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Responses to hypoxia and recovery: repayment of oxygen debt is not associated with compensatory protein synthesis in the Amazonian cichlid, *Astronotus ocellatus*

J. M. Lewis[1,*], I. Costa[1], A. L. Val[2], V. M. F. Almeida-Val[2], A. K. Gamperl[1] and W. R. Driedzic[1]

[1]*Ocean Sciences Centre, Memorial University of Newfoundland, St. John's, NL, A1C 5S7, Canada and* [2]*Laboratory of Ecophysiology and Molecular Evolution, Instituto Nacional de Pesquisas da Amazônia, Alameda Cosme Ferreira, 1756, 69.083-000, Manaus, Amazonas, Brazil*

*Author for correspondence (e-mail: jmlewis@mun.ca)

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Summary

Oxygen consumption, as an indicator of routine metabolic rate (RoMR), and tissue-specific changes in protein synthesis, as measured by ^{3}H-labelled phenylalanine incorporation rates, were determined in *Astronotus ocellatus* to investigate the cellular mechanisms behind hypoxia-induced metabolic depression and recovery. RoMR was significantly depressed, by approximately 50%, when dissolved oxygen levels reached 10% saturation (0.67±0.01 mg l^{-1} at 28±1°C). This depression in RoMR was accompanied by a 50–60% decrease in liver, heart and gill protein synthesis, but only a 30% decrease in brain protein synthesis. During recovery from hypoxia, an overshoot in RoMR to 270% of the normoxic rate was observed, indicating the accumulation of an oxygen debt during hypoxia. This conclusion was consistent with significant increase in plasma lactate levels during the hypoxic exposure, and the fact that lactate levels rapidly returned to pre-hypoxic levels. In contrast, a hyperactivation of protein synthesis did not occur, suggesting the overshoot in oxygen consumption during recovery is attributed to an increase in cellular processes other than protein synthesis.

Key words: hypoxia, recovery, routine metabolic rate, protein synthesis, lactate production, *Astronotus ocellatus*.

Introduction

Dissolved oxygen is one of the most important environmental factors affecting the survival of animals that rely on aquatic respiration, and animals that are exposed to periods of hypoxia show adaptations at the behavioural, morphological and/or physiological level. At the physiological level, animals commonly resort to one of two strategies: (1) maintenance of low levels of activity, which is fuelled by anaerobic metabolism or (2) depression of metabolism, accomplished by decreasing ATP producing and consuming processes (Boutilier, 2001; Lutz and Nilsson, 1997). The latter approach allows survival for longer periods of hypoxia/anoxia because of the conservation of energy and the limited accumulation of toxic end products, such as lactate. However, there is a trade-off to this approach as deep metabolic depression impairs the animal's ability to respond to external stimuli and leaves the animal vulnerable to predators.

The majority of successful oxyconformers are ectotherms that survive short bouts of hypoxia at warm temperatures, but require seasonal or behaviourally regulated decreases in environmental/body temperature to survive extended anoxia. Such animals are the crucian carp (*Carassius carassius*), goldfish (*Carassius auratus*), common frog (*Rana temporaria*) and two species of freshwater turtle (*Chrysemys picta bellii* and *Trachemys scripta elegans*) (Boutilier, 2001). In these animals a depression of metabolic rate by 70–95% occurs during hypoxia/anoxia, based on oxygen consumption rates or calorimetry (Van Waversveld et al., 1989; Jackson, 1968). This depression at the whole animal level is accompanied by tissue-specific decreases in protein synthesis of 50% in crucian carp (Smith et al., 1996) and 70 to >90% in freshwater turtles (Land et al., 1993; Bailey and Driedzic, 1996; Fraser et al., 2001). Protein synthesis is one of the major energy consuming processes, accounting for 18–26% of cellular energy expenditure (Hawkins, 1991). As such, the downregulation of protein turnover is one of the major contributing factors to the depression in ATP turnover and metabolic depression at the whole animal level (Guppy et al., 1994).

Animals that are exposed to a prolonged period of oxygen deprivation accumulate an oxygen debt that is repaid during recovery by a substantial increase in oxygen consumption. This oxygen debt has been shown to occur at both the whole animal and tissue levels in goldfish after extended hypoxia exposure (Van den Thillart and Verbeek, 1991; Johansson et

al., 1995). Johansson et al. (Johansson et al., 1995) predicted that a substantial increase in protein turnover would accompany the repayment of the oxygen debt, but a consistent pattern in protein synthesis during recovery from hypoxia has not been found. For example, an *in vitro* study on turtle hepatocytes exposed to 12 h of anoxia showed a significant overshoot in protein synthesis rates to 160% of normoxic levels during recovery (Land et al., 1993). However, *in vivo* studies on crucian carp and a freshwater turtle species did not show hyperactivation of protein synthesis during post-anoxic recovery (Smith et al., 1996; Fraser et al., 2001).

The Amazonian cichlid, the oscar or acará-açu *Astronotus ocellatus*, is an ideal species to study hypoxia-induced metabolic depression without the confounding variable of decreased temperature. During periods of high water, Amazon várzeas become flooded and the surfaces of the lakes become densely covered with floating marcrophytes (Val and Almeida-Val, 1995). The dense surface vegetation causes extreme diurnal variation in dissolved oxygen levels, with supersaturation occurring at midday when photosynthesis is at its maximum and levels dropping close to zero during the night (MacCormack et al., 2003). *A. ocellatus* undergoes a significant decrease in routine metabolic rate (RoMR; ~30%) when oxygen levels in the water reach 20% saturation and only reverts to anaerobic metabolism once oxygen levels drop below 6% saturation consumption, which is accompanied by a decrease in RoMR of approximately 60% (Muusze et al., 1998). These results suggest that *A. ocellatus*, like the crucian carp and freshwater turtles, is able to maintain aerobic metabolism in situations of oxygen deprivation by decreasing the rate of ATP turnover until near anoxic conditions are reached. Until recently, little was known about the cellular mechanisms behind the hypoxia-induced metabolic depression in *A. ocellatus* and of its response during recovery from severe hypoxia beyond the behavioural and physiological responses of *A. ocellatus* to hypoxia (Muusze et al., 1998; Sloman et al., 2006). Recent studies have begun investigation into the various ATP-consuming processes that contribute to the whole animal metabolic depression. These studies have shown significant reduction in Na^+,K^+-ATPase in gill and kidney during hypoxia exposure (Richards et al., 2007) which is accompanied by a reduction of ion exchange at the gills and an overall reduction in metabolic nitrogenous waste production (urea and ammonia) (Wood et al., 2007). The decrease in these ATP consuming processes are not accompanied by changes in concentration of ATP (Richards et al., 2007), indicating that *A. ocellatus* is able to successfully tolerate extended hypoxia exposure because of the reduction in key ATP-consuming processes.

The objectives of this study were to expand our knowledge of the biochemical processes behind the hypoxia-induced metabolic depression and post hypoxia recovery in *A. ocellatus* through investigation of the tissue-specific protein synthesis rates in relation to the whole animal metabolic depression. In addition, the present study is the first to obtain measurements of whole animal metabolic rate and protein synthesis under similar experimental conditions on the same population of fish.

Materials and methods

Experiments were conducted at the Laboratory of Ecophysiology and Molecular Evolution, INPA, Brazil. A population of *Astronotus ocellatus* (Agassiz 1831) was held in an outdoor holding tank with aerated well water (O_2 saturation of 80–100%) at 28°C and fed commercial food once daily, until transferred to experimental tanks (for protein synthesis studies) or respirometers (for measurements of routine metabolic rate, RoMR). In total, eight fish were used for measurement of RoMR (156–225 g, average 186±10.0 g) and 44 fish were used for the measurement of protein synthesis (70–160 g, average 95.8±3.8 g). All fish were held without feeding for 48 h prior to beginning measurements for RoMR and protein synthesis. In fish used for analysis of RoMR and protein synthesis, measurements were taken under normoxic and hypoxic conditions as well as during the recovery from acute hypoxia exposure. Normoxic conditions were identical to those of the holding tank (O_2 saturation of 80–100%), and the hypoxic challenge consisted of a step down decrease of the dissolved oxygen (DO) level. This was accomplished by bubbling nitrogen directly into the water of the experimental tank, or into the reservoir that supplied water to the respirometer. Water oxygen levels were stepped down from 100 to 70, 50, 30, 20 and 10%, with O_2 levels maintained at each step for 1 h, and the fish held at 10% O_2 saturation for 3 h prior to re-oxygenation. Re-oxygenation was achieved by bubbling air vigorously into the water, and water O_2 levels returned to normoxic levels within 30–45 min.

Measurement of routine metabolic rate

Individual fish were transferred to a specially designed Plexiglas® respirometer (15×20×40 cm; 11.875 l) supplied with oxygen-saturated water (80–100%) from a 100 l reservoir and allowed to acclimate for 48 h before the beginning of the experiment. Water from the reservoir was continuously pumped through the respirometer using a submersible pump (model NK-1, Little Giant Co., Vernon Hills, IL, USA). Water temperature and oxygen concentration were monitored through a circuit composed of tubing with extremely low gas permeability (Tygon® Food & LFL, Cole Palmer, Inc., Oklahoma City, OK, USA) using a peristaltic pump (Masterflex L/S model 77200-12, Cole-Palmer) and flow-through chambers (D201, WTW, Weilheim, Germany) containing oxygen probes (model CellOx 325, WTW) positioned in the inflow and outflow tubing of the respirometer. Measurements of water oxygen levels and water flow rate (range 0.8–1.0 l min^{-1}) were taken at hourly intervals during the experiment (i.e. during normoxia, hypoxia and recovery from hypoxia), as well as before the placement of the fish and immediately after the removal of the fish from the respirometer

in order to correct for bacterial O_2 consumption. Bacterial O_2 consumption was consistently less than 2% of the RoMR of the fish and was therefore considered to be negligible. The RoMR of each fish was calculated at each measurement interval as:

$$\text{RoMR} = [(C_{O_2}(i)–C_{O_2}(o)] \times \dot{V}w \times 60 / M_b$$

where RoMR is in mg O_2 kg h^{-1}; $C_{O_2}(i)$ is the O_2 concentration in inflowing water (mg O_2 l^{-1}); $C_{O_2}(o)$ is the O_2 concentration in outflowing water (mg O_2 l^{-1}); $\dot{V}w$ is the water flow rate through the respirometer (l min^{-1}) and M_b is the mass of fish (kg) [modified from Cech (Cech, 1990)].

Protein synthesis

Normoxia

Twelve fish were removed from the holding tank, weighed, tagged for individual recognition and transferred to a separate experimental tank under identical environmental conditions. After 48 h, fish were injected intraperitoneally, without anaesthetic, with 1.0 ml 100 g^{-1} of [2,3-^{3}H]phenylalanine (Amersham International) solution. This injection solution consisted of 135 mmol l^{-1} phenylalanine in a solution containing 125 mmol l^{-1} NaCl, 3 mmol l^{-1} KCl, 1 mmol l^{-1} $MgSO_4.7H_2O$, 1.5 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} Hepes (sodium salt), 5 mmol l^{-1} glucose, 2 mmol l^{-1} Na_2HPO_4, pH 7.8 at 28°C, in addition to sufficient [2,3-^{3}H]phenylalanine to ensure a dosage of 100 μCi ml^{-1}. Following injection, fish were returned immediately to the experimental tank and after an incubation time of 1, 2 or 3 h, groups of four fish were killed by a blow to the head and immediate severing of the spine. Brain, heart, liver, white muscle and gill tissue were excised in that order, and frozen in liquid nitrogen. All samples were kept at –70°C until analysis.

Hypoxia

In this treatment, 12 fish were weighed, tagged and transferred to the experimental tank. After a 48-h acclimation period, fish were exposed to a stepwise decrease in dissolved oxygen levels as described previously. Fish were injected immediately once water oxygen saturation reached 10%, and four fish were sampled (as above) at 1, 2 and 3 h after injection with DO levels maintained at 10% for the 3 h hypoxia exposure.

Recovery

To assess changes in protein synthesis rates during recovery from hypoxia, 20 fish were exposed to an acute hypoxia challenge, as described above. After holding fish at 10% DO for 3 h, air was bubbled into the experimental tank allowing the dissolved O_2 level to return to normoxic levels (80–100%). Groups of five fish were injected at hourly intervals, starting when O_2 saturation levels returned to normoxic levels (group 1) and ending 4 h after O_2 returned to normoxic levels (group 4). Each group of fish was sampled 1 h post-injection allowing protein synthesis to be tracked over a 4-h time period during the post-hypoxic recovery. Tissues were excised and stored as previously described.

Blood sampling for lactate

Blood samples were obtained from as many fish as possible during the protein synthesis experiment, resulting in N=4, normoxic; N=8, hypoxic; and N=7, recovery. Blood was drawn from the caudal vein with a heparinized syringe prior to sampling the fish for protein synthesis analysis. Blood samples were centrifuged and plasma was stored at –70°C for lactate analysis.

Sample preparation and scintillation counting

The protocol used for the analysis of protein synthesis followed that of Treberg et al. (Treberg et al., 2005), with techniques modified from the original paper that presents the flooding dose approach to measure rates of protein synthesis (Garlick et al., 1980). Samples were homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA) in nine volumes of 6% perchloric acid (PCA) except for liver, which was homogenized in four volumes of PCA. Homogenized samples were left on ice for 10–15 min and a 1 ml aliquot was transferred to a microcentrifuge tube. Excess homogenate (liver, muscle and in some cases brain samples), was stored at –70°C for the analysis of lactate. The 1 ml aliquot of homogenate for protein synthesis was centrifuged for 5 min at 15,600 ***g***, after which the supernatant was removed and frozen at –20°C for analysis of the total free phenylalanine content and its radioactivity.

The protein pellet was washed by manually re-suspending the pellet in 1.0 ml of 6% PCA, vortexing, centrifuging as described above, and then discarding the supernatant. This wash step was repeated until the radioactivity in the discarded supernatant was at background levels to ensure only protein bound [^{3}H]phenylalanine was being measured in the protein pellet. After sufficient washing, 1.0 ml of 0.3 mol l^{-1} NaOH was added to the tube containing the protein pellet. The protein pellet was incubated in a water bath held at 37°C until fully dissolved. The dissolved protein was stored at –20°C until analysis for protein content and protein-bound radioactivity.

Aliquots of the original supernatant from the homogenized tissue and the dissolved protein were added to 10 ml of Ultima Gold^{TM} liquid scintillation cocktail and counted on a Beckman Coulter LS6500 liquid scintillation counter to obtain the [2,3-^{3}H]phenylalanine content of the free and protein-bound phenylalanine pools of the tissues, respectively. Phenylalanine specific activity was considered to be c.p.m./phenylalanine content (nmol). This assumes negligible conversion of radiolabelled phenylalanine to other radiolabelled components, which has been proved with HPLC analysis in a species of mollusc (Pakay et al., 2002). This assumption is considered to be acceptable for the current study given the elevated and constant c.p.m./phenylalanine ratio in all tissues over the time course of the study and the linear rate of incorporation of radiolabel into the protein pool for all tissues.

Biochemical assays

Free pool phenylalanine content was measured from the PCA extraction supernatant and phenylalanine standards in 6% PCA

using a fluorometric assay following the protocol described in McCaman and Robins (McCaman and Robins, 1962). Protein content of the tissue was determined from the NaOH-solubilized protein pellet by using the BioRad D_c kit (Bio-Rad Laboratories, Hercules, CA, USA) using standards made from bovine serum albumin. Lactate was measured in standards in 6% PCA, plasma, liver, white muscle and brain tissue *via* the reduction of NAD^+ to NADH at 340 nm using a Sigma diagnostics kit.

Statistical analyses

Comparison of oxygen consumption data was carried out by using a repeated measures ANOVA followed by a Dunnett's *post-hoc* test, to compare all values with the normoxic (control) value. Lactate concentrations for normoxia, hypoxia and recovery treatments were compared using a one-way ANOVA, with Tukey's *post-hoc* test for multiple comparisons. In the protein synthesis experiment, mean tissue phenylalanine content and specific activity over the incubation time were compared using a one-way ANOVA with Tukey's *post-hoc* test for multiple comparisons, and the incorporation of radioactivity into protein was examined by linear regression. Once data were confirmed to fit the validation criteria, phenylalanine incorporation rates for each tissue were compared using a one-way ANOVA followed by a Tukey's *post-hoc* test. In all cases $P<0.05$ was considered significant.

Results

Routine metabolic rate

The RoMR of *A. ocellatus* under normoxic conditions was 138.7±16.5 mg kg^{-1} h^{-1} (Fig. 1). Despite decreasing oxygen saturation of the water, there was no significant decrease in RoMR until water O_2 levels reached 10% saturation (0.67±0.005 mg l^{-1} at 28±1°C). At this level, O_2 consumption was decreased to 65.1±2.0 mg kg^{-1} h^{-1}, a value approximately 50% of the normoxic rate. Although RoMR at 1 h post-hypoxia significantly increased to 305.5±29.7 mg kg^{-1} h^{-1}, 270% of rates obtained under normoxia, RoMR returned to pre-hypoxic levels (174.0±3.1 mg kg^{-1} h^{-1}) by 2 h post-hypoxia and remained at similar levels for the remainder of the experiment.

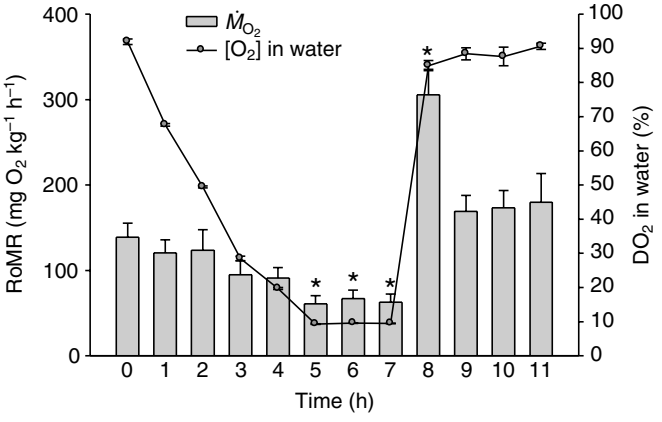

Fig. 1. Routine metabolic rate (RoMR) in *A. ocellatus* in relation to changing levels of O_2 saturation in the water. RoMR ($\dot{M}_{O_2}$) measurements are shown as means ± s.e.m., N=8 fish. *Significant differences in RoMR from time 0 (normoxia) values ($P<0.05$).

Lactate concentration

Lactate concentrations under normoxic conditions in the various tissues were 0.04±0.03 μmol ml^{-1} for plasma (N=4) and 0.25±0.06, 0.84±0.11 and 2.63±0.30 μmol g^{-1} tissue for liver (N=11), brain (N=12) and white muscle (N=6), respectively (Fig. 2). As there were no significant differences in lactate concentration in any of the tissues during the 3-h hypoxia and 4-h recovery periods, results were pooled within each treatment to give a mean value for hypoxic and recovery samples. During the 3-h hypoxic exposure, only plasma exhibited a significant increase in lactate concentration (1.13±0.27 μmol ml^{-1}, N=8). During the post-hypoxic recovery period, plasma lactate returned to levels (0.42±0.30 μmol ml^{-1}, N=7) that were not significantly different from pre-hypoxic values. Finally, significant decreases in lactate concentrations in liver (0.02±0.01 μmol g^{-1} tissue, N=20) and brain 0.55±0.06 μmol g^{-1} tissue, N=20) occurred during the recovery period, whereas lactate concentration in white muscle was maintained at similar concentrations over all three treatments.

Validation of protein synthesis methodology

In order to accurately interpret protein synthesis rates obtained *via* the flooding dose methodology several validation criteria must be met: (1) the injection dose must be shown to be sufficient to elevate the free phenylalanine pool of the various tissues; (2) the specific activity of the free

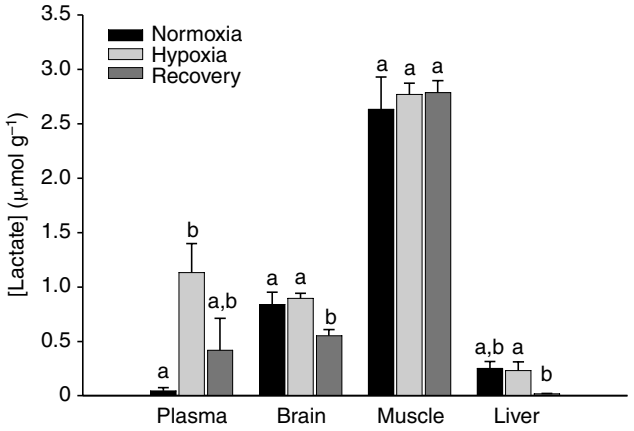

Fig. 2. Lactate concentration in plasma, brain, white muscle and liver tissue of *A. ocellatus* during normoxia, hypoxia and recovery from hypoxia. Values are means ± s.e.m.; numbers of samples/fish were: plasma normoxic (N=4), hypoxic (N=8), recovery (N=7); brain normoxic (N=6), hypoxic (N=10), recovery (N=20); muscle normoxic (N=12), hypoxic (N=12), recovery (N=20); liver normoxic (N=11), hypoxic (N=10), recovery (N=9). Significant differences within each tissue are indicated by different letters, $P<0.05$.

phenylalanine must increase rapidly post-injection and remain stable throughout the time protein synthesis is measured; and (3) the rate of phenylalanine incorporation must be linear and begin immediately after injection. As shown in the following sections, all three criteria were fulfilled during this experiment.

Elevation of free phenylalanine pool content

The concentration of free pool phenylalanine in the various tissues was not significantly different between the three treatments or between sample times within treatments. Therefore, results for normoxic, hypoxic and post-hypoxic fish were pooled and referred to as injected fish (N=44). Injected fish had free phenylalanine levels of 0.55±0.04, 0.16±0.01, 0.08±0.01, 0.20±0.01 and 0.19±0.01 mmol phe g^{-1} fresh tissue (N=44) for liver, white muscle, brain, heart and gill, respectively. When compared to levels of free phenylalanine in un-injected fish (0.12, 0.08, 0.03, 0.05, 0.09 mmol phenylalanine g^{-1} fresh tissue; N=1), levels were twofold higher in brain, white muscle and gill, and fivefold higher in liver and heart tissue. The baseline levels of free phenylalanine from un-injected oscars and the increase achieved *via* the flooding dose of phenylalanine are comparable to levels measured in crucian carp (Smith et al., 1999).

Intracellular free pool phenylalanine specific activity

Intracellular specific activity of the free phenylalanine pool for both normoxic and hypoxic fish was elevated 1 h post-injection, and remained constant over the 3 h that protein synthesis was measured (Fig. 3). On average, the specific activity for normoxic fish was 1039±79, 641±27, 711±37, 788±61 and 656±22 c.p.m. $nmol^{-1}$ phenylalanine for liver, white muscle, brain, heart and gill, respectively (N=12). For hypoxia-exposed fish, the specific activity for the same tissues was 708±44, 642±17, 696±26, 622±34 and 620±14 c.p.m. $nmol^{-1}$ phenylalanine (N=12). As there was no significant difference in the specific activity between post-hypoxia recovery sampling times, results were pooled for each tissue to give mean values of 830±23, 641±12, 702±7.0, 665±10 and 672±17 c.p.m. $nmol^{-1}$ phenylalanine for liver, white muscle, brain, heart and gill, respectively (N=20).

Phenylalanine incorporation into tissue protein

Protein synthesis rates were expressed as nmol phenylalanine incorporated per mg protein. Regression equations calculated over the 3-h sampling time demonstrated significant and linear incorporation of phenylalanine into liver, brain, heart and gill tissue of both normoxic and hypoxic fish (Fig. 4). As the intercepts of the regression lines were not significantly different from zero (i.e. time 0), it can be assumed that the incorporation of phenylalanine into liver, brain, heart and gill tissues began immediately following injection. Rates of protein synthesis for white muscle in both normoxic and hypoxic treatments were below detectable levels. As linear incorporation rates were achieved for both normoxic and hypoxic fish over the three time points and phenylalanine incorporation began immediately post-injection, rates of protein synthesis during recovery were determined only at 1 h post-injection.

Tissue-specific rates of phenylalanine incorporation

Rates of phenylalanine incorporation under normoxic conditions were 0.92±0.13, 0.80±0.05, 0.90±0.16 and

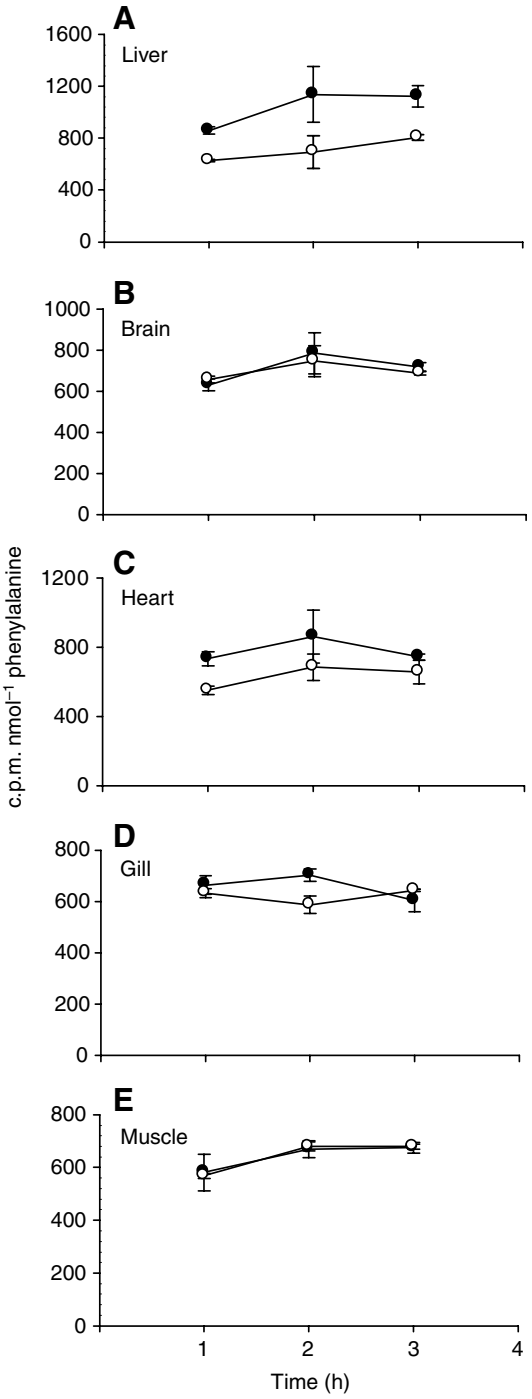

Fig. 3. Post-injection changes in the specific activity of the intracellular free phenylalanine pool in (A) liver, (B) brain, (C) heart, (D) gill and (E) white muscle of *A. ocellatus* during normoxia (closed circles) and hypoxia (open circles). Values are means ± s.e.m., N=4.

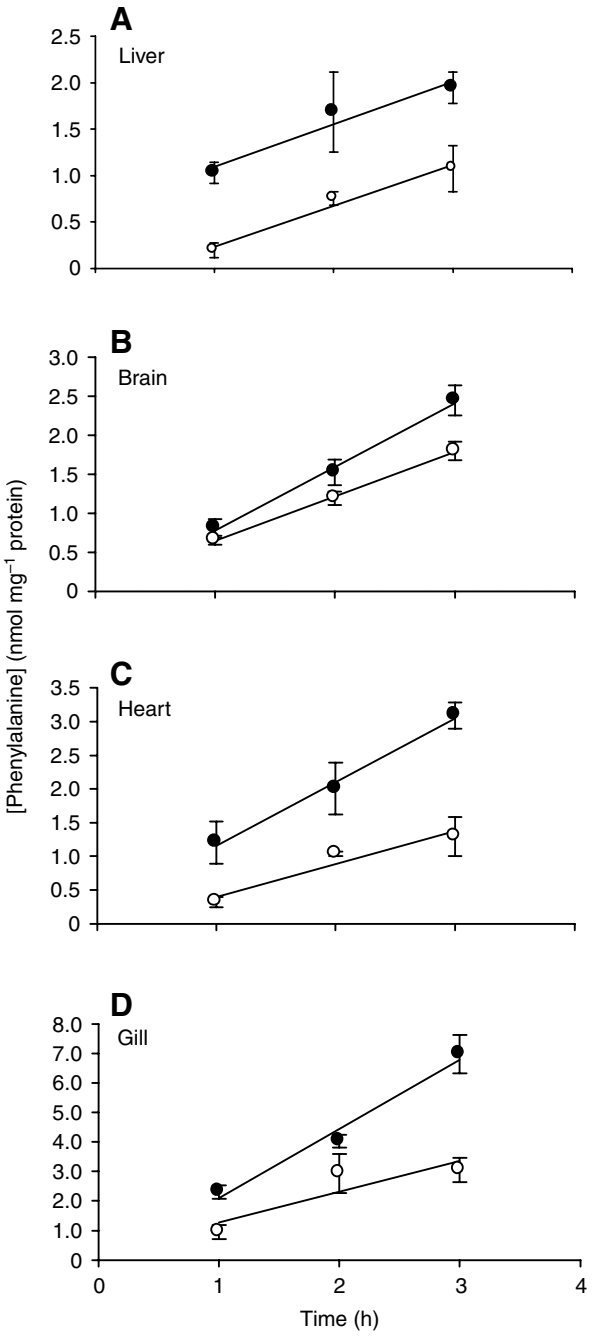

Fig. 4. Post-injection time course for the incorporation of radiolabelled phenylalanine into protein in *A. ocellatus* during normoxia (closed circles) and severe hypoxia (open circles). (A) Liver (y=0.47x+0.62, r^2=0.60, P_r=0.05, P_y=0.22; y=0.44x–0.22, r^2=0.80, P_r=0.003, P_y=0.39); (B) brain (y=0.82x–0.05, r^2=0.92, P_r=2.35×10^{-5}, P_y=0.84; y=0.57x+0.07, r^2=0.95, P_r=2.97×10^{-6}, P_y=0.61); (C) heart (y=0.94x+0.20, r^2=0.80, P_r=0.005, P_y=0.71; y=0.55x–0.16, r^2=0.84, P_r=6.78×10^{-4}, P_y=0.53); (D) gill (y=2.34–0.24, r^2=0.93, P_r=1.56×10^{-5}, P_y=0.72; y=1.05x+0.21, r^2=0.68, P_r=0.015, P_y=0.79). For each tissue regression equations refer to normoxic and hypoxic fish, respectively. All r^2 values are significant (P_r<0.05), all y-intercept values are not significant from zero (P_y>0.05). Values are means ± s.e.m., N=4, except for hypoxia-exposed liver 2 h sample, where N=3. White muscle not shown because rates of incorporation were not detectable.

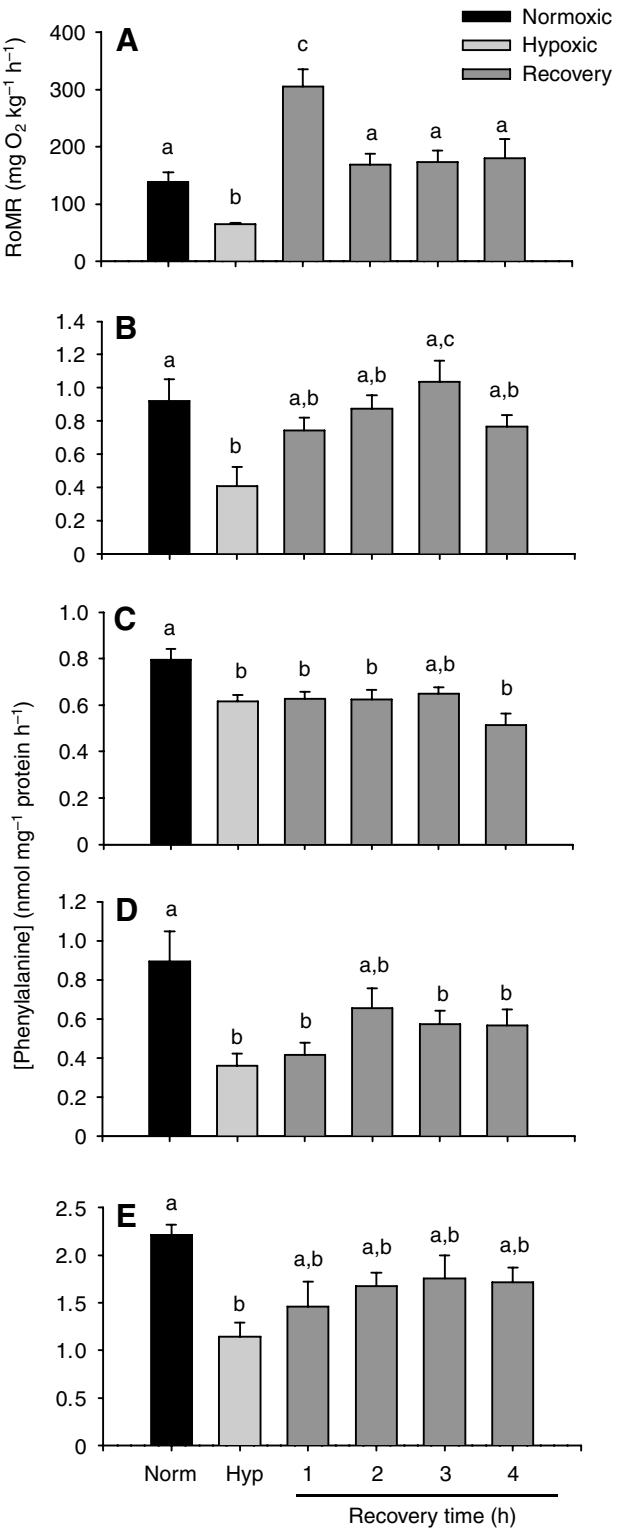

Fig. 5. (A) Routine metabolic rate RoMR and (B–E) tissue phenylalanine incorporation in liver (A), brain (B), heart (C) and (D) gill, in *A. ocellatus* exposed to normoxia, severe hypoxia, and during recovery from hypoxia. Values are means ± s.e.m., N=8 for routine metabolic rate measurements. For phenylalanine incorporation measurements, N=12 for normoxia and hypoxia exposure, and N=4 for each time point during recovery. Different lowercase letters indicate a significant difference; P<0.05.

2.2±0.11 nmol phenylalanine mg^{-1} protein h^{-1} for liver, brain, heart and gill, respectively. During exposure to hypoxia, rates of protein synthesis decreased to 0.41±0.12, 0.62±0.03, 0.36±0.06, and 1.14±0.15 nmol phe mg^{-1} protein h^{-1}, a depression of 56% for liver, 27% for brain, 60% for heart and 50% for gill (Fig. 5). During recovery from acute hypoxia, no hyperactivation of protein synthesis occurred, and two different patterns in post-hypoxic phenylalanine incorporation were observed. In liver and gill, rates of phenylalanine incorporation (0.74±0.08 and 1.46±0.27 nmol phenylalanine mg^{-1} protein h^{-1}, respectively) were not significantly different from normoxic levels by 1 h post-hypoxia, and remained at similar levels for the duration of the recovery period. By contrast, phenylalanine incorporation took longer to return to normoxic levels in brain (3 h) and heart (2 h), and in both these tissues phenylalanine incorporation was significantly less than normoxic values for the remainder of the recovery period. This latter result suggesting that full recovery of protein synthesis in brain and heart tissues takes longer than 4 h.

Discussion

Hypoxia-induced metabolic depression

Routine metabolic rate

In the present study, routine metabolic rate (RoMR) was maintained at normoxic rates until dissolved oxygen levels in the water decreased to 10%. At this time, RoMR underwent a 50% depression, which was maintained for the full 3 h of hypoxia exposure. This response to hypoxia was similar to that obtained previously for oscars, in which a depression in RoMR of approximately 50% was observed once water O_2 levels reached 10% oxygen saturation, and a reduction of 60% was measured when water O_2 levels approached those of anoxic conditions (Muusze et al., 1998). At the lowest level of hypoxia tested in the present study (10% DO), lactate levels were only significantly increased in plasma. However, this increase only brought lactate levels to one-fifth of levels obtained in *A. ocellatus* at 6% DO (Muusze et al., 1998). These results, in combination with the absence of lactate accumulation in white muscle, indicate anaerobic metabolism is only beginning to be employed to supplement energy demands at this level of oxygen deprivation, and metabolic depression is an effective way of conserving ATP until *A. ocellatus* is faced with almost anoxic conditions. In other studies with comparable lengths of hypoxia exposure, levels of lactate increased to a greater extent in plasma [4.9-fold (Richards et al., 2007); 11–16-fold (Wood et al., 2007)] and white muscle [2–3-fold (Richards et al., 2007; Wood et al., 2007)] than in the current study. This discrepancy in lactate accumulation during hypoxic exposure is most likely a result of the quick entry into hypoxia (~1 h) for these two studies as compared to the gradual transition into hypoxia of our study (~5 h).

The level of metabolic depression achieved by *A. ocellatus* is similar to that of goldfish and crucian carp, both of which decrease metabolic rate by approximately 70% under anoxia (Van Waversveld et al., 1989), but not as great as demonstrated by freshwater turtles (90–95% reduction) (Jackson, 1968). Metabolic depression is less in teleosts as a result of maintenance of ion exchange with the environment and low levels of predator avoidance. For example, in their natural environment *A. ocellatus* are susceptible to predation from air-breathing fish and aerial predators, and laboratory experiments show that they split their time equally between unprotected normoxic environments and sheltered hypoxic environments (Sloman et al., 2006). Turtles, however, are essentially a closed system as they retreat into their protective shell and enter a comatose-like state during periods of oxygen deprivation.

Protein synthesis

The use of the flooding dose methodology to measure *in vivo* protein synthesis requires that several validation criteria be fulfilled. The results from this study show that the injection dosage used successfully flooded the free phenylalanine pool during both normoxia and 3 h of hypoxic exposure, causing a two- to fivefold increase in phenylalanine concentration in the various tissues. As well, the specific activity of the free phenylalanine pool was elevated 1 h post-injection and remained stable at this level for the 3 h over which protein synthesis was measured. The final validation criterion requires the incorporation of radiolabelled phenylalanine into the tissues to be linear post-injection. This was shown for all tissues in both normoxia- and hypoxia-exposed fish, except for white muscle (Fig. 4). The radioactivity of protein-bound phenylalanine in white muscle was below detectable levels, indicating rates of protein synthesis in this tissue to be minimal. Given that rates of protein synthesis in fish white muscle are extremely low as compared to mammals (Fauconneau et al., 1995), and *A. ocellatus* has a much lower mass specific oxygen uptake than other teleosts, including tropical species (Almeida-Val et al., 2006), it is not surprising that protein synthesis was undetectable in the white muscle of *A. ocellatus*.

The role of protein synthesis in hypoxia-induced metabolic depression in ectothermic animals has been previously described in freshwater turtles (specifically *Trachemys scripta elegans* and *Chrysemys picta bellii*) and in the crucian carp (*Carassius carassius*), and these studies show that the extent to which protein synthesis is depressed is positively linked with the degree to which activity is curtailed. For example, rates of protein synthesis were suppressed by approximately 70% in the heart of *T. scripta elegans* (Bailey and Driedzic, 1996) and by >95% in various tissues in *C. picta bellii*; both these species enter a comatose-like state during anoxia (Land et al., 1993; Fraser et al., 2001). By contrast, the crucian carp, which maintains low levels of activity during hypoxia/anoxia exposure, exhibits a depression in protein synthesis of approximately 50% in heart and white muscle, 95% in liver tissue, but no significant depression in the brain (Smith et al., 1996). Similar to the crucian carp, *A. ocellatus* exhibited tissue-specific depression in protein synthesis when exposed to acute hypoxia exposure. Rates of protein synthesis in liver, heart and

gill were depressed by 50–60%, whereas rates of protein synthesis in the brain were only depressed by 27%. Thus, our results reinforce the idea that fish need to maintain protein synthesis in the brain to prevent damage to neural tissue, and to sustain appropriate brain functions so that predators can be effectively avoided. The depression of protein synthesis in gills is accompanied by a simultaneous decrease in Na^+ pumping and leak rates in the gills, as shown by measurement of Na^+,K^+-ATPase activity and Na^+ flux (Richards et al., 2007; Wood et al., 2007).

One of the suggested mechanisms controlling the depression of protein synthesis, and therefore depression of metabolic rate, is a decrease in pH (Hochachka and Somero, 2002; Richards et al., 2007). The reduction of protein synthesis has been linked to an increase in recombinant elongation factor 2 kinase (EF2K) caused by exposure to low pH (Dorovkov et al., 2002). The significant reduction of protein synthesis in liver, brain, heart and gill tissues in hypoxia-exposed *A. ocellatus*, observed in the present study, combined with the decreases in extracellular and intracellular pH in *A. ocellatus* exposed to comparable hypoxic conditions (Richards et al., 2007) strengthens the argument of Richards et al., that pH may have a direct effect on protein synthesis and therefore, metabolic rate in *A. ocellatus*.

Recovery from acute hypoxia exposure

A significant overshoot in oxygen consumption to 270% of normoxic rates was observed during the first hour of recovery, indicating that the 3-h hypoxic exposure was substantial enough to cause the fish to accumulate an oxygen debt. Crucian carp have also been shown to accumulate a substantial oxygen debt during periods of hypoxia (Van den Thillart and Verbeek, 1991), and it has been suggested the hyperactivation of metabolic rate during anoxic/severe hypoxic recovery is associated with the restoration of phosphocreatine, the conversion of lactate into glycogen, and possibly an increase in protein synthesis (Johansson et al., 1995). Although there was no accumulation of lactate in the white muscle in the present study, Richards et al. (Richards et al., 2007) have shown a 30% decrease in creatine phosphate in white muscle after a 4 h exposure to hypoxia, which returned to pre-hypoxia exposure levels during recovery. As such, the substantial increase in oxygen consumption seen in our study during the first hour of recovery may be linked to the restoration of phosphocreatine. An *in vitro* study on turtle hepatocytes exposed to 12 h of anoxia has shown a significant increase in protein synthesis (to 160% of normoxic rates) during the first hour of recovery (Land et al., 1993). However, the present study, which measured *in vivo* protein synthesis rates, did not show any hyperactivation of protein synthesis in the various tissues during the recovery period. These results agree with other *in vivo* studies showing a hyperactivation of protein synthesis does not occur in anoxic exposed turtles (Fraser et al., 2001) or crucian carp (Smith et al., 1996). There were two distinct patterns observed in post-hypoxic phenylalanine incorporation in *A. ocellatus*. In tissues that are a main source for protein synthesis, liver and gill, phenylalanine incorporation returned to pre-hypoxic rates by 1 h post-hypoxia. By contrast, protein synthesis in brain and heart take longer than 4 h post-hypoxia to fully recover. The slow recovery in brain tissue is particularly interesting as its hypoxia-induced reduction in protein synthesis is half of that shown by the other tissues. The reasons for this remain elusive; however, it may be linked to the removal of a dietary source of amino acids (due to the cessation of feeding), requiring *A. ocellatus* to rely on the recycling of existing protein (i.e. protein turnover) to replenish diminished supplies due to the decrease of protein synthesis during metabolic depression.

Conclusions

The present study was successful in furthering the insight into the biochemical adaptations of *A. ocellatus* to conditions of extreme low oxygen to include a description of the role protein synthesis plays in contributing to the whole animal metabolic depression. The response of *A. ocellatus* to acute hypoxia and subsequent recovery, at both the physiological and biochemical level, was similar to that of the well studied anoxia-tolerant teleost, the crucian carp. However, there are tissue-specific differences in the magnitude of the hypoxia-induced depression of protein synthesis (brain 20%, other tissues 50–60%), which suggest that brain function is maintained during hypoxia to facilitate active predator avoidance. As well, this study demonstrated that an acute (3 h) exposure to severe hypoxia is substantial enough to cause *A. ocellatus* to accumulate an oxygen debt, but the repayment of this oxygen debt is not accompanied by a compensatory hyperactivation in protein synthesis. This latter finding indicates the high metabolic rate *A. ocellatus* during the first hour of recovery is attributed to cellular processes other than the assimilation of protein. Combining the results from the current work with recent discoveries from comparable studies on the behavioural, physiological and biochemical adaptations of *A. ocellatus* (Muusze et al., 1998; Almeida-Val et al., 2000; Sloman et al., 2006; Richards et al., 2007; Wood et al., 2007) it can now be concluded the metabolic depression observed behaviourally is achieved through a decrease in physical activity, activation of anaerobic metabolism and the reduction of energy consuming processes (nitrogenous waste production, ion exchange and protein synthesis). These biochemical adaptations enable *Astronotus ocellatus* to maintain stable ATP levels, and therefore extend survival time when faced with conditions of extreme low oxygen.

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