

## RESEARCH ARTICLE

# Interactions between hypoxia tolerance and food deprivation in Amazonian oscars, *Astronotus ocellatus*

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### SUMMARY

Oscars are often subjected to a combination of low levels of oxygen and fasting during nest-guarding on Amazonian floodplains. We questioned whether this anorexia would aggravate the osmo-respiratory compromise. We compared fed and fasted oscars (10–14 days) in both normoxia and hypoxia (10–20 Torr, 4 h). Routine oxygen consumption rates ( $\dot{M}_{O_2}$ ) were increased by 75% in fasted fish, reflecting behavioural differences, whereas fasting improved hypoxia resistance and critical oxygen tensions ( $P_{crit}$ ) lowered from 54 Torr in fed fish to 34 Torr when fasting. In fed fish, hypoxia reduced liver lipid stores by approximately 50% and total liver energy content by 30%. Fasted fish had a 50% lower hepatosomatic index, resulting in lower total liver protein, glycogen and lipid energy stores under normoxia. Compared with hypoxic fed fish, hypoxic fasted fish only showed reduced liver protein levels and even gained glycogen (+50%) on a per gram basis. This confirms the hypothesis that hypoxia-tolerant fish protect their glycogen stores as much as possible as a safeguard for more prolonged hypoxic events. In general, fasted fish showed lower hydroxyacylCoA dehydrogenase activities compared with fed fish, although this effect was only significant in hypoxic fasted fish. Energy stores and activities of enzymes related to energy metabolism in muscle or gills were not affected. Branchial  $Na^+$  uptake rates were more than two times lower in fed fish, whereas  $Na^+$  efflux was similar. Fed and fasted fish quickly reduced  $Na^+$  uptake and efflux during hypoxia, with fasting fish responding more rapidly. Ammonia excretion and  $K^+$  efflux were reduced under hypoxia, indicating decreased transcellular permeability. Fasted fish had more mitochondria-rich cells (MRC), with larger crypts, indicating the increased importance of the branchial uptake route when feeding is limited. Gill MRC density and surface area were greatly reduced under hypoxia, possibly to reduce ion uptake and efflux rates. Density of mucous cells of normoxic fasted fish was approximately fourfold of that in fed fish. Overall, a 10–14 day fasting period had no negative effects on hypoxia tolerance in oscars, as fasted fish were able to respond more quickly to lower oxygen levels, and reduced branchial permeability effectively.

Key words: respiration, critical oxygen tension, energy metabolism, ionoregulation, ion flux.

Received 20 November 2012; Accepted 2 September 2013

### INTRODUCTION

The Amazon basin is a large body of extremely soft water. The oscar *Astronotus ocellatus* (Agassiz 1831) (acará-açu) often migrates from the main river into the Amazonian floodplains (várzea) when water levels are high. These floodplains are rich in organic material, but fluctuations in oxygen concentrations can be substantial and hypoxic waters are very common. Studies on several fish species, including oscars, have identified multiple adaptive strategies to survive hypoxia and even anoxia (Almeida-Val and Hochachka, 1995; Muusze et al., 1998). Strategies involve substantial metabolic depression and increased use of anaerobic metabolic pathways (Muusze et al., 1998; Almeida-Val et al., 2000; Chippari-Gomes et al., 2005) rather than increased oxygen extraction efficiency (Scott et al., 2008), traits that scale positively with fish size (Almeida-Val et al., 2000; Sloman et al., 2006). Ventilation does increase under hypoxia (Chippari-Gomes et al., 2005; Scott et al., 2008), which puts the fish at risk of losing more ions to their extremely dilute

environment due to the osmo-respiratory compromise (Randall et al., 1972; Nilsson, 2007). However, previous research has shown that under these circumstances oscars are capable of reducing their net ion losses efficiently, thereby decreasing the need for active ion uptake (Wood et al., 2007; Wood et al., 2009). Because branchial ionoregulation is an energetically demanding process responsible for 2–20% of the energy metabolism (Febry and Lutz, 1987), it is important to reduce this cost as much as possible under hypoxic or anoxic conditions; correspondingly, activities of gill and kidney  $Na^+/K^+$ -ATPases are substantially reduced under severe hypoxia (Richards et al., 2007; Wood et al., 2007).

However, ions are not only absorbed through branchial uptake routes, as dietary uptake can also play an important role. In freshwater salmonids, NaCl uptake from food may potentially exceed uptake from the water across the gills (Smith et al., 1989), a fact that was often overlooked until recently (reviewed by Wood and Bucking, 2011). Under the Amazonian soft water conditions,

food might be an important source of ions, especially when the energy available for branchial uptake processes is limited under severe hypoxia.

Fasting is another challenge routinely faced by oscars. On the floodplain, male and female oscars guard their nests vigorously from spawning until hatching (3 days) and while the fry are in the yolk sac phase (4 days), a period where they do not spend any time feeding. Afterwards, they continue to defend their offspring: fry stay together in a school and any that leave are collected in the parents' mouths and 'spit' back into the group. We questioned whether this period of fasting would compromise their abilities to deal with hypoxia and to ionoregulate under the ion-poor conditions of the Amazon. The aim of our research was to examine the impact of fasting (10–14 days) on energy availability and use (oxygen consumption rates, energy reserves, key enzymes in energy metabolism), excretion of metabolic wastes (ammonia, urea) and ionoregulation ( $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$  fluxes). Because both feeding and hypoxia can have a drastic effect on branchial ion uptake, we additionally looked at plasticity in gill morphology, under both normoxia and hypoxia.

Taking into account that oscars voluntarily undergo a period of anorexia during their breeding season, our starting hypotheses were threefold. Firstly, we postulated that oscars would be able to cope well with a period of fasting, possibly by depressing energy metabolism. Secondly, we hypothesized that during this fasting period, branchial ion transport would be upregulated to compensate for the lack of ion uptake from food. Finally, we hypothesized that fasted fish would be less able to handle an additional hypoxic challenge for two reasons: a lack of readily available energy supply for anaerobic metabolism (glycogen stores) and an inability to reduce the hypothesized upregulation of branchial ion fluxes.

## MATERIALS AND METHODS

### Experimental animals

Adult oscars (*Astronotus ocellatus*; 23–197 g) were purchased from Sítio dos Rodrigues (km 35, Rod. AM-010, Brazil). At the Ecophysiology and Molecular Evolution Laboratory of the Instituto Nacional de Pesquisas da Amazônia (INPA), in Manaus, Brazil, the fish were held under a natural photoperiod in groups of approximately 30 fish in 500 l tanks. The holding and experimental waters were taken from a well on the INPA campus (concentrations in  $\mu\text{mol l}^{-1}$ :  $\text{Na}^+$  35,  $\text{Cl}^-$  36,  $\text{K}^+$  16,  $\text{Ca}^{2+}$  18,  $\text{Mg}^{2+}$  4; dissolved organic carbon, 0.6 mg C  $\text{l}^{-1}$ ; pH 6.5) with partial recirculation and continuous filtration, at  $28 \pm 1.5^\circ\text{C}$ . INPA well water is very soft and can be considered typical of Amazonian water (Duncan and Fernandes, 2010). During this period, the fish were fed a daily 1% body mass ration of commercial pellets (Nutripeixe Tr 36, Purina Co, São Paulo, SP, Brazil; nutrient composition available from the manufacturer). Our analysis yielded the following major electrolyte concentrations in the food:  $\text{Na}^+$  657,  $\text{Cl}^-$  457,  $\text{K}^+$  333,  $\text{Ca}^{2+}$  418,  $\text{Mg}^{2+}$  150 mmol  $\text{kg}^{-1}$ . For the fish designated as fed, feeding was suspended 24 h prior to experiments. For the fish designated as fasted, food was withheld for 10–14 days. Fish were always weighed at the end of an experiment, rather than at the start, so as to avoid disturbance. All experimental procedures complied with Brazilian and INPA animal care regulations.

### Respirometry

For respirometry, oscars ( $N=12$  for fed and  $N=11$  for fasted fish) with a mean mass of  $98 \pm 3$  g were used. Experimental chambers were 1.75 l Nalgene kitchen containers. A rubber stopper ensured that the lids accommodated a portable  $\text{O}_2$  probe through an opening without any

leakage (WTW Oxi325 Oximeter, Weilheim, Germany). The 16 chambers were mounted on a trough, which served as an external water bath for temperature control. The entire trough drained into a vigorously aerated 310 l reservoir from which water was pumped back to the individual chambers at approximately  $200 \text{ ml min}^{-1}$ . Water in the reservoir was replaced daily. Each chamber was fitted with an individual water line for flushing, and with an individual air-stone for aeration. Black plastic shielding minimized visual disturbance. Oscars were left overnight to settle with continuous water flow-through and aeration. At the start of measurements, the air-stones and water lines of four respirometers at a time were disconnected and all air bubbles were carefully removed before the  $\text{O}_2$  electrodes (WTW Oxi325 Oximeter, Weilheim, Germany) were inserted and sealed. Measurements were taken every 5 min until  $\text{O}_2$  levels dropped below 5% saturation, after which time measurements were terminated and aeration and water flow were turned back on. Total time for a typical trial was around 2 h (1.5–2.5 h).

When taking a measurement, the oxygen probe was gently stirred in a circular way without disturbing the fish to provide a water current over the membrane until a stable measurement was obtained. To avoid possible effects of stress caused by turning off water flow and aeration and inserting the  $\text{O}_2$  electrode at the start of the experiment, we excluded measurements from the first 15–20 min of the experiment in our calculations. Because  $P_{\text{crit}}$  was found to be below 55 Torr (7332.71 Pa; 1 Torr=133.32 Pa), we considered oxygen consumption rates ( $\dot{M}_{\text{O}_2}$ ) from water  $\text{O}_2$  concentrations above 55 Torr to be normoxic. Therefore, we averaged all values between 15 and 20 min after onset of the measurements until 55 Torr was reached for the calculation of  $\dot{M}_{\text{O}_2}$ .  $\dot{M}_{\text{O}_2}$  ( $\mu\text{mol g}^{-1} \text{ h}^{-1}$ ) was calculated using the formula:

$$\dot{M}_{\text{O}_2} = \frac{\Delta\text{O}_2 \times V}{M}, \quad (1)$$

where  $\Delta\text{O}_2$  is the change in dissolved  $\text{O}_2$  concentration over time ( $\mu\text{mol l}^{-1} \text{ h}^{-1}$ ),  $V$  is respirometer volume (l) and  $M$  is individual fish mass (g).

$P_{\text{crit}}$  was calculated according to the BASIC program developed by Yeager and Ultsch (Yeager and Ultsch, 1989), which uses stepwise regression to calculate the two best-fit regression lines, and then determines the point (water oxygen level) at which they intersect.

### Ion fluxes

Flux experiments were performed in a similar setup as described above for respirometry. Oscars ( $N=11$  for fed and  $N=12$  for fasted fish) with a mean mass of  $111 \pm 7$  g were used. Fish were placed in their individual containers the evening before an experiment and left overnight to settle. In this setup, experimental chambers were Nalgene kitchen containers, again shielded with black plastic, and fitted with an individual water line for flushing and an individual air-stone for air or  $\text{N}_2$ -gassing. These chambers fitted the laterally compressed morphology of the fish and had a volume of 2.5 l. The lids could accommodate one of the portable  $\text{O}_2$  probes as described above. The chambers were again mounted on a trough that served as a water bath for temperature control. This time the trough drained into a vigorously aerated 800 l reservoir, from which water was pumped back to the individual chambers at approximately  $200 \text{ ml min}^{-1}$ . Water in the reservoir was replaced daily and experimental temperature was  $28 \pm 1.5^\circ\text{C}$ .

At the start of a flux experiment, the water flow was stopped but aeration continued, and the level was set to a nominal volume of 1.5 l. Exact volumes were determined by subtracting the mass of the fish

when they were weighed at the end of the experiment. An aliquot of  $^{22}\text{Na}$  (manufactured by New England Nuclear, Dupont, Boston, MA, USA, and supplied by REM, São Paulo, Brazil) was added to each container ( $2\ \mu\text{Ci l}^{-1}$ ) and allowed to equilibrate for 1 h. Flux rates were measured by withdrawing  $2\times 10\ \text{ml}$  water samples for assay at the start of the experiment (0 h) and at subsequent 1 h intervals. Water samples were analyzed for  $^{22}\text{Na}$  radioactivity, and the concentrations of total  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ , ammonia and urea. The standard protocol was a 3 h control period of normoxia, followed by a 4 h period of acute hypoxia, with simultaneous hourly measurements of unidirectional  $\text{Na}^+$  fluxes with  $^{22}\text{Na}$ , and net  $\text{K}^+$ ,  $\text{Cl}^-$ , ammonia and urea fluxes. Normoxia ( $\dot{P}_{\text{O}_2} > 130\ \text{Torr}$ ) was maintained by vigorous aeration during the normoxic periods, and water  $\dot{P}_{\text{O}_2}$  was checked once per hour in each chamber with the portable  $\text{O}_2$  probe. Acute hypoxia was induced by changing the vigorous gassing to  $\text{N}_2$ , and then maintaining the  $\dot{P}_{\text{O}_2}$  between 10 and 20 Torr with more gentle gassing with  $\text{N}_2$  or air as required. During the hypoxia period, water  $\dot{P}_{\text{O}_2}$  was checked in each chamber every 15 min.

Water total ammonia and urea concentrations were measured colorimetrically by the salicylate hypochlorite assay (Verdouw et al., 1978) and the diacetyl monoxime assay (Rahmatullah and Boyde, 1980), respectively.  $\text{Na}^+$  and  $\text{K}^+$  concentrations in water were measured by flame atomic absorption spectrophotometry (AAAnalyst 800, Perkin-Elmer, Wellesley, MA, USA) and  $\text{Cl}^-$  concentrations by the colorimetric assay of Zall et al. (Zall et al., 1956). Radioactivity measurements ( $^{22}\text{Na}$ ) were made by scintillation counting (LS6500, Beckman Coulter, Fullerton, CA, USA) on sample volumes of 5 ml added to 5 ml of Packard Ultima Gold AB Fluor (Perkin-Elmer). Internal standardization tests demonstrated that quenching was constant, so no correction was necessary.

Net flux rates ( $\mu\text{mol kg}^{-1}\ \text{h}^{-1}$ ) of  $\text{Na}^+$  ( $J_{\text{net}}^{\text{Na}}$ ),  $\text{K}^+$ ,  $\text{Cl}^-$ , total ammonia and urea were calculated using the formula:

$$J_{\text{net}}^{\text{Na}} = \frac{\Delta[S] \times V}{M}, \quad (2)$$

where  $\Delta[S]$  is the change in concentration of the measured substance over time ( $\mu\text{mol l}^{-1}\ \text{h}^{-1}$ ),  $V$  is respirometer volume (l) and  $M$  is individual fish mass (kg). We employed the traditional method for determining unidirectional  $\text{Na}^+$  fluxes (Kirschner, 1970; Wood, 1992), which measures influx ( $J_{\text{in}}^{\text{Na}}$ , by convention positive) directly by disappearance of  $^{22}\text{Na}$  radioactivity from the external water:

$$J_{\text{in}}^{\text{Na}} = \frac{([\text{CPM}_i] - [\text{CPM}_f])(V)}{(\text{SA}_{\text{ext}})(T)(M)}, \quad (3)$$

and calculates efflux ( $J_{\text{out}}^{\text{Na}}$ , by convention negative) by difference using the conservation equation:

$$J_{\text{in}}^{\text{Na}} + J_{\text{out}}^{\text{Na}} = J_{\text{net}}, \quad (4)$$

where  $\text{CPM}_i$  and  $\text{CPM}_f$  are the initial and final  $^{22}\text{Na}$  radioactivity in the water ( $\text{cpm l}^{-1}$ ) at the start and end of the flux period, respectively;  $V$  is the volume of water (l);  $\text{SA}_{\text{ext}}$  is the mean external specific activity ( $^{22}\text{Na}$  per total  $\text{Na}^+$ ) in the water ( $\text{cpm } \mu\text{mol}^{-1}$ ), calculated from measurements of water  $^{22}\text{Na}$  radioactivity and total water  $[\text{Na}^+]_{\text{ext}}$  at the start and end of the flux period;  $T$  is the time of flux period (h); and  $M$  is the mass of the fish (kg).

#### Tissue sampling for gill morphology, energy stores and enzyme analysis

For terminal tissue sampling, 24 fish were used ( $N=12$  for each feeding regime) with a mean mass of  $60\pm 4\ \text{g}$ . Oscars were placed in the respirometry containers the evening before an experiment and left

overnight to settle with continuous water flow-through and aeration. Fish were then killed under normoxia ( $N=6$  for each feeding regime) or after 3 h of hypoxia ( $\dot{P}_{\text{O}_2}=10\text{--}20\ \text{Torr}$ , imposed as above;  $N=6$  for each feeding regime). The fish were anaesthetized in  $0.5\ \text{g l}^{-1}$  MS-222 neutralized with  $1.0\ \text{g l}^{-1}$   $\text{NaHCO}_3$ , and then killed by cephalic concussion. Fish were weighed, the second gill arch from the right-hand side of each fish was excised and the middle parts of the arches ( $\sim 5\text{--}8\ \text{mm}$ ) were cut out. Samples were quickly rinsed in water, then immediately placed in cold Karnovsky's fixative for storage at  $4^\circ\text{C}$ . The samples were later shipped to San Diego State University, CA, USA, for examination by scanning electron microscopy (see below). The remaining gill arches were excised, and filaments were separated from the arch, flash-frozen in liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$  until later analysis for energy stores and enzymes. The liver was removed, weighed so as to provide the hepato-somatic index (HIS; % of liver mass compared with total mass), and a piece of white muscle was excised between the midline and the dorsal fin. Both tissues were flash-frozen in liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$  until later analysis for energy stores and enzymatic activities.

#### Scanning electron microscopy

After arriving at San Diego State University, the middle parts of the gills were quickly rinsed in PBS buffer, post-fixed for 1 h in 1% osmium tetroxide, dehydrated in a series of ascending concentrations of ethanol from 30 to 100%, critical-point dried with liquid  $\text{CO}_2$ , mounted on stubs, sputter-coated with gold-palladium, and examined with a Hitachi S 2700 electron microscope (Tokyo, Japan) at the accelerating voltage of 20 kV.

The surface ultrastructure of the trailing (afferent) edge of the filaments located below respiratory lamellae was examined. Quantification of the density of mitochondria-rich cells (MRCs; number of MRCs per  $\text{mm}^2$ ) and of mucous cells (MCs; number of MCs per  $\text{mm}^2$ ) was determined on SEM micrographs ( $2000\times$  magnification) by counting the number of apexes of MRCs and pores of MCs on five randomly selected rectangular areas of filament epithelium of six fish (total number of measurements=30). The surface areas of 30 individual MRCs were calculated on photographs at  $6000\times$  magnification according to the shape of their apical openings. These openings varied from circular to oval, triangular and roughly trapezoidal.

#### Metabolite and enzyme assays

A portion of each gill, liver and white muscle sample was homogenized on ice in four volumes of ice-cold Tris buffer ( $10\ \text{mmol l}^{-1}$  Tris-HCl,  $85\ \text{mmol l}^{-1}$  NaCl, pH 7.4). Aliquots were analyzed for protein using Bradford's reagent (Bradford, 1976) and glycogen using Anthrone reagent (Roe and Dailey, 1966), and total lipids were extracted and measured following Bligh and Dyer (Bligh and Dyer, 1959). For enzyme analysis, the remaining tissue was homogenized in nine volumes of a medium containing  $75\ \text{mmol l}^{-1}$  Tris and  $1\ \text{mmol l}^{-1}$  EDTA at pH 7.6. All assays were conducted on crude homogenates or dilutions thereof. Maximal enzyme activity levels were measured at  $22\text{--}25^\circ\text{C}$ , using either a Shimadzu UV-240 or a Gilford 2600 spectrophotometer. All enzyme assays had a final cuvette volume of 1 ml. Lactate dehydrogenase (LDH), glutamate dehydrogenase (GDH) and hydroxyacylCoA dehydrogenase (HOAD) enzyme activities were measured following the oxidation of NADH at 340 nm, and citrate synthase (CS) enzyme activities were based on the increase of free coenzyme A combined with DTNB [5,5-dithio-bis(2-nitrobenzoic acid)] measured at 412 nm. Assay conditions were based on well-established protocols for fish tissues (Sidell et al., 1987).



Compositions of the assay media were as follows (in concentrations of  $\text{mmol l}^{-1}$ ): (1) LDH (EC 1.1.1.27): 50 imidazole, 1 KCN and 0.15 NADH, pH 7.4; the reaction was initiated with 1  $\text{mmol l}^{-1}$  pyruvate; (2) HOAD (EC 1.1.1.35): 50 imidazole, 1 EDTA, 1 KCN and 0.15 NADH, pH 7.5; the reaction was initiated with 0.1  $\text{mmol l}^{-1}$  acetoacetyl CoA; (3) CS (EC 4.1.3.7): 75 Tris, 0.25 DTNB and 0.4 acetyl CoA, pH 8.0; the reaction was initiated with 0.5  $\text{mmol l}^{-1}$  oxaloacetate; and (4) GDH (EC 1.4.1.3): 50 imidazole, 250 ammoniumacetate, 0.1 EDTA, 1 ADP, 0.1 NADH and 10 2-oxoglutarate, pH 7.2.

### Statistics

All data are reported as means  $\pm$  1 s.e.m. ( $N$ =number of fish). Relationships were assessed by two-way ANOVA followed by an uncorrected Fisher's least significant difference (LSD) test (GraphPad Prism 6.00, GraphPad Software, La Jolla, CA, USA). Student's two-tailed  $t$ -test was used for single comparisons between respiration data. A significance level of  $P \leq 0.05$  was used throughout.

## RESULTS

### Effects of fasting and hypoxia on energy metabolism

Normoxic routine  $\dot{M}_{\text{O}_2}$  was significantly higher in fasted oscars compared with fed oscars, with rates of  $4.09 \pm 0.20 \mu\text{mol g}^{-1} \text{h}^{-1}$  for fasted fish and  $2.81 \pm 0.18 \mu\text{mol g}^{-1} \text{h}^{-1}$  for fed fish ( $P < 0.001$ ), respectively. This seemed to be largely due to behavioural differences. Fed oscars rested calmly on one side in the respirometers, while fasted fish were upright, and used continuous fin movement to maintain position. This difference in behaviour was not observed in the holding tanks, where fed and fasted oscars were equally active. Despite the higher  $\dot{M}_{\text{O}_2}$  under normoxia, fasting seemed to have a protective effect during hypoxia, as fasted oscars were able to regulate their  $\dot{M}_{\text{O}_2}$  down to lower environmental  $\text{O}_2$  levels, resulting in a significantly lower  $P_{\text{crit}}$ :  $33.6 \pm 3.7$  compared with  $53.8 \pm 8.3$  Torr, respectively ( $P < 0.05$ ; Fig. 1).

Fasting or short-term hypoxia had no effect on energy stores in muscle or gill (Table 1). In normoxic fish, fasting alone did not have much effect on liver energy stores when expressed on a per gram basis (Table 2). However, the HSI decreased drastically from  $1.9 \pm 0.1$  to  $0.9 \pm 0.1$  ( $P < 0.001$ ), meaning that overall energy stores in the liver were reduced to close to half of their original level. Total liver protein ( $P < 0.001$ ), glycogen ( $P < 0.01$ ) and lipid ( $P < 0.01$ ) all decreased substantially (Table 2). When comparing hypoxic fed and fasted fish, liver protein was reduced in fasted compared with fed oscars ( $P < 0.05$  when expressed as  $\text{mg g}^{-1}$  tissue,  $P < 0.001$  when expressed per fish). On a  $\text{mg g}^{-1}$  basis, liver glycogen increased ( $P < 0.05$ ) in hypoxic fasted fish. However, this increase was no longer present when taking into account the reduced HSI.

The effects of hypoxia were much smaller. When looking at energy stores in normoxic fish compared with hypoxic fish, liver lipids were decreased by acute hypoxia in fed fish ( $P < 0.05$  when expressed as  $\text{mg g}^{-1}$  tissue,  $P < 0.01$  when expressed per fish). Because of the already reduced energy stores in fasting fish, no significant differences occurred between normoxic and hypoxic fish. There were no interactive effects between feeding status and oxygen level.

Enzyme activities in liver and muscle were not significantly affected by hypoxia (Table 3). Although fasting caused HOAD activities in liver to decrease by approximately 50%, this effect was only significant in hypoxic fish ( $P < 0.05$ ).

### Effects of fasting and hypoxia on ion and metabolite fluxes

Under normoxia, branchial  $\text{Na}^+$  influx was on average more than double in fasted fish compared with fed fish, whereas

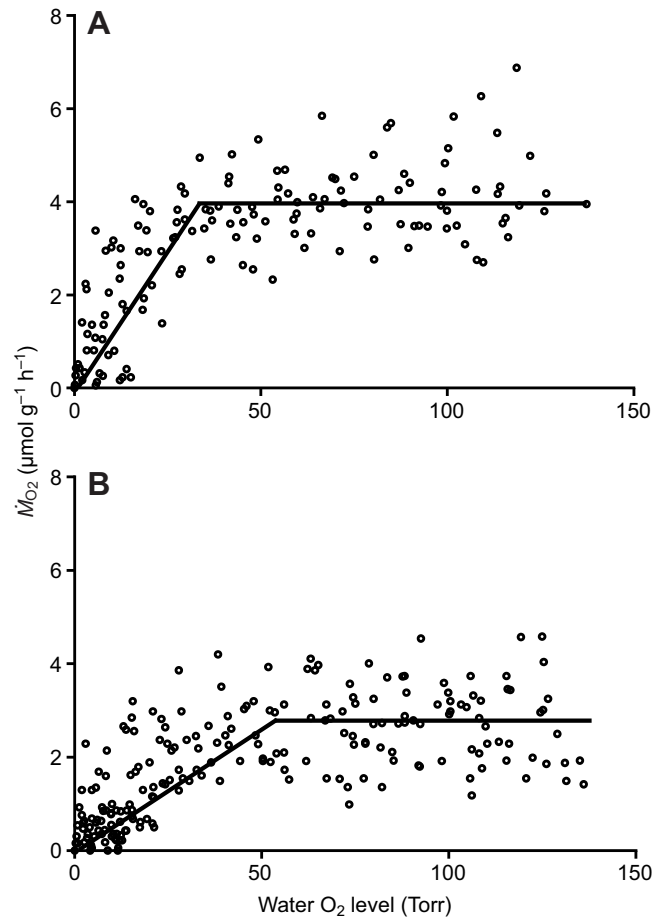


Fig. 1. Relationship between water  $\text{O}_2$  level (Torr) and oxygen consumption rate ( $\dot{M}_{\text{O}_2}$ ;  $\mu\text{mol g}^{-1} \text{h}^{-1}$ ). Regression lines show the average critical oxygen tensions ( $P_{\text{crit}}$ ) calculated from individual  $P_{\text{crit}}$  of oscars that were (A) fasted for 10–14 days ( $P_{\text{crit}} = 33.61 \pm 3.73$  Torr,  $N = 10$ ) or (B) fed up to 24 h before the start of the experiment ( $P_{\text{crit}} = 53.80 \pm 8.26$  Torr,  $N = 8$ ). Data points of all fish are shown.

unidirectional efflux rates were approximately equal (Fig. 2). This resulted in a net  $\text{Na}^+$  gain for fasted oscars, while fed oscars actually experienced a net loss of  $\text{Na}^+$  at the gills. Exposure to hypoxia caused an immediate decrease in  $\text{Na}^+$  influx in fasted fish followed by a significant, but smaller decrease in  $\text{Na}^+$  efflux from the second hour of exposure onwards. As a result, fasted fish started to experience a small net loss in  $\text{Na}^+$  during hypoxia, albeit to a lesser extent than in fed fish. Fed oscars had a lower  $\text{Na}^+$  influx to start with, and this was not affected by hypoxia until the third hour of exposure. Influx and efflux were subsequently reduced in a similar manner, and fed fish seemed to be more capable than fasted fish of reducing  $\text{Na}^+$  efflux. As a result, branchial net  $\text{Na}^+$  flux experienced little change, resulting in a steady net  $\text{Na}^+$  loss.

Under normoxia, fed oscars lost significantly less  $\text{K}^+$  to the water on a net basis than fasted oscars (Fig. 3). Both groups greatly reduced  $\text{K}^+$  loss during hypoxia, but losses remained larger in fasted fish than fed fish. Net  $\text{Cl}^-$  fluxes (Fig. 4) were much more variable compared with  $\text{Na}^+$  and  $\text{K}^+$  fluxes. However, under normoxic conditions, fasted fish ( $N = 11$ ) showed on average a net  $\text{Cl}^-$  uptake ( $+13.3 \pm 20.8 \mu\text{mol kg}^{-1} \text{h}^{-1}$ ) while fed fish ( $N = 10$ ) lost  $\text{Cl}^-$  ( $-46.0 \pm 24.7 \mu\text{mol kg}^{-1} \text{h}^{-1}$ ) on a net basis. Under hypoxic conditions, both fasted and fed oscars experienced net  $\text{Cl}^-$  losses

Table 1. Energy stores (mg g<sup>-1</sup>) in muscle and gill tissue of fed and fasted oscars exposed to normoxia or acute hypoxia

	Fed		Fasted	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Protein				
Muscle	95.6±18.2	63.7±11.2	68.8±9.7	69.8±16.8
Gill	60.8±2.9	66.9±3.9	60.6±4.2	66.9±4.8
Glycogen				
Muscle	1.9±0.4	2.6±0.5	1.8±0.6	3.2±0.7
Gill	3.6±0.2	3.7±0.2	3.6±0.1	4.3±0.4
Lipid				
Muscle	5.2±0.7	3.6±0.4	4.9±0.5	5.4±1.0
Gill	30.3±4.2	25.8±3.9	35.5±4.8	32.8±3.7

Values are means ± s.e.m. (N=6). No significant differences were detected.

averaging  $-54.1 \pm 13.9$  and  $-86.5 \pm 15.5 \mu\text{mol kg}^{-1} \text{h}^{-1}$ , respectively. Only the change in fasted fish was significant ( $P < 0.02$ ).

As with oxygen consumption, ammonia excretion rates were significantly greater in fasted fish compared with fed fish under normoxic conditions (Fig. 5). Under hypoxic conditions, oscars reduced ammonia excretion immediately. Fasted fish did this more efficiently during the first hours of hypoxia exposure with a  $\pm 85\%$  drop compared with normoxic excretion rates in fasted fish, while fed fish showed only a 60% drop. Thereafter, the difference between the two groups gradually disappeared because both reduced excretion rates to approximately 20% of their respective pre-hypoxic rate after 4 h of hypoxia. Interestingly, urea-N fluxes, which were less than 20% of ammonia fluxes, showed the opposite trend under normoxia, with more excretion in fed fish compared with fasted fish (Fig. 6). Excretion rates were downregulated under hypoxic conditions in fed fish, while no significant change occurred in fasted fish.

#### Effects of fasting and hypoxia on gill morphology

Although general gill morphology was similar in fasted and fed oscars (Fig. 7A,D), fasting did alter the density and surface area of MRCs. Fasted oscars had a significantly higher density of MRCs

on both the filament epithelium and the lamellar epithelium (Table 4). These MRCs also exhibited larger apical crypts (Fig. 7B,C), sometimes even clustering together. This resulted in a 45% larger surface area (Table 4) exposed to the surrounding water in fasted relative to fed oscars. Fasted oscars also had numerous large MCs bulging out into the water (Fig. 6C, Table 4). These were much less obvious in fed oscars (Fig. 6F).

Exposure to hypoxia greatly reduced the number of exposed MRCs (Fig. 8, Table 4), which now only appeared on the filament epithelium in both fed and fasted oscars. The decrease in number was more pronounced in fed oscars, with a 47% drop compared with a 38% drop in fasted oscars (Table 4). Additionally, the surface area of the apical crypts decreased dramatically in both fasted and fed oscars (Fig. 8C,F). Again, this was more pronounced in fed fish, with a 65% decrease compared with a 40% decrease for fasted fish (Table 4). Fig. 8C and 8F clearly show the reduced surface area and the deep, highly concave MRC apical crypts.

#### DISCUSSION

We originally hypothesised that oscars would be able to handle a period of fasting rather well, possibly by depressing energy

Table 2. Liver energy stores (mg), liver energy content (kJ) and hepatosomatic index (HSI) in fed and fasted oscars exposed to normoxia and acute hypoxia

	Fed		Fasted	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Energy stores (mg g <sup>-1</sup> )				
Protein	93.4±6.5	98.5±8.4	89.6±5.4	75.3±4.4 <sup>#</sup>
Glycogen	221.5±15.0	172.2±39.0	257.2±21.6	260.2±15.2 <sup>#</sup>
Lipid	75.6±16.0	35.7±6.0 <sup>*</sup>	58.7±6.2	35.4±7.0
Energy content (kJ g <sup>-1</sup> )				
Protein	1.64±0.11	1.73±0.15	1.58±0.10	1.33±0.08 <sup>#</sup>
Glycogen	3.79±0.26	2.95±0.67	4.40±0.37	4.45±0.26 <sup>#</sup>
Lipid	2.94±0.62	1.39±0.21 <sup>*</sup>	2.28±0.24	1.38±0.27
Total energy (kJ g <sup>-1</sup> )	8.37±0.77	5.84±0.75 <sup>**</sup>	8.26±0.29	7.15±0.33
HSI		1.9±0.1		0.9±0.1 <sup>###</sup>
Energy stores (mg fish <sup>-1</sup> )				
Protein	107.8±7.5	113.7±9.7	47.7±2.9 <sup>###</sup>	40.1±2.3 <sup>###</sup>
Glycogen	255.6±17.3	198.8±45.0	136.9±11.6 <sup>###</sup>	138.5±8.1
Lipid	87.3±18.4	41.2±6.3 <sup>**</sup>	31.2±3.3 <sup>###</sup>	18.9±3.7
Energy content (kJ fish <sup>-1</sup> )				
Protein	1.90±0.13	2.00±0.17	0.84±0.05 <sup>###</sup>	0.71±0.04 <sup>###</sup>
Glycogen	4.37±0.30	3.40±0.77	2.34±0.20 <sup>###</sup>	2.37±0.14
Lipid	3.40±0.72	1.60±0.25 <sup>**</sup>	1.21±0.13 <sup>###</sup>	0.73±0.14
Total energy (kJ fish <sup>-1</sup> )	9.67±0.89	6.73±0.86 <sup>**</sup>	4.39±0.16 <sup>###</sup>	3.81±0.18 <sup>###</sup>

Values are expressed as mg or kJ per gram liver and as mg or kJ per standard 60 g fish (fed fish mass was  $62.7 \pm 6.1$  g and fasted fish mass was  $59.2 \pm 6.5$  g).

Values are means ± s.e.m. (N=6). <sup>#</sup>Significant difference between fed and fasted within the same oxygenation level; <sup>\*</sup>significant difference between normoxia and hypoxia within the same feeding ration (<sup>#</sup> $P < 0.05$ ; <sup>###</sup> $P < 0.01$ ; <sup>####</sup> $P < 0.001$ )

Table 3. Enzyme activities ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein) in tissues of fed and fasted oscars exposed to normoxia or acute hypoxia

	Fed		Fasted	
	Normoxia	Hypoxia	Normoxia	Hypoxia
CS				
Liver	0.789±0.118	0.702±0.105	0.625±0.077	0.626±0.054
Muscle	1.293±0.102	1.141±0.177	1.071±0.095	1.232±0.149
GDH				
Liver	0.317±0.112	0.195±0.061	0.368±0.185	0.233±0.012
Muscle	0.245±0.066	0.282±0.088	0.192±0.035	0.264±0.031
LDH				
Liver	0.201±0.097	0.171±0.031	0.198±0.032	0.183±0.020
Muscle	0.010±0.005	0.014±0.006	0.015±0.006	0.007±0.002
HOAD				
Liver	2.311±0.605	2.996±0.634	1.269±0.535	1.230±0.314 <sup>#</sup>
Muscle	3.466±1.378	2.823±1.422	2.285±1.311	1.371±0.513

CS: cytrate synthase; GDH: glutamate dehydrogenase; LDH: lactate dehydrogenase; HOAD: hydroxyacylCoA dehydrogenase.

Values are means  $\pm$  s.e.m. ( $N=6$ ). <sup>#</sup>Significant difference between fed and fasted within the same oxygenation level ( $P<0.05$ ).

metabolism. From our results we can conclude that oscars indeed coped well with 10–14 days of food deprivation. However, they did not do this by depressing their energy metabolism, but rather by using liver lipid and protein stores and thus conserving glycogen stores. Conserving glycogen stores seems to be a general strategy in hypoxia-tolerant fish, as was already noted in crucian carp and goldfish (Nilsson, 1990; Liew et al., 2012). Our second assumption, that fasting oscars would upregulate branchial ion uptake under normoxic conditions, was confirmed. Fasting fish increased  $\text{Na}^+$  influx, leading to a net  $\text{Na}^+$  uptake, which was associated with an increased number and surface area of MRCs. Finally, and contrary to our third hypothesis, fasting oscars were able to withstand hypoxia rather well, with a lower  $P_{\text{crit}}$  than fed fish. As a result, the regression line for oxygen consumption below  $P_{\text{crit}}$  has a much steeper slope in the fasted fish, indicating that diffusion of  $\text{O}_2$  was higher at low environmental oxygen levels compared with that in fed fish. Nevertheless, we measured a quicker reduction in transcellular permeability and a faster reduction in ammonia excretion. It seems that transcellular ion transport is reduced, while oxygen diffusion is not.

In mammals and birds, metabolic transitions in food deprivation have been divided into three phases, and the transition to starvation occurs somewhere around the end of phase II or by the start of phase III. In phase I, the post-absorptive stage, metabolism is largely

fuelled by glycogen depletion of liver stores and fatty acids from adipose tissue. Phase II starts when glycogen stores are depleted and gluconeogenesis becomes necessary to fuel glucose-requiring organs such as the brain. At first, amino acids from protein are used, and subsequently glycerol from adipose tissues starts to play an important role, although protein degradation remains slow compared with phase III. Phase III is detrimental, and starts when all adipose tissue is depleted and muscle protein starts to be degraded, with high nitrogen excretion levels as a consequence (Wang et al., 2006). Although not thoroughly investigated for ectothermic vertebrates, it appears that the overall progression in metabolic adaptation is similar for most vertebrates, but occurs at a slower time scale in ectotherms (Wang et al., 2006).

Voluntary anorexia, such as in breeding oscars, should be seen as fasting rather than starvation, and it rarely enters phase III. Oscars fasted for 10–14 days in our study had only used approximately 40% of their liver glycogen, and approximately 60% of both liver protein and lipids. Although liver glycogen was not depleted, and was not the primary substrate of choice, we would place these oscars at the beginning of phase II, with protein and lipid degradation both playing an equally important role. This is in contrast with the general belief that in fish, most species use fat before protein is degraded (Jobling, 1980; Mendez and Wieser, 1993; Brett, 1995; Cook et al.,

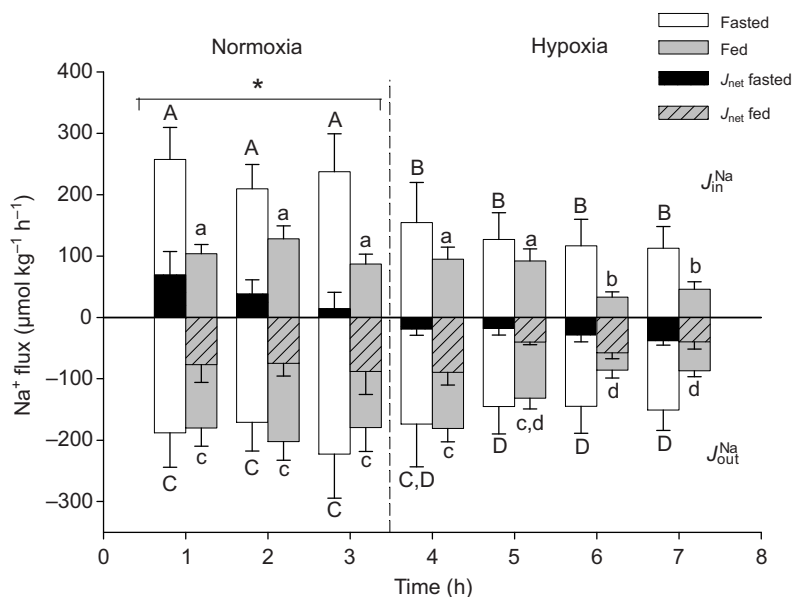


Fig. 2. Changes in unidirectional ( $J_{\text{out}}^{\text{Na}}$ ,  $J_{\text{in}}^{\text{Na}}$ ) and net flux rates ( $J_{\text{net}}^{\text{Na}}$ ) of  $\text{Na}^+$  in oscars that were either fasted for 10–14 days or fed up to 24 h before the start of the experiment.  $J_{\text{out}}^{\text{Na}}$  values are downward bars (white for fasted and grey for fed fish),  $J_{\text{in}}^{\text{Na}}$  values are upward bars (white for fasted and grey for fed fish) and  $J_{\text{net}}^{\text{Na}}$  values are black (fasted fish) or grey hatched bars (fed fish). The fish were exposed to normoxia ( $P_{\text{O}_2}=130\text{--}150$  Torr) followed by acute hypoxia ( $P_{\text{O}_2}=10\text{--}20$  Torr). Data are means  $\pm$  1 s.e.m. ( $N=9\text{--}12$ ). Asterisk indicates a significant difference ( $*P\leq 0.05$ ) between normoxic fed and fasted fish at the same time period, and letters indicate a significant difference ( $P\leq 0.05$ ) between normoxic and hypoxic flux rates (uppercase for fasted fish, lowercase for fed fish).

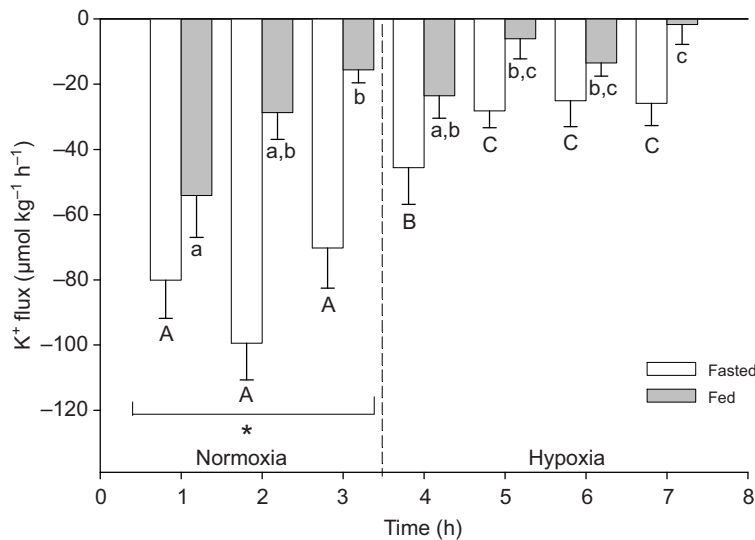


Fig. 3. Changes in net flux rates of K<sup>+</sup> in oscar fish that were either fasted for 10–14 days (white bars) or fed up to 24 h before the start of the experiment (grey bars). The fish were exposed to normoxia (P<sub>O<sub>2</sub></sub>=130–150 Torr) followed by acute hypoxia (P<sub>O<sub>2</sub></sub>=10–20 Torr). Data are means ± 1 s.e.m. (N=9–12). Asterisk indicates a significant difference (\*P≤0.05) between normoxic fed and fasted fish at the same time period, and different letters indicate a significant difference (P≤0.05) between normoxic and hypoxic flux rates (uppercase for fasted fish, lowercase for fed fish).

2000, Figueiredo-Garutti et al., 2002), or that glycogen is used as a first energy source even before lipid and protein, as in mammals (Navarro and Gutierrez, 1995). In fact, HOAD activity was reduced by 50% in the liver of fasting oscar fish, indicating a reduction in mitochondrial beta oxidation, and thus supporting the conclusion that protein rather than fat was the prime source of energy at this stage. However, it is important to realise that the compositional method measures the fuels depleted, not necessarily the fuels burned, because of possible interconversion and excretion of fuels, as was clearly demonstrated by Lauff and Wood (Lauff and Wood, 1996). In general, it seems that hypoxia-/anoxia-tolerant fish are different in that they tend to conserve liver glycogen when fasted in anticipation of possible hypoxic/anoxic events where glycogenolysis is essential for survival, as was unambiguously described in the anoxia-tolerant crucian carp (Nilsson, 1990). A recent comparative study between fasting common carp and goldfish also proved that fasting almost completely depleted glycogen stores in common carp, but not in the anoxia-tolerant goldfish (Liew et al., 2012). In that study, both species also used liver protein for basal metabolism during fasting. Overall, it seems that fish that fast during migration, such as salmonids and eels, tend to conserve protein and use glycogen and lipids, while anoxia-tolerant species, such as crucian

carp, goldfish and oscar fish, conserve glycogen when fasting and use lipids and protein instead.

Notably, the capacity of oscar fish to deal with hypoxia was not compromised by food deprivation as fasted fish had a lower P<sub>crit</sub> than fed oscar fish, and thus managed to maintain their oxygen consumption rates down to lower environmental oxygen levels. The remaining glycogen stores seemed sufficient to fuel the depressed energy metabolism under hypoxia. Very few changes occurred in energy stores under the few hours of severe hypoxia, except for a decrease in total liver lipid and total energy content. Earlier studies on *A. ocellatus* and *Astronotus crassipinnis* exposed to hypoxia levels between 7 and 15 Torr for 8–20 h showed a depressed energy metabolism with increased plasma glucose and lactate and muscle lactate levels, but without many changes in tissue energy stores (Chippari-Gomes et al., 2005; Richards et al., 2007). In *A. crassipinnis*, liver glycogen dropped after 3 h of hypoxia at 7 Torr, but not at 15 Torr (Chippari-Gomes et al., 2005), and in *A. ocellatus* liver glycogen levels returned to control levels after 20 h, following an initial increase at 7 Torr (Richards et al., 2007). There was no change in LDH activity either, despite the elevated lactate levels. This made the authors wonder about the metabolic fuel that was utilised to support the suppressed metabolism (Richards et al., 2007).

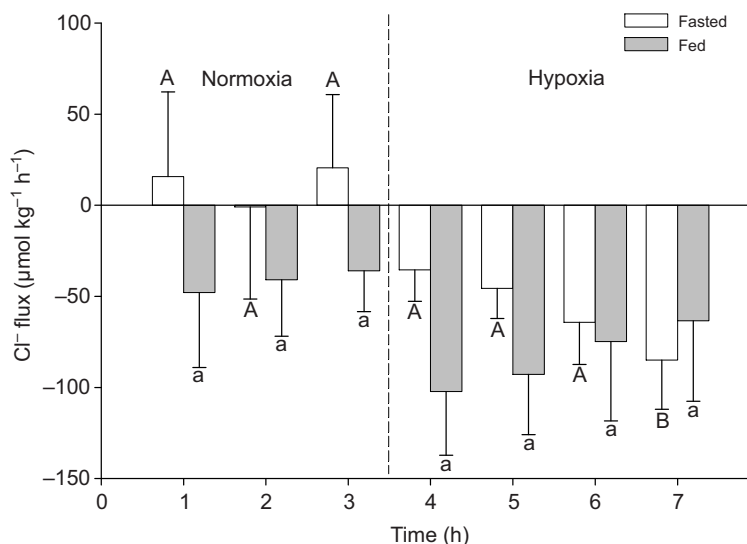


Fig. 4. Changes in net flux rates of Cl<sup>-</sup> in oscar fish that were either fasted for 10–14 days (white bars) or fed up to 24 h before the start of the experiment (grey bars). The fish were exposed to normoxia (P<sub>O<sub>2</sub></sub>=130–150 Torr) followed by acute hypoxia (P<sub>O<sub>2</sub></sub>=10–20 Torr). Data are means ± 1 s.e.m. (N=9–12). Different letters indicate a significant difference (P≤0.05) between normoxic and hypoxic flux rates (uppercase for fasted fish, lowercase for fed fish).

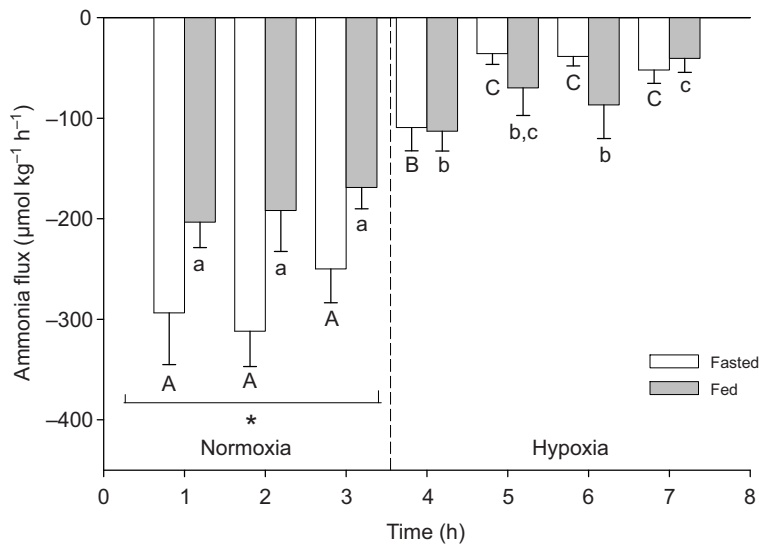


Fig. 5. Changes in net excretion rates of ammonia in oscars that were either fasted for 10–14 days (white bars) or fed up to 24 h before the start of the experiment (grey bars). The fish were exposed to normoxia ( $P_{O_2}=130\text{--}150$  Torr) followed by acute hypoxia ( $P_{O_2}=10\text{--}20$  Torr). Data are means  $\pm$  1 s.e.m. ( $N=9\text{--}12$ ). Asterisk indicates a significant difference ( $P\leq 0.05$ ) between normoxic fed and fasted fish at the same time period, and different letters indicate a significant difference ( $P\leq 0.05$ ) between normoxic and hypoxic flux rates (uppercase for fasted fish, lowercase for fed fish).

Perhaps the drop in liver lipids seen in our study provides the answer here.

The fact that hardly any changes occurred in enzyme activities as a result of 10–14 days of fasting reinforces that this experience is not particularly stressful for this species, and confirms the early phase of fasting/starvation. This agrees with earlier data in carp, where no changes in gene expression were seen in the first 16 days of food deprivation (Hung, 2005).

In our study, oxygen consumption rates of fasted oscar were actually higher than those of fed oscars, yet they tended to gain  $Na^+$  on a net basis. Fasting oscars exhibited an increased  $Na^+$  influx compared with fed fish under normoxia, most likely to compensate for the fact that they could not absorb  $Na^+$  from food. Efflux rates of  $Na^+$  were not different between fed and fasting fish. For fasting fish, this resulted in a net  $Na^+$  uptake, while fed fish showed a net  $Na^+$  loss. Also, net  $Cl^-$  fluxes were positive in fasting oscars in contrast to fed individuals under normoxia. Thus, both  $Na^+$  and  $Cl^-$  homeostasis seemed to be in balance for the fasted fish under normoxia, despite the higher  $\dot{M}_{O_2}$ . Regrettably, we did not measure ventilation rates or gill perfusion. Therefore, we cannot conclude whether the increased  $\dot{M}_{O_2}$  was related to increased water and/or blood flow, which would aggravate the osmorepiratory compromise (Wood et al., 2009), or whether it

was caused by an increased oxygen demand and improved diffusion.

When the oscars were exposed to severe hypoxia, both  $Na^+$  influx and efflux were reduced, as was seen in earlier studies by Wood et al. (Wood et al., 2007; Wood et al., 2009). This response was much faster in fasted fish, with an immediate reduction in  $Na^+$  influx (first hour of hypoxia) and a subsequent reduction in  $Na^+$  efflux (second hour of hypoxia). This resulted in a small net loss of  $Na^+$  for hypoxic fasting fish. In fed fish the response was slower, and both  $Na^+$  influx and efflux were only significantly reduced from the third hour of hypoxia onwards. Their responses resulted in an unchanged net  $Na^+$  loss. Overall, fasting fish seemed to be better prepared to immediately counteract any possible  $Na^+$  loss. The same response was not seen for net  $Cl^-$  fluxes, and both fasted and fed fish experienced a net  $Cl^-$  loss under hypoxia, possibly related to anion imbalance associated with lactacidosis and a corresponding retention of bicarbonate that impedes  $Cl^-$  uptake.

The reduction of  $Na^+$  fluxes is seen as a dual process, with an immediate effect on gill transcellular permeability, including concomitant reductions in water exchange rates, net  $K^+$  loss rates, and ammonia and urea excretion rates (Wood et al., 2007; Wood et al., 2009), followed by a subsequent slower 60–65% reduction in  $Na^+/K^+$ -ATPase activity (Richards et al., 2007; Wood et al., 2007).

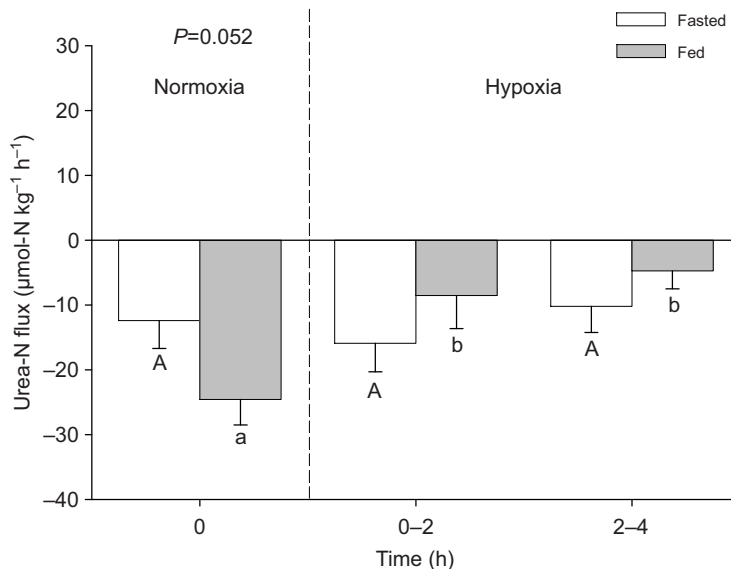


Fig. 6. Changes in net excretion rates of urea-N in oscars that were either fasted for 10–14 days (white bars) or fed up to 24 h before the start of the experiment (grey bars). The fish were exposed to normoxia ( $P_{O_2}=130\text{--}150$  Torr) followed by acute hypoxia ( $P_{O_2}=10\text{--}20$  Torr). Data are means  $\pm$  1 s.e.m. ( $N=9\text{--}12$ ). Different letters indicate a significant difference ( $P\leq 0.05$ ) between normoxic and hypoxic flux rates (uppercase for fasted fish, lowercase for fed fish).



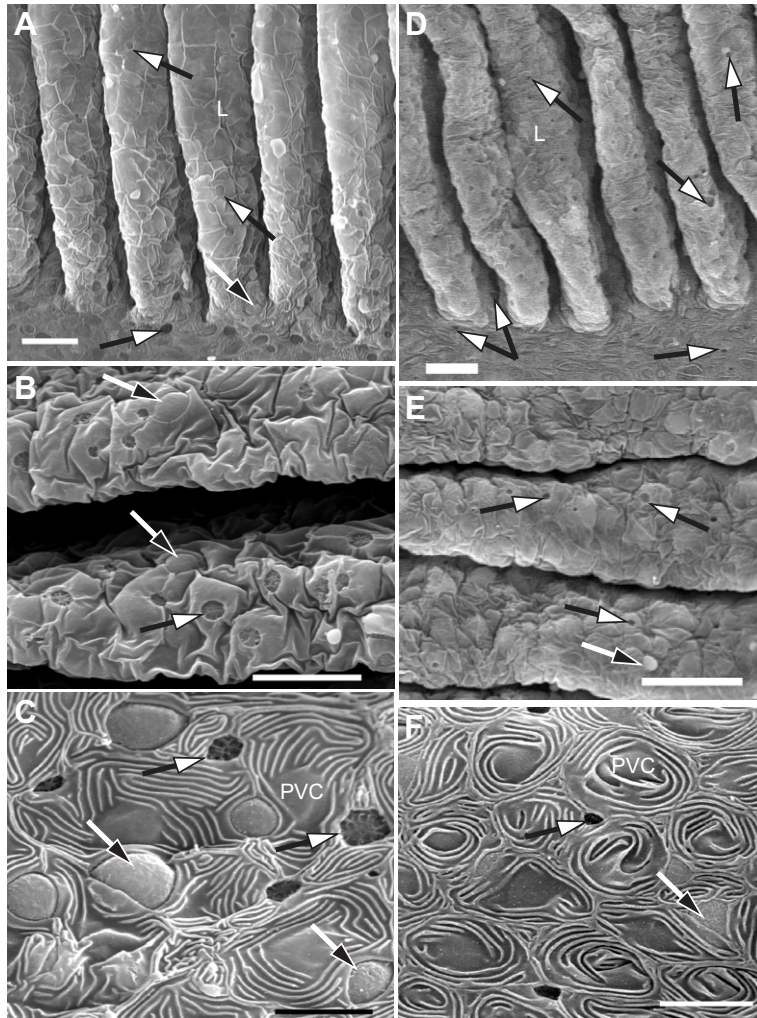


Fig. 7. Representative scanning electron micrographs of gill surface of fasted and fed oscars in normoxic conditions. (A) Fasted fish, 10 days of fasting. General view of the trailing edge of filament and lamellae. Note numerous large apical crypts of mitochondria-rich cells (MRCs) and openings of mucous cells (MCs). Here and in Fig. 8 white-headed arrows designate MRCs and black-headed arrows designate MCs. (B) Fasted fish, 10 days of fasting. Higher magnification of the lamella edges demonstrates abundant MRCs and several MCs. (C) Fasted fish, 10 days of fasting. High magnification of filament epithelium surface shows apical crypts of MRCs with sieve-like surface structure and large openings of MCs filled with mucus. (D) Fed fish. General view of the trailing edge of filament and lamellae. Note numerous small crypts of MRCs. (E) Fed fish. Higher magnification of the lamella edges showing abundant crypts of MRCs and rare MC openings. (F) Fed fish. High magnification of filament epithelium surface shows small MRC crypts and MC openings. Scale bars: (A,D) 25  $\mu\text{m}$ ; (B,E) 30  $\mu\text{m}$ ; (C,F) 10  $\mu\text{m}$ . L, lamella; PVC, pavement cell.

These processes are not driven by oxygen limitations for the branchial MRCs because they perform well down to oxygen levels of 5 Torr (Scott et al., 2008). More likely, they fit with the occurrence of metabolic depression that these fish use when environmental oxygen levels drop below their  $P_{\text{crit}}$  [34–54 Torr in the present study, 50–70 Torr in Sloman et al. (Sloman et al., 2006)], as well as into rapid changes in gill morphology, which are thought to reduce transcellular permeability (see below). There is no evidence for reduction in paracellular permeability across the gills during hypoxia (Wood et al., 2009). Below these oxygen levels, oscars not only reduce ionoregulatory costs, but also protein synthesis, another metabolically costly process (Lewis et al., 2007).

Under normoxia, ammonia excretion and  $\text{K}^+$  efflux were higher in fasted fish, suggesting that dietary nitrogen and  $\text{K}^+$  are shunted into growth and protein synthesis in fed fish (Lewis et al., 2007).

Fasted fish were burning body protein, as can be seen from reduced liver protein levels, explaining the higher ammonia excretion rates under normoxia. Although ammonia/urea excretion and oxygen consumption rates were not measured in the same individual fish, the calculated nitrogen quotient remained approximately constant (0.072) between fed and fasted fish. By standard metabolic theory (see Lauff and Wood, 1996), both groups were therefore relying on protein to fuel approximately 26% of aerobic metabolism.

Hypoxia reduced both ammonia excretion and  $\text{K}^+$  efflux, in accordance with the interpretation of decreased transcellular permeability. Again, for  $\text{K}^+$  this happened more quickly in fasted fish compared with fed fish, indicating that they shut down transcellular transport more efficiently. Ammonia excretion was immediately reduced in both fed and fasted fish during hypoxia, but to a larger extent in the fasted oscars. Fed oscars also relied

Table 4. Density of mucous cells (MC) and density and apical surface area of mitochondria-rich cells (MRC) in gills of fed and fasted oscars exposed to normoxia or acute hypoxia

	Fed		Fasted	
	Normoxia	Hypoxia	Normoxia	Hypoxia
MC density (no. $\text{mm}^{-2}$ )	478 $\pm$ 29	579 $\pm$ 31	1866 $\pm$ 48####	867 $\pm$ 50####,***
MRC density (no. $\text{mm}^{-2}$ )	1691 $\pm$ 24	897 $\pm$ 15***	1785 $\pm$ 29##	1112 $\pm$ 19####,***
MRC surface area ( $\mu\text{m}^2$ )	5.6 $\pm$ 0.1	2.0 $\pm$ 0.2***	8.1 $\pm$ 0.2####	4.9 $\pm$ 0.2####,***

Values are means  $\pm$  s.e.m. ( $N=5-6$ ). #Significant difference between fed and fasted within the same oxygenation level; \*significant difference between normoxia and hypoxia within the same feeding ration (## $P\leq 0.01$ , #### $P\leq 0.001$ ).

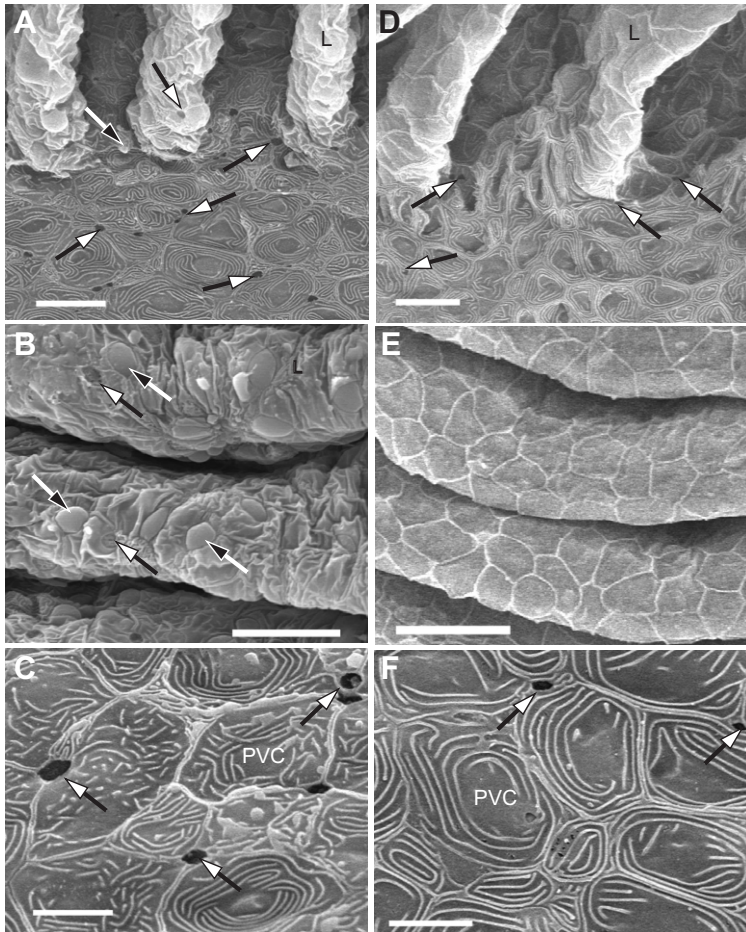


Fig. 8. Representative scanning electron micrographs of gill surface of fasted and fed oscars after 3 h exposure to acute hypoxia.

(A) Fasted fish. General view of the trailing edge of filament and lower parts of lamellae. Note less abundant and smaller crypts of MRCs and fewer openings of MCs. (B) Fasted fish. Respiratory lamellae. Note abundance of large openings of MCs distributed along the edge of lamellae and rare appearance of MRC crypts. (C) Fasted fish. High magnification of filament epithelium demonstrates few small crypts of MRCs, few and smaller openings of MCs, and deposition of the excessive mucus on the epithelial surface. (D) Fed fish. General view of the trailing edge of filament and lower parts of lamellae. Note few small apical crypts of MRCs and openings of MCs. (E) Fed fish. Respiratory lamellae. Outermost layer of lamellar epithelium is composed of pavement cells. No MRCs or MCs are seen on the lamella surface. (F) Fed fish. Few small MRC crypts and MC openings seen under high magnification of filament epithelium surface. Scale bars: (A,C,D,F) 10  $\mu\text{m}$ ; (B,E) 30  $\mu\text{m}$ .

more on urea for excretion of nitrogen wastes than fasted oscars, and this was also reduced under hypoxia. This corresponds well with the reduced urine flow rate that was observed under hypoxic conditions (Wood et al., 2009).

Gill morphology can be quite plastic under changing environmental conditions such as oxygen levels or temperature. In some species, such as the anoxia-tolerant crucian carp (*Carassius carassius*) and the goldfish (*Carassius auratus*), it can lead to gross morphological changes (Sollid et al., 2003; Sollid et al., 2005a; Sollid et al., 2005b; Sollid and Nilsson, 2006). In oscars, changes under hypoxia have been shown to be more modest but very rapid, including a reduced number and apical surface area of MRCs due to pavement cell expansion from the first hour of exposure onwards (Wood et al., 2009; Matey et al., 2011). This is thought to be the morphological correlate, and indeed perhaps the cause, of decreased transcellular permeability. Hypoxia-sensitive rainbow trout, *Oncorhynchus mykiss*, showed the opposite effects, with MRCs that were increasing in size and bulging out, and pavement cell retraction (Ifitkar et al., 2010; Matey et al., 2011). Fed fish in our study show the same response under hypoxia as seen before (Wood et al., 2009; Matey et al., 2011): MRCs at the gill decreased in number and surface area, and MCs increased slightly in number. Especially on the lamellae, MRCs seemed to totally disappear. Interestingly, normoxic fasted fish not only showed an increased number of MRCs, but also had a significantly larger surface area corresponding to the higher  $\text{Na}^+$  uptake rates discussed above. Most striking was the difference in MCs, almost four times the number seen in fed oscars. Additional MCs and mucous production likely assisted in reducing ion losses in food-deprived oscars. Under hypoxia, MRC number and surface area decreased, concomitant with

the decreasing ion fluxes, but remained above the numbers for fed fish – as did the  $\text{Na}^+$  fluxes. So overall there seems to be a positive relationship between  $\text{Na}^+$  flux and the number and surface area of the MRCs. MCs in fasted fish decreased in number by half under hypoxia, and clearly released their content on the surface, possibly in an attempt to increase diffusion distance and boundary layer effects, and thereby reduce ion loss.

Contrary to our expectation, we can conclude that a short-term fasting period (10–14 days), such as during breeding, does not affect the capacity of oscars to handle short periods of severe hypoxia.  $P_{\text{crit}}$  in fasted fish was reduced compared with fed fish, indicating a greater resistance to hypoxia. As hypothesised, ionoregulation was maintained by higher branchial  $\text{Na}^+$  uptake rates and a quicker response in reducing transcellular permeability during acute hypoxia. Gills were equipped with more MRCs with larger apical surfaces to provide the additional ion uptake under normoxia.

#### LIST OF SYMBOLS AND ABBREVIATIONS

$\text{CPM}_f$	final $^{22}\text{Na}$ radioactivity in the water
$\text{CPM}_i$	initial $^{22}\text{Na}$ radioactivity in the water
GDH	glutamate dehydrogenase
HOAD	hydroxyacylCoA dehydrogenase
HSI	hepatosomatic index
$J_{\text{in}}^{\text{Na}}$	sodium influx
$J_{\text{out}}^{\text{Na}}$	sodium efflux
$J_{\text{net}}$	net flux
LDH	lactate dehydrogenase
$M$	mass
MC	mucous cell
$M_{\text{O}_2}$	oxygen consumption rate



MRC	mitochondria-rich cell
$P_{crit}$	critical oxygen tension
$\dot{P}_{O_2}$	oxygen pressure
PVC	pavement cell
SA <sub>ext</sub>	mean external specific activity
$T$	time of flux period
$V$	volume
$\Delta O_2$	change in dissolved $O_2$ concentration over time
$\Delta[S]$	change in concentration of the measured substance over time

### ACKNOWLEDGEMENTS

We thank Fabiola X. Valdez Domingos, Rafael Mendonça Duarte and all members of the Val lab for their hospitality.

### AUTHOR CONTRIBUTIONS

G.D.B. designed and performed the experiments, analysed the data, interpreted results and wrote the paper. C.M.W. designed and performed the experiments, analysed the data, interpreted results and corrected the manuscript. F.I.L., G.R.S. and K.A.S. helped to perform the experiments and corrected the manuscript. V.M. performed the gill morphology analysis, interpreted results and corrected the manuscript. M.d.N.P.d.S. and V.M.F.A.-V. performed enzymatic analysis, interpreted results and corrected the manuscript. A.L.V. contributed lab facilities/materials/analysis tools, interpreted results and corrected the manuscript.

### COMPETING INTERESTS

No competing interests declared.

### FUNDING

G.D.B. was supported by a grant from the Research Foundation of Flanders (FWO) and an International Collaboration Grant from the Research Council of the University of Antwerp. This work was funded by an NSERC Discovery Grant to C.M.W. and National Research Council (CNPq) of Brazil/Amazon State Research Foundation (FAPEAM) PRONEX and INCT-ADAPTA grants to A.L.V. C.M.W. is supported by the Canada Research Chair Program, and his travel was funded in part by the International Congress of Comparative Physiology and Biochemistry. A.L.V. and V.M.F.A.V. are recipients of research fellowships from CNPq. K.A.S. was supported by a grant from the Association for the Study of Animal Behaviour and the Royal Society (UK). G.R.S. was the recipient of a JEB travelling fellowship for this work.

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