e.g., Fig. 1, *B* and *E*), whereas mass-specific J^{Amm} and J^{Urea} were several-fold higher in small oscars (e.g., Fig. 1, *C* and *F*). Large oscars were also generally closer to achieving Na^+ balance (i.e., $J^{Na}net$ close to zero, Fig. 1*F*), whereas small oscars usually exhibited negative $J^{Na}net$ (Fig. 1*B*). Negative balance is typical of Amazonian fish in ion-poor native waters, and it is probable that the deficit is normally supplied by dietary Na^+ (15, 16). The oscars had been fasted 2 days before test. Our initial intention was to expose both small fish and large fish to the same severity of hypoxia (~10 mmHg; e.g., Fig. 1*D*), but in preliminary experiments, it was found that some small oscars succumbed at this level, so a less severe hypoxia (~20 mmHg) was used for the latter (e.g., Fig. 1*A*).

Na^+ flux responses to hypoxia and normoxic recovery. In the first hour of severe hypoxia exposure, large oscars exhibited significant declines of about 50% in both $J^{Na}in$ and $J^{Na}out$, such that $J^{Na}net$ was little affected, and the responses persisted through 3 h of hypoxia (Fig. 1*E*). Similar responses were seen in small oscars, but developed more slowly (Fig. 1*B*). Upon return to normoxia, the inhibition of both $J^{Na}in$ and $J^{Na}out$ persisted over the first hour in both size groups, despite the restoration of normal water P_{O_2} (Fig. 1, *B* and *E*). In view of the 7-h limitation dictated by radioisotopic back-flux considerations, only the first hour of normoxic recovery was examined in the experiments of Fig. 1. Therefore, separate experiments were performed in which flux measurements started during severe hypoxia and continued for 4 h of normoxic recovery. These trials confirmed that J^{Na} in remained depressed during the first hour of normoxic recovery but was fully restored throughout the subsequent 3 h of normoxia (Fig. 2*B*). In the large fish experiment shown in Fig. 2*B*, $J^{Na}out$ was not significantly depressed during the first hour of normoxic restoration, in contrast to both the large fish and small fish experiments in Fig. 1, *E* and *B*, respectively. However, in a comparable small fish experiment (not shown), both $J^{Na}out$ and $J^{Na}in$ remained low during the first hour of return to high P_{O_2} (as in Fig. 1*B*) and then increased to stable, typical normoxic values thereafter. Thus there was some variability in the rate of $J^{Na}out$ recovery.

The experiment performed with large oscars and water pH measurements at 30-min intervals demonstrated that water pH did not change appreciably during the hypoxic regime. Representative data ($n = 5$) are normoxia, 6.75 ± 0.06; hypoxia, −1 h, 6.97 ± 0.13; hypoxia −3 h, 7.14 ± 0.10; normoxia restoration −1 h, 6.75 ± 0.07; normoxia restoration −3 h, 6.91 ± 0.08.

An experiment was carried out with large fish to determine whether the inhibition of $J^{Na}in$ and $J^{Na}out$ would persist during prolonged hypoxia. Indeed, the responses appeared to intensify over time, such that the reduction of $J^{Na}in$ was greater at 7 and 8 h of severe hypoxia than it had been at 1 h and 2 h (Fig. 3*B*). Similarly, the reduction of $J^{Na}out$ was also greater, at least at 8 h.

As a step change to severe hypoxia may not be environmentally realistic, experiments were performed in which the water P_{O_2} was allowed to fall more gradually. The same reductions in $J^{Na}in$ and $J^{Na}out$ were seen, with the first significant decreases occurring at a threshold P_{O_2} of about 40 mmHg in large fish (Fig. 4*B*). Small fish exhibited very similar responses, although the first significant reductions in $J^{Na}in$ and $J^{Na}out$ did not occur until a threshold of about 20 mmHg (not shown). Notably, in all of these responses (Figs. 1–4), reductions in $J^{Na}in$ and $J^{Na}out$ were of similar magnitude, so $J^{Na}net$ was little affected.

Kinetic analysis of the concentration dependence of Na^+ uptake was performed only for large oscars. Under normoxia (P_{O_2} = 144 ± 3 mmHg), the relationship exhibited typical Michaelis-Menten saturation kinetics (Fig. 5*A*). Under hypoxia (P_{O_2} = 17.0 ± 0.9 mmHg), the Michaelis-Menten relationship persisted, but the position of the curve exhibited a marked downward shift. Jmax was significantly reduced by about 60% during hypoxia (from 502 ± 128 to 218 ± 26 $\mu mol \cdot kg^{-1} \cdot h^{-1}$), but Km was not significantly altered (780 ±

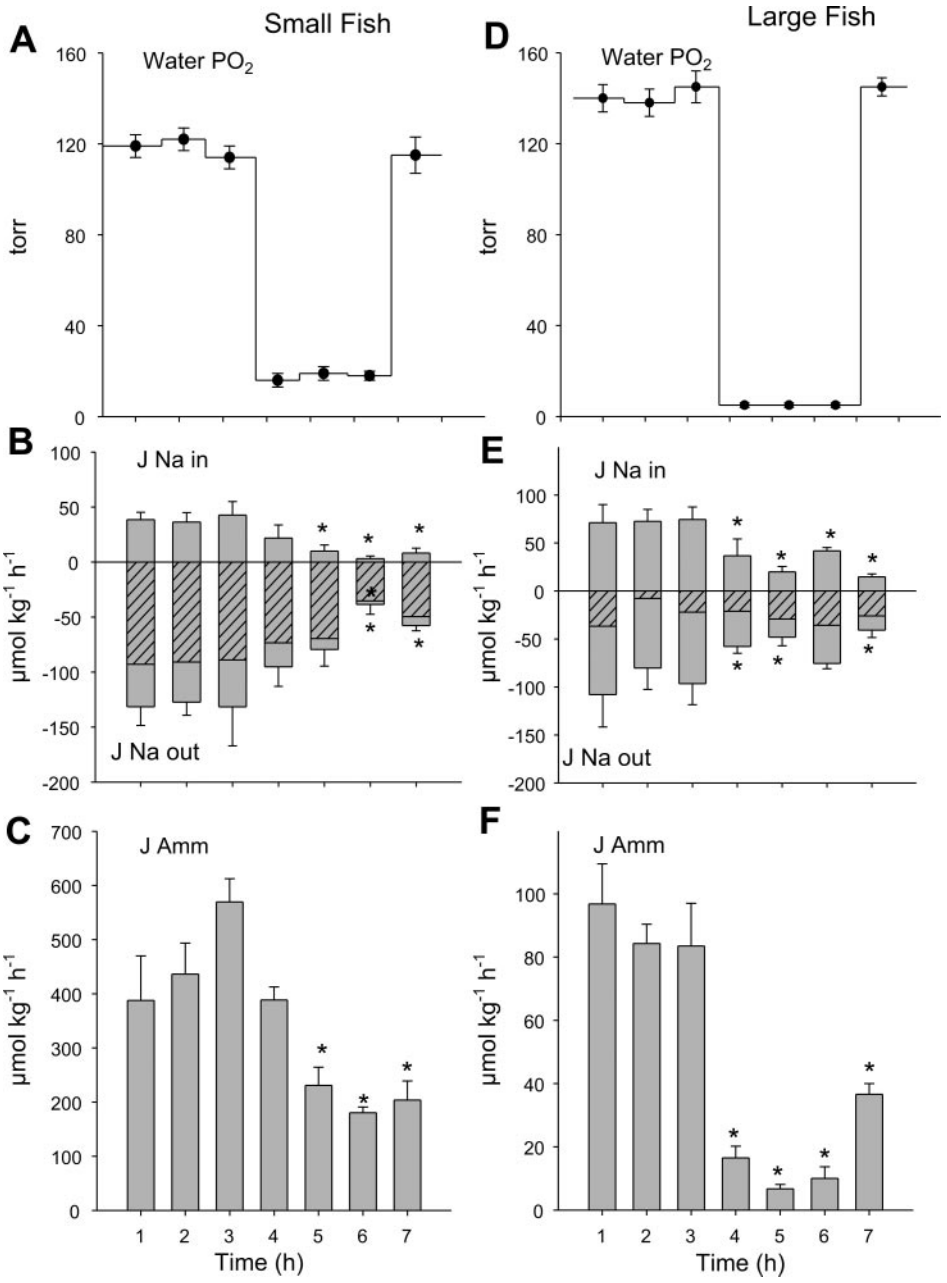

Fig. 1. The responses of small oscars (*A*, *B*, *C*; $n = 5$) and large oscars (*D*, *E*, *F*; $n = 7$) to an acute induction of severe hypoxia for 3 h followed by an acute restoration of normoxia. (*A*, *D*) Water O_2 tension; (*B*, *E*) Na^+ unidirectional influx (J^{Na}in, upward bars), Na^+ efflux (J^{Na}out, downward bars), and Na^+ net flux rates (J^{Na}net hatched bars); and (*C*, *F*) net excretion rate of ammonia (J^{Amm}). Values are expressed as means ± SE. $*P \leq 0.05$ relative to the mean value of the first 3 h (normoxia).

252 vs. 470 ± 118 µmol/l). This response suggests that hypoxia acts like a noncompetitive inhibitor, reducing the number (Jmax) of functioning Na^+ transport sites.

Ammonia flux responses to hypoxia and normoxic recovery. Simultaneous measurement of the ammonia fluxes (Fig. 5*B*) during the Na^+ kinetic analysis (Fig. 5*A*) demonstrated a strong Michaelis-Menten dependence of J^{Amm} on environmental [Na^+] in large oscars during normoxia. The apparent Km (in terms of [Na^+]) was 103 ± 20 µmol/l, while Jmax was 434 ± 23 µmol·kg^{-1}·h^{-1}. Notably, this Km value was significantly lower (i.e., higher apparent affinity for environmental [Na^+]) than for the J^{Na}in relationship in these same fish (780 ± 252 µmol/l), but the Jmax was very similar (502 ± 128 µmol·kg^{-1}·h^{-1}). The difference in Km suggests that any coupling of J^{Na}in with J^{Amm} must be indirect.

The kinetic experiment was repeated on the same large oscars 2 days later during hypoxia (P_{O_2} = 17.0 ± 0.9 mmHg). The coupling of J^{Amm} to environmental [Na^+] was entirely blocked by hypoxia (Fig. 5*B*).

Kinetic experiments with small oscars (not shown) demonstrated a very similar relationship between J^{Amm} and environmental [Na^+] during normoxia (P_{O_2} = 125 ± 6 mmHg), with an identical Km (122 ± 49 µmol/l) to that in large oscars, but a much larger mass-specific Jmax (1,319 ± 156 µmol·kg^{-1}·h^{-1}), as would be anticipated from allometric considerations. However, J^{Na}in values could not be determined in these small fish experiments (see MATERIALS AND METHODS). Notably, the coupling of J^{Amm} to environmental [Na^+] was again eliminated by hypoxia (P_{O_2} = 29 ± 4 mmHg).

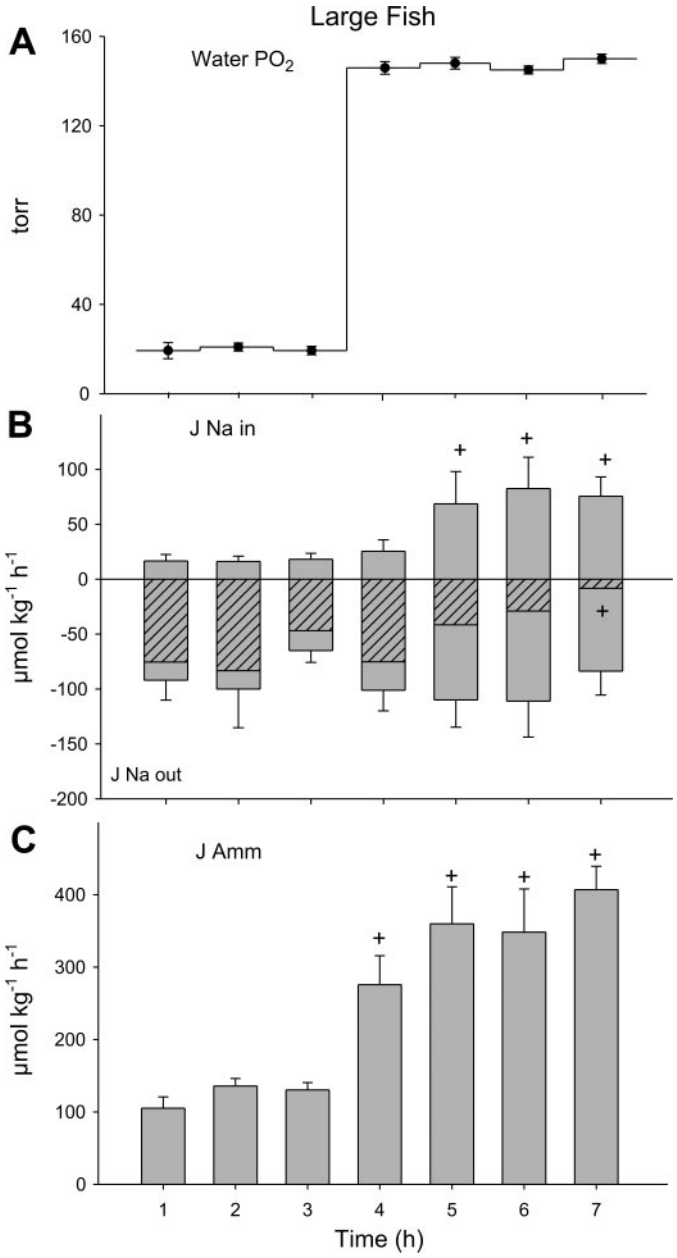

Fig. 2. The responses of large oscars ($n = 8$) to acute restoration of normoxia after 3 h of acute exposure to severe hypoxia. *A*: water O_2 tension. *B*: J^{Na}in, upward bars, J^{Na}out (downward bars), and J^{Na}net (hatched bars) of Na^+. *C*: J^{Amm}. Values are expressed as means ± SE. $+P \leq 0.05$ relative to the mean value of the first 3 h (severe hypoxia).

J^{Amm} was measured in all time-course experiments (e.g., Figs. 1–4) and invariably declined during hypoxia and increased again after restoration of normoxia. Responses were generally more pronounced in large fish (e.g., Fig. 1, *C* and *F*). The trends were therefore qualitatively similar to those in J^{Na}in and J^{Na}out, but the relative changes in J^{Amm} were often larger than in the Na^+ fluxes, and the time courses of the responses were not always matched, again suggesting that any coupling must be indirect. Thus in the acute and gradual hypoxia exposures of Figs. 3*C* and 4*C*, respectively, the initial declines in J^{Amm} lagged behind the initial declines in J^{Na}in (Figs. 3*B* and 4*B*), but the reductions in J^{Amm} did become more intense over time during prolonged severe hypoxia (Fig. 3*C*). During the first hour of normoxia restoration, J^{Amm} exhibited either no recovery (e.g., Fig. 1*C* and an additional small fish experiment not shown) or only partial recovery (e.g., Figs. 1*F* and 2*C*), but thereafter was always fully restored to the original normoxia level.

J^{Urea} was also measured in all time course experiments (not shown). As with J^{Amm}, J^{Urea} was several-fold larger on a mass-specific basis in small fish relative to large fish, as expected from allometry. Under normoxia, J^{Urea} was only

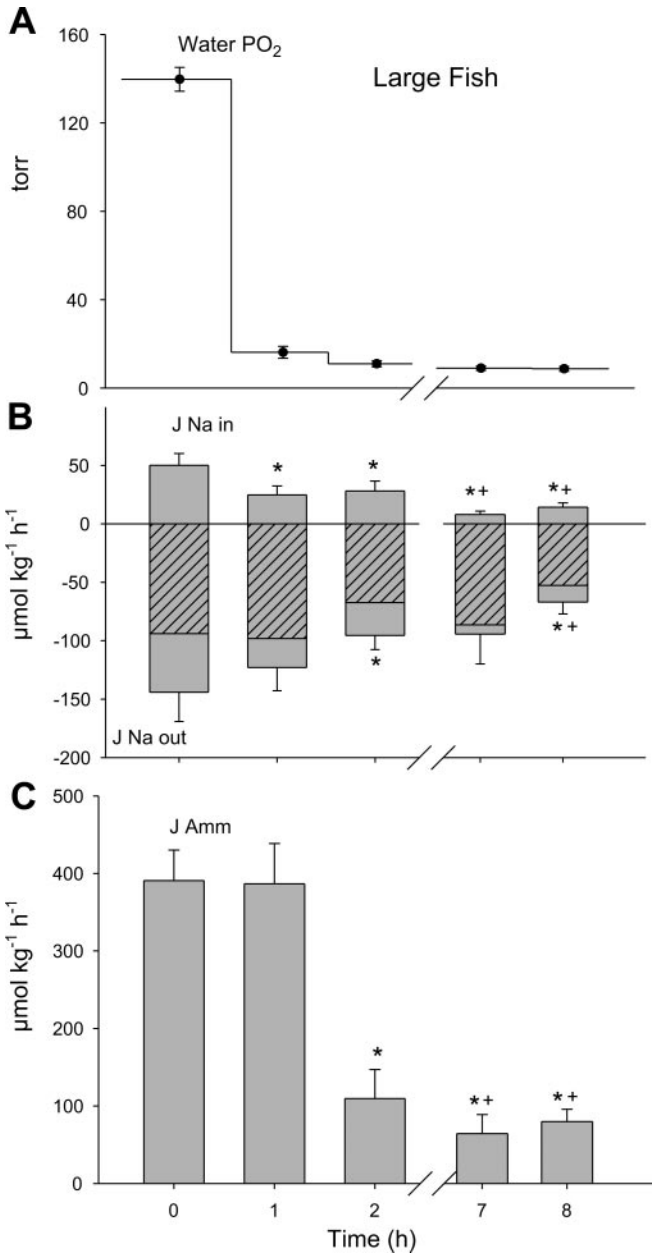

Fig. 3. The responses of large oscars ($n = 5$) to prolonged exposure to severe hypoxia. Flux measurements were made during the first 2 h of acute exposure, and then again at 7 and 8 h of prolonged exposure. *A*: water O_2 tension. *B*: J^{Na}in (upward bars), J^{Na}out (downward bars), and J^{Na}net (hatched bars) of Na^+. *C*: J^{Amm}. Values are expressed as means ± SE. $*P \leq 0.05$ relative to the normoxia value of the first hour. $+P \leq 0.05$ relative to the mean value of the first 2 h of severe hypoxia.

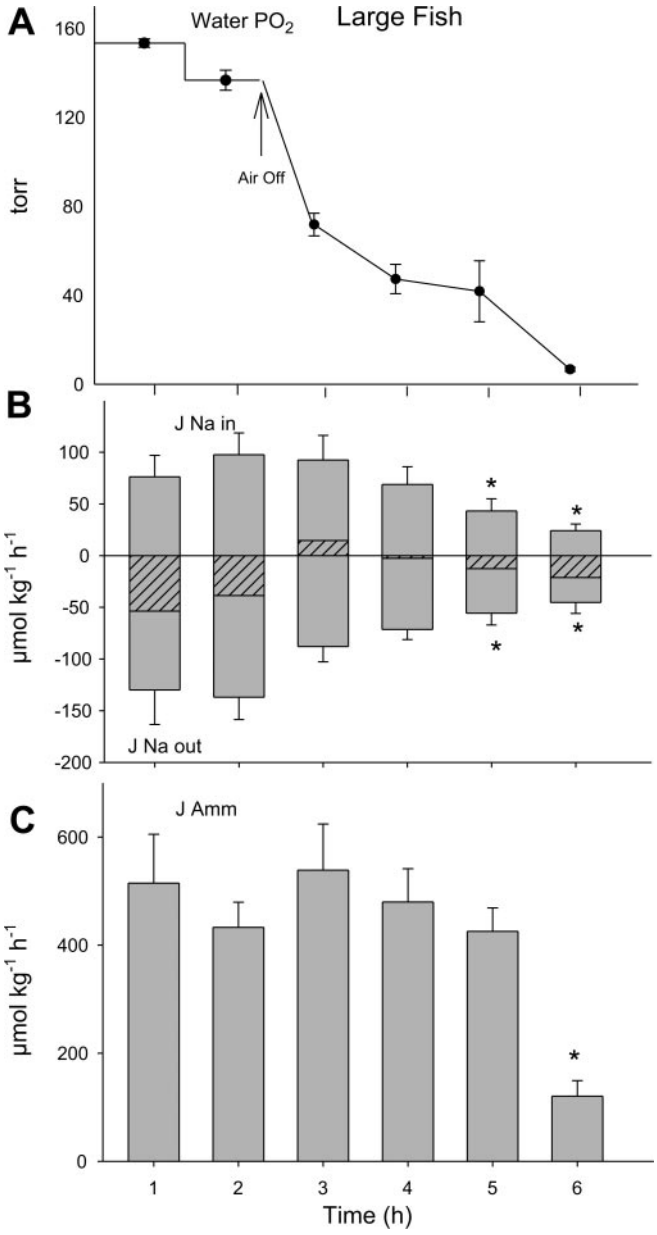

Fig. 4. The responses of large oscars ($n = 7$) to a gradual induction of severe hypoxia. Aeration was stopped after the first 2 h (normoxia control period), and the O_2 tension was allowed to fall gradually over the following 4 h. *A*: water O_2 tension. *B*: J^{Na}in (upward bars), J^{Na}out (downward bars), and J^{Na}net (hatched bars) of Na^+. *C*: J^{Amm}. Values are expressed as means ± SE. *$P \leq$ 0.05 relative to the mean value of the first 2 h (normoxia).

10.9 ± 2.5% ($n = 34$) of J^{Amm} on a per unit N basis, and during hypoxia, declines in J^{Urea} almost always occurred in parallel to declines in J^{Amm}. Thus, as with J^{Amm}, decreases in J^{Urea} were relatively larger in large oscars than in small oscars. However, the relationship between J^{Urea} and J^{Amm} was not one of direct proportionality (Fig. 6). The slope of the regression relating percentage change in urea-N excretion to that in ammonia-N excretion was only 0.57, and the intercept was significantly greater than zero. Therefore, at greater than about 50% inhibition, the relative declines in J^{Amm} were greater than the relative declines in J^{Urea}. Under severe hypoxia, when J^{Amm} was greatly depressed, J^{Urea} was 27.2 ± 5.9% ($n = 34$) of J^{Amm} on a per unit-N basis.

Plasma and tissue responses to hypoxia and normoxic recovery. Large oscars were terminally sampled at various times during a regime consisting of normoxia, 3 h acute severe hypoxia, and 3 h of normoxia restoration (Fig. 7*A*). There were no declines in plasma electrolytes, and indeed plasma [Na^+] and [Cl^-] increased significantly at 1 h (Fig. 7*B*), while [Mg^{2+}] increased significantly at both 1 h and 3 h of acute hypoxia (Table 1). The rise in plasma [Ca^{2+}] was not significant (Table 1). All of these changes were reversed during normoxia restoration. Gill Na^+-K^+-ATPase activity remained unchanged at 1 h but declined by 60% at 3 h of severe hypoxia (Fig. 7*C*). This depression was completely reversed by 1 h of normoxic recovery. Plasma total ammonia level rose significantly by 45% after 3 h of hypoxia but was similarly corrected by 1 h of normoxic recovery (Fig. 7*D*).

Plasma glucose fell by about 40% after 3 h of hypoxia but was no longer significantly depressed at both sampling times after return to normoxia (Table 1). Plasma lactate concentration rose rapidly during severe hypoxia, with 11-fold and

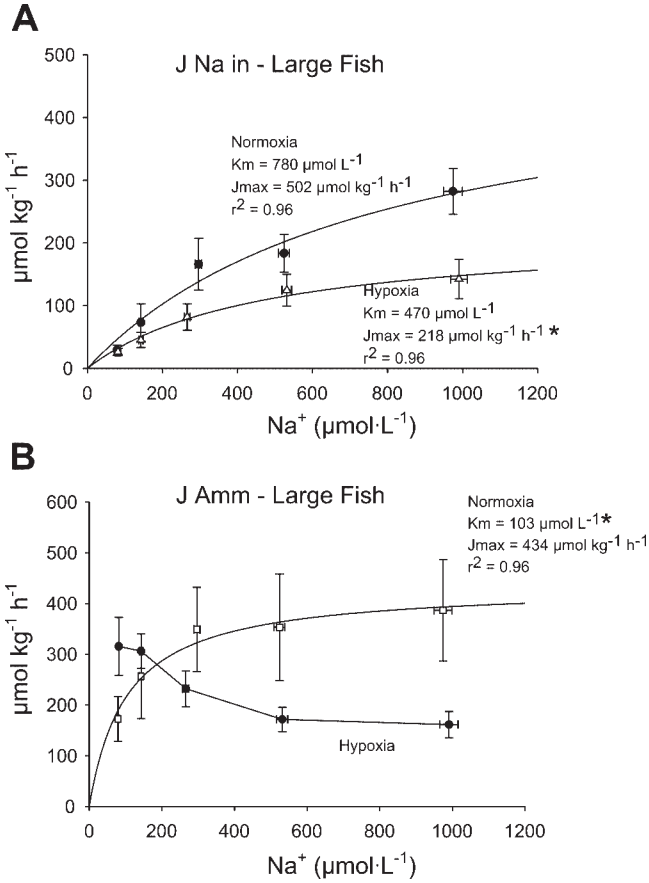

Fig. 5. *A*: Michaelis-Menten kinetics of Na^+ influx rate (J^{Na}in) as a function of external Na^+ concentration in large oscars ($n = 8$) during normoxia and during severe hypoxia. Note the decrease in Jmax without significant change in Km during hypoxia. *B*: Michaelis-Menten kinetics of J^{Amm} as a function of external Na^+ concentration in the same large oscars during normoxia. Note the lack of a Michaelis-Menten relationship during severe hypoxia. Note also the difference in Km, with no difference in Jmax between the J^{Na}in relationship (*A*) and the J^{Amm} relationship (*B*) in normoxia. Values are expressed as means ± SE.

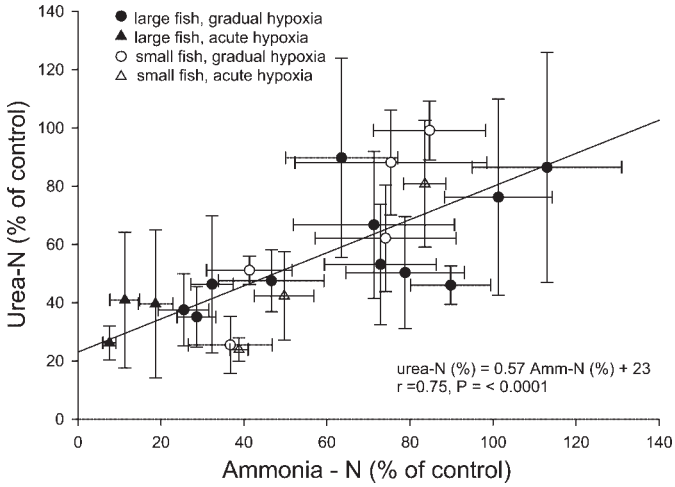

Fig. 6. The relationship between the mean relative urea-N excretion rate (J^{Urea}, *y*, as a percentage of the normoxic control value) and the mean relative ammonia-N excretion rate (J^{Amm}, *x*, as a percentage of the normoxic control value) in individual periods of various hypoxic exposure experiments with large and small oscars. Values are presented as means ± SE (n = 5–9 at each point). The equation of the regression line is $y = 0.57\ x + 23$ ($r = 0.75$, $P \leq 0.0001$).

16-fold increases, respectively, after 1 and 3 h of acute exposure (Table 1). Lactate was rapidly cleared from the plasma and was no longer significantly elevated at 1 h after restoration of normoxia. These plasma lactate changes coincided with significant increases in muscle lactate concentration from 7.6 ± 0.8 mmol/kg (n = 7) under normoxia to 17.3 ± 1.9 (n = 7) and 17.4 ± 1.6 mmol/kg (n = 7) at 1 and 3 h of hypoxia, respectively; the normoxic recovery samples were lost. Plasma cortisol exhibited a biphasic pattern, doubling at 1 h of hypoxia and later falling to 50% of the original control level at 1 h of normoxia restoration, before returning to normal after 3 h of recovery (Table 1).

DISCUSSION

Overview. Some of our original hypotheses were supported, but others were not. Active Na^+ influx rates from the water ($J^{Na}in$) and ammonia excretion rate (J^{Amm}) were both greatly depressed during hypoxia, and J^{Amm} became uncoupled from $J^{Na}in$. J^{Urea} also fell, indicating a general decrease in N metabolism, although the fall was not as large as in J^{Amm}. In accord, plasma total ammonia concentration rose modestly, suggesting that the ammonia excretion rate was inhibited to a greater extent than the ammonia production rate. However, contrary to our original predictions based on the respiratory-osmoregulatory compromise, $J^{Na}out$ actually decreased rather than increased during hypoxia. As a consequence, $J^{Na}net$ remained largely unchanged, and plasma ion levels did not fall. Factors other than the respiratory-osmoregulatory compromise, such as regulated channel arrest, may instead come into play. In summary, under severe hypoxia, oscars suffer no obvious ionoregulatory imbalance, and only a moderate disturbance of ammonia regulation. In addition to exhibiting impressive adaptations in its behavior, anaerobic potential, and aerobic regulation capacity (1, 2, 32, 42), *Astronotus ocellatus* is clearly well adapted to maintain ionoregulatory homeostasis and N balance in the severe O_2 regimes that are part of its normal lifestyle in the Amazon floodplain.

Small vs. large oscars. Although Na^+ influx kinetics were measured only for large oscars, they fit the pattern of relatively high Km (i.e., low Na^+ affinity) and low influx and efflux rates (i.e., low permeability) reported for other Amazonian cichlids (reviewed in Ref. 16), very different from the characids, which are abundant in the same environment and which exhibit low Km (i.e., high Na^+ affinity) and much higher Na^+ turnover

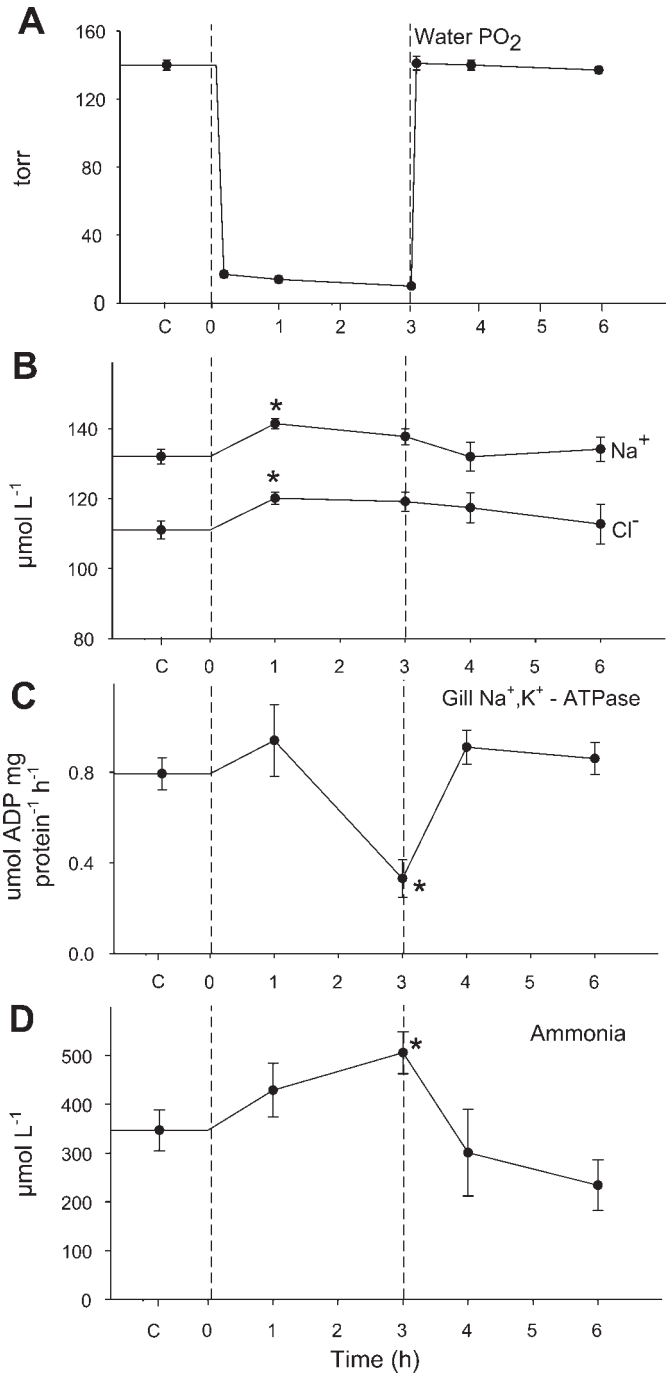

Fig. 7. Plasma and tissue responses of large oscars (n = 7 at each point) to an acute induction of severe hypoxia for 3 h followed by an acute restoration of normoxia for 3 h. *A*: water O_2 tension. *B*: plasma Na^+ and Cl^- concentrations. *C*: gill Na^+, K^+-ATPase activity. *D*: plasma total ammonia concentration. Values are presented as means ± SE. *$P \leq 0.05$ relative to the normoxic control value (C).

Table 1. *Changes in plasma chemistry of large oscars (Astronotus ocellatus) during 3 h of acute exposure to severe hypoxia, followed by 3 h of acute normoxia restoration*

		Hypoxia		Normoxia Restoration	
	Normoxia Control	1 h	3 h	4 h	6 h
P_{O_2}, mm Hg	140±3	14±2*	10±1*	141±4	137±2
Ca^{2+}, mmol/l	3.16±0.33	3.25±0.26	3.76±0.22	3.40±0.30	3.43±0.25
Mg^{2+}, mmol/l	1.02±0.07	1.31±0.09*	1.38±0.05*	1.20±0.05	1.10±0.05
glucose, mmol/l	15.73±1.58	10.54±1.56	9.57±0.86*	11.65±1.21	10.40±1.63
lactate, mmol/l	0.84±0.22	9.40±2.61*	13.27±2.78*	3.84±1.85	1.09±0.30
cortisol, μg/l	71±9	138±20*	104±18	34±6*	113±18

Values are expressed as means ± SE; $n = 7$ at each time point. *$P < 0.05$ relative to normoxic control value.

rates (14, 15). The cichlid strategy is presumably more economical because it necessitates less active ion pumping. The fact that mass-specific Na^+ turnover rates are not greater in small oscars than large oscars in the expected allometric fashion (e.g., Ref. 4) may in itself represent a cost-saving adaptive strategy for these small fish in ion-poor water. However, mass-specific ammonia excretion rates (J^{Amm}) were higher in small fish than in large fish (Fig. 1, *C* and *F*), and the differences were relatively larger than in MO_2 (42), suggesting that small oscars rely on protein oxidation to a greater extent (23, 47).

The responses to hypoxia, including reductions in J^{Na}in, J^{Na}out, J^{Amm}, and J^{Urea}, were qualitatively similar between large and small oscars and differed only in quantitative detail. The reductions in J^{Na}in and J^{Na}out during hypoxia became significant more rapidly in large fish (first hour vs. second or third hour), and the inhibitions of J^{Amm} and J^{Urea} were more pronounced than in small fish (Fig. 1). Note, however, that a less severe level of acute hypoxia was used for small fish (~20 vs. ~10 mmHg in large fish) because of their lower hypoxic tolerance. These responses should be interpreted with respect to the metabolic responses of large and small oscars to hypoxia, as determined through respirometry in a parallel study under very similar conditions (42). Sloman et al. (42) reported that MO_2 was around 2,200 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ under normoxic conditions in large fish and remained stable down to a P_{O_2} of 50 mmHg, where it was significantly depressed (by 26%). In contrast, MO_2 was around 4,900 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ under normoxia in small oscars and tended to fall progressively as P_{O_2} declined, with the first significant decrease (by 27%) occurring at 70 mmHg. In both large and small fish, MO_2 fell steadily with P_{O_2} below these thresholds. On the basis of model equations fitted to these data (42), a 200-g fish would have exhibited a 75% decline in MO_2 at 10 mmHg, whereas a 15-g fish would have exhibited a 68% decline at the higher P_{O_2} of 20 mmHg and a 79% decline had 10 mmHg been used.

During the gradual hypoxia trials, in large fish, reductions in both J^{Na}in and J^{Na}out first became significant at a threshold P_{O_2} of about 40 mmHg (Fig. 4, *A* and *B*; corresponding to a decline in MO_2 of only 37%), slightly below the threshold P_{O_2} (P_{O_2}crit = 50 mmHg), at which aerobic metabolic rate depression started. This suggests that reduced ion turnover at the gills is part of the strategy for metabolic rate depression in large oscars and is likely regulated in some fashion. However, in small fish, the P_{O_2} threshold for significant reduction in unidirectional Na^+ fluxes was about 20 mmHg, corresponding to a 68% decline in MO_2, whereas P_{O_2}crit was 70 mmHg. In small oscars, physiological regulation may be less developed, so that direct effects of O_2 starvation on the gills (with later onset) may be the dominant mechanism. In summary, it appears that large oscars are better able to implement these cost-saving responses more quickly, at a higher P_{O_2}, without compromising net Na^+ balance, another indicator of their better hypoxia tolerance relative to small oscars (cf. Refs. 2 and 42). Interestingly, Lewis et al. (24) recently reported that protein synthesis rates in the gills of large oscars were reduced by 50% during a comparable hypoxic exposure but recovered without overshoot within 1 h after restoration of normoxia.

Influence of hypoxia on ionoregulation. Contrary to predictions based on the respiratory-osmoregulatory compromise, J^{Na}out and J^{Na}net did not increase, and plasma ions did not fall during severe hypoxia. The situation is very different than during exercise, where increases in MO_2 are associated with increases in ion losses in a number of species (12, 13, 57, 58). However, unlike exercise, where MO_2 clearly increases, there is a marked decrease in MO_2 during hypoxia (32, 42). Gill blood flow changes may be rather different during hypoxia, because in contrast to the overall branchial vasodilation that accompanies exercise, there is a branchial vasoconstriction and an increase in the arterio-venous flow, which returns O_2 to the heart (17, 44, 45). At present, we have no evidence that increased lamellar blood flow actually occurs during hypoxia in *Astronotus ocellatus*. This is an important area for future investigation, as are possible changes in the perfusion of the secondary circulation to the skin, and alterations in the relative surface areas and diffusion distances from water to blood of the gills vs. other parts of the body during the hypoxic exposure.

The only study with a comparable focus is that of Thomas et al. (46), in which rainbow trout, a hypoxia-intolerant species, were acutely exposed to a P_{O_2} of 40 mmHg. Thomas et al. (46) were able to measure net flux rates of Na^+ and Cl^- with the water in only a few trout due to fecal contamination problems but concluded that these remained approximately stable during hypoxia, in accord with the present study. Furthermore, plasma [Na^+] and [Cl^-] actually increased, again in agreement with the present results (Fig. 7*B*), attributed to a fluid shift into the intracellular compartment (i.e., hemoconcentration), as well as a preferential movement of Na^+ into the extracellular fluid to balance the lactate anion. Comparable explanations likely apply for the oscar, as all plasma ions tended to increase, there was a rapid appearance of lactate in the extracellular fluid (Table 1) and a marked rise in muscle lactate occurred (the measured concentration of 17.4 mmol/kg at 1–3 h of hypoxia would have amounted to about 24 mmol/l on an intracellular

fluid basis). This rapid response of lactate production, as well as the moderate fall in plasma glucose (Table 1), are in accord with previous reports on the well-developed anaerobic potential of white muscle in large oscars (2, 32). Lactate production from glycogen stores would have driven a shift of fluid out of the extracellular compartment into muscle by osmosis. The rapid appearance of lactate and its equally rapid clearance after return to normoxia may have been associated with the sharp increases and decreases in cortisol at these times (Table 1; see Refs. 10 and 30). Very recently, Richards et al. (37) have reported a detailed biochemical study on *Astronotus ocellatus* exposed to a similar hypoxic regime and have confirmed many of the trends seen in the present study, including lactate accumulation, glycogen depletion, hemoconcentration, and apparent fluid shift to the intracellular compartment.

Three possible explanations, which are by no means mutually exclusive, come to mind for the observed decreases in J^{Na}out during hypoxia (Figs. 1, *B* and *E*, 2*B*, 3*B*, and 4*B*). The first is channel arrest, a well-documented phenomenon at the cellular level in hypoxia-tolerant species (6, 7, 19). If this occurred at the gills, it could reduce both J^{Na}in and J^{Na}out, and perhaps also J^{Amm}. The second possibility, a different interpretation of the respiratory-osmoregulatory compromise, is that a hypoxia-tolerant species such as the oscar actually reduces gill area and permeability during severe hypoxia by reducing lamellar perfusion so as to reduce ionoregulatory costs in a situation in which the potential for MO_2 uptake from the water has become very slight. This might occur in association with depressed protein synthesis in the gills (24), with a change in the pattern of cardiac output (the well-documented bradycardia without fall in stroke volume) and/or a possible change in circulatory pattern in the gills during hypoxia (17, 18, 44, 45), as discussed earlier. Measurements of water flux across the gills would illuminate whether effective branchial water permeability is turned down in parallel to apparent branchial Na^+ permeability, which would support this explanation. The third possibility is that an exchange diffusion transport system occurs at the gills and is turned down during hypoxia, so that the reduced J^{Na}out is directly coupled to the reduced J^{Na}in. Exchange diffusion has been seen during normoxia in many freshwater teleosts and crustaceans (e.g., 40, 58), including about half of the Amazonian teleosts surveyed by Gonzalez et al. (15), but to our knowledge, it has never been studied during hypoxia.

In both large and small oscars, J^{Na}in declined substantially during hypoxia (Figs. 1–4), in accord with our original hypothesis that this expensive process would be limited by O_2 availability. However, several lines of evidence point to, at least, a partial temporal disconnection between the O_2 regime and the simultaneously measured ion fluxes, suggesting that two (or more) mechanisms may be causing the reduction in Na^+ turnover during hypoxia.

The first is that in the large fish experiment of Fig. 7*C*, branchial Na^+-K^+-ATPase activity was fully maintained at the end of 1 h of hypoxia, despite the fact that Na^+ influx and efflux rates had already declined over the first hour in a comparable series (Fig. 1*E*). Na^+-K^+-ATPase activity did fall greatly (by about 60%) by 3 h of hypoxia but had fully recovered by 1 h of normoxia reestablishment (Fig. 7*C*), yet unidirectional flux rates remained depressed during this first hour (Fig. 1*E*). An important caveat here is the recent demonstration that in trout hepatocytes, low P_{O_2} can reduce the transport activity of Na^+-K^+-ATPase before its ATP hydrolytic capacity (the parameter measured in the assay) is affected (5). It must be remembered, that Na^+-K^+-ATPase activity is assayed under optimal conditions in vitro, whereas in vivo, the intracellular milieu of the enzyme may change during hypoxia. This is an important area for future investigation. Second, during prolonged exposure to severe hypoxia, both J^{Na}in and J^{Na}out were further reduced at later times (Fig. 3*B*), again pointing to the involvement of a second mechanism that intensified the effect of the initial inhibition. Third, in the kinetic analysis of J^{Na}in, Jmax was significantly depressed by about 60% during prolonged hypoxia (Fig. 5*A*), as might be expected from the observed 60% reduction in branchial Na^+-K^+-ATPase activity (Fig. 7*C*), that is, a reduction in the number of transport sites without a change in their affinity for Na^+.

In summary, the data are consistent with downregulation of Na^+ influx (and efflux) rates by at least two mechanisms during hypoxia. One is clearly by a delayed reduction in Na^+-K^+-ATPase hydrolytic activity. Notably, this appears to occur by posttranslational modification of enzyme activity, because specific Na^+-K^+-ATPase mRNA and protein abundance did not fall in a comparable experiment on *Astronotus ocellatus*, in which branchial Na^+-K^+-ATPase hydrolytic activity was depressed by about 50% after 4 h of hypoxia (37). There is also clearly another mechanism(s), which is (are) more rapid and more persistent, but additional work will be required to determine the relative contributions of O_2 starvation on Na^+-K^+-ATPase transport activity, on channel closing, on changes in lamellar perfusion, and on alterations in the activity of other proteins (e.g., H^+-ATPase, Na^+/H^+, and Na^+/Na^+ exchangers) in the observed responses. Regardless, the bottom line is that *Astronotus ocellatus* can withstand severe hypoxia without a marked disturbance of internal ion status, by simultaneously reducing Na^+ pumping and leak rates at the gills.

The finding that external water pH did not change appreciably during hypoxia, and normoxia restoration demonstrates that the observed decreases in J^{Na}in and J^{Na}out during hypoxia, and later increases during normoxic recovery were not an artifact of changing water pH. Furthermore, recent measurements of internal pH during a comparable hypoxia regime in the same species demonstrate that this is not a confounding factor either. Richards et al. (37) reported moderate acidosis of ~0.15 units in both the blood plasma (extracellular fluid) and the white muscle (intracellular compartment) during hypoxia. By standard theory, this moderate internal acidification would be expected to "increase" Na^+ influx (by stimulated Na^+/H^+ exchange, or stimulated H^+-ATPase/Na^+ channel activity; Refs. 3, 21, 25, 27, 34) so as to correct the acidosis, whereas we observed a "decrease" in Na^+ influx during hypoxia.

Influence of hypoxia on ammonia excretion. Ammonia excretion rates (J^{Amm}) were much higher on an absolute basis than Na^+ influx rates (J^{Na}in) (Figs. 1–4), so any linkage of J^{Amm} to J^{Na}in, if it occurs in the oscar, must be indirect. During normoxia, J^{Amm} exhibited a Michaelis-Menten type dependence on external Na^+ concentration (Fig. 5*B*), but the apparent Km was significantly lower (i.e., affinity for Na^+ was higher) than for the simultaneously determined Michaelis-Menten dependence of J^{Na}in on external Na^+ concentration,

whereas the Jmax values were similar (Fig. 5*A*). Again, this result suggests that the coupling must be indirect rather than through a direct 1:1 Na^+/NH_4^+ exchanger (e.g., 21, 27, 60). Regardless, the coupling is rapidly effective, such that experimental increases in water $[Na^+]$ quickly constrain an increase in J^{Amm}. This is not seen in the rainbow trout (39), where arguments have been made for linkage either via a nonobligatory Na^+/NH_4^+ exchanger (39) or via "diffusion-trapping" of NH_3-linked to an H^+-pump/Na^+-channel system (3, 54).

During hypoxia, J^{Amm} was reduced in a similar fashion to $J^{Na}in$ and $J^{Na}out$, and as with these Na^+ fluxes, the inhibition became more intense during prolonged hypoxia and usually persisted during the first hour of restoration of normoxia (Figs. 1–4), again arguing for some sort of common mechanism. However, the responses often differed in exact time course and magnitude, and the apparent kinetic coupling of J^{Amm} to external Na^+ concentration was completely lost during prolonged hypoxia (Fig. 5*B*). It remains to be determined whether the declines in J^{Amm} during hypoxia were driven primarily by a downregulation of the ammonia production rate (47, 48), or by specific hypoxia-induced blockade of the branchial ammonia excretion mechanism (e.g., by down-regulation of a Na^+-linked transport system or channel arrest).

For several reasons, it appears likely that both phenomena were involved and that the latter predominated. Urea-N excretion (J^{Urea}), although it represented only a small fraction of N-waste excretion, was reduced whenever J^{Amm} was reduced during hypoxic exposures (Fig. 6). Urea arises from different metabolic pathways than ammonia in teleost fish (uricolysis or arginolysis rather than transdeamination or adenylate breakdown; see Refs. 51 and 56 for reviews). This suggests that a general reduction in metabolic N-waste production occurs during hypoxia, in parallel with depressed protein synthesis in many tissues (24). Likely, oxidation of N-rich fuels is reduced, while carbohydrate utilization is increased during hypoxia in accord with a general suppression of aerobic metabolic rate, reduction in protein turnover, and activation of anaerobic metabolism. However, the relative reduction in J^{Amm} was greater than in J^{Urea} (Fig. 7). Second, if the reduction in J^{Amm} were simply a consequence of reduced production, it seems unlikely that it should persist during the first hour of normoxia restoration when aerobic metabolism was likely restored. And most cogently, plasma total ammonia concentration increased significantly by 3 h of hypoxic exposure (Fig. 7*D*), suggesting that the excretion mechanism was inhibited to a greater extent than the production mechanism. Nevertheless, the change in plasma ammonia was not large, so again the hypoxia-tolerant oscar appears to be rather good at maintaining internal homeostasis during severe O_2 limitation.

ACKNOWLEDGMENTS

We especially thank Sylvia Wood, Maria de Nazeré Paula da Silva, Gudrun de Boeck, Marisa Fernandes Castilho, Ana Cristina Leite Menezes, Linda Diao, and Sunita Nadella for help with experiments and analyses.

GRANTS

Financial support was provided by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants to C. M. Wood and P. J. Walsh, by a National Research Council (CNPq) of Brazil/Amazon State Research Foundation (FAPEAM) Projects of the Nucleus of Excellence Grant to A. L. Val, and by a U.S. National Science Foundation Grant IOB 0455904 to P. J. Walsh. C. M. Wood and P. J. Walsh are supported by the Canada Research Chair Program and V. M. F. Almeida-Val and A. L. Val are supported by CNPq and FAPEAM and are recipients of research fellowships from CNPq. K. A. Sloman received a travel grant from the Royal Society, and M. Kajimura received travel grants from the Society of Experimental Biology and the American Fisheries Society.

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