



Hypoxia tolerance in two amazon cichlids: mitochondrial respiration and cellular metabolism adjustments are result of species environmental preferences and distribution

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Abstract The amazon fishes' responses to hypoxia seem to be related to the Amazon basin diversity of aquatic environments, which present drastic daily and seasonal variations in the dissolved oxygen concentration. Among these fishes' adaptation to hypoxia, behavioral, metabolic, physiological, and biochemical responses are well known for some species. In this work, we aimed to identify how two different aquatic environments, normoxic forest streams and hypoxic lakes, dictate the responses to hypoxia for two cichlid species, *Mesonauta festivus* and *Aequidens pallidus*. In our results, we found that *A. pallidus* is less tolerant to hypoxia, which seems to be related to this animal's natural normoxic environment. Even though this species modulated the mitochondrial respiration in order to improve the oxygen use, it also showed a lower decrease in metabolic rate when exposed to hypoxia and no activation of the anaerobic metabolism. Instead, *M. festivus* showed a higher decrease in metabolic rate and an activation of the anaerobic metabolism. Our data reveal that the natural dissolved oxygen influences the hypoxia tolerance and the species' tolerance is related to its ability to perform metabolic depression. The interest results are the absence of mitochondrial respiration influences in

these processes. The results observed with *A. pallidus* bring to light also the importance of preserving the forests, in which streams hold very specialized species acclimated to normoxia and lower temperature. The importance of hypoxia tolerance is, thus, important to keep fish assemblage and is thought to be a strong driver of fish biodiversity.

Keywords Phenotypic plasticity · Hypoxia tolerance · Cellular metabolism · Mitochondrial respiration · Amazon · Cichlids

Introduction

Several aquatic environments undergo different dissolved oxygen concentration variations worldwide, presenting from moderate to severe hypoxia, during shorter and longer periods (Mandica and Regan 2018). The Amazon River basin is diverse in aquatic environments, and most of these environments endure daily and seasonal fluctuation in water oxygen concentration fluctuation. These environments have different daily and seasonal dynamics such as *várzeas*, lakes, flooded forests, and rivers main (Junk et al. 1983). The variations of oxygen levels in these environments can go from moderate to severe and last for short and long periods, bringing considerable differences in O₂ availability between these environments (Val and Almeida-Val 1996). Even though most of the Amazonian aquatic environments go through oxygen

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level fluctuation, some environments have a relatively stable oxygen concentration, like the main streams of small forests, which are normoxic year-round (Val and Almeida-Val 1996). Some first orders streams, located at highland forests, show high oxygen levels and no variation along the year; these streams also have lower temperatures and oxygen or temperature variations that occur only in small ponds along the streams that are temporarily formed due to the rainy regime of the forest. Thus, these small areas may vary through the year, influenced by rainy season, but are well determined and located, such as these lateral ponds (Araújo-Lima et al. 1995; Sabino and Zuanon 1998). The stability in these forest aquatic environments is mainly provided by the vegetal canopy's coverage and rainy season (Pazin et al. 2006) and both features are currently threatened by global warming (Costa et al. 2020). In order to live in these environments and cope with these variations, fishes developed the most diverse responses which seem to be explained by their different life styles and usage these animals have in their environments (Mandic and Regan 2018).

Different environments lead to different responses in animals, by adaptations of the said population to endure the local conditions. Natural stressors can impose several threats to animals. Fishes can be adapted to different temperatures (McBryan et al. 2016), different water pH (Araújo et al. 2017), and different dissolved oxygen concentrations (Val and Almeida-Val 1996). Mostly, different oxygen concentrations can bring differences in the hypoxia tolerance in zebrafish, *Danio rerio* (Ho and Burggren 2012), provided by differences in the expression of genes that are highly responsive to hypoxia stress, such as *hif-1 α* , which in this and other works, the expression is related to the natural oxygen levels that these animals are exposed to (Thomas et al. 2007; Kodama et al. 2012).

Most of the Amazonian fishes' responses to hypoxia seem to be related to their environment characteristics, responding at several biological levels, from behavioral to respiratory, physiological, biochemical, and genetic adjustments (Val et al. 1998). Although some species chose to avoid hypoxic environments, others present physiological and biochemical adaptations to endure such stress. During development, as a defense against predator, some species can use hypoxic environments as refuge

(Anjos et al. 2008). When exposed to hypoxia, the characidae *Colossoma macropomum* can extend its lower lip in order to extract the water from the upper water column, which is more oxygenated (Saint-Paul 1984; Val and Almeida-Val 1996). When exposed to hypoxia, Amazonian fishes can show different strategies: (1) decrease the ATP demand by decreasing the aerobic respiration, or (2) increase the anaerobic metabolism (Almeida-Val et al. 2000; Hochachka and Somero 2002), even though some species show both strategies, which seem to be related to their tolerance levels (Almeida-Val et al. 1993; Almeida-Val and Hochachka 1995; Chippari-Gomes et al. 2005). For instance, while decreasing the overall metabolic rate in order to decrease energy demand, the cichlid *Astronotus ocellatus*, which is one of the vertebrate species that can cope with hypoxia and anoxia for long periods of time and survives under oxygen deprivation in nature, increases its anaerobic metabolism, increasing blood levels of lactate, as lactate dehydrogenase is recruited to increase its levels (Almeida-Val et al. 2000; Sloman et al. 2006). These results have been confirmed by several other works with the Oscar species, *Astronotus crassipinnis*, which results in this species high tolerance to hypoxia (Heinrichs-Caldas et al. 2019).

Even though mitochondrial activity in hypoxia is not well studied in Amazon fishes, it is known from studies in other species that its respiration and ROS production play a role in mitochondrial responses to low oxygen levels (Solaini et al. 2010), but it is not well established how the mitochondrial responses are related to these animals' hypoxia tolerance. The mitochondria of the estuarine killifish *Fundulus heteroclitus*, when acclimated to hypoxia, show a regulation in mitochondrial P_{50} (Partial pressure of oxygen where mitochondrial respiration is half maximal), endured reoxygenation after anoxia, without changing the respiration, and reduced the rate of reactive oxygen species (ROS) emission (Du et al. 2016). Hickey et al. (2012) showed that the epaulette shark (*Hemiscyllium ocellatum*), a hypoxia-tolerant species, has the mitochondrial oxidative phosphorylation unaffected by acute hypoxia exposure, while the hypoxia-intolerant shovelnose ray (*Aptychotrema rostrata*) has the oxidative phosphorylation decreased when exposed to low oxygen levels. From recent studies with Amazonian fishes, it is known that the heart mitochondrial respiratory capacity is related to the animal's respiratory

strategies (Campos et al. 2020), showing that the different ways to face hypoxia modulate the mitochondrial respiration. Even though it is clear that mitochondrial respiration changes among species (Moyes et al. 1992), it is unclear if mitochondrial respiration can be related to local adaptation and hypoxia tolerance in tropical species.

Since low oxygen levels bring a potential oxidative stress, most of the aquatic animals that go through hypoxia developed a high antioxidant defense system. Antioxidant defenses can act throughout enzymatic activities, such as catalase (CAT) and superoxide dismutase (SOD), that work with other enzymes in order to keep normal levels of ROS in the cells (Chowdhury and Saikia 2020). For instance, during anoxia, the goldfish, *Carassius auratus*, increases liver catalase activity, in order to keep the oxidative stress manageable (Lushchak et al. 2001). In another work, Víg and Nemcsók 1989 found that the carp, *Cyprinus carpio*, increased the SOD activity in the liver after several hours in hypoxia. When exposing *Astronotus ocellatus* and *Colossoma macropomum* to long-term hypoxia, Marcon (1996) showed that *A. ocellatus* presented minimal changes in antioxidant defenses and no cellular damage in the liver and blood, while *C. macropomum* showed to be more susceptible to oxidative damage after hypoxia exposure. This author also suggested that the responses observed for both species are related to its hypoxia tolerance and metabolic preference, with *C. macropomum* relying mostly in the aerobic metabolism.

Several works assessing fish tolerance to different stressors show that cichlids are the most hypoxia-tolerant Amazonian fishes and different genera/species may adjust better to hypoxia and others may not (Almeida-Val et al. 1995). Herein, we studied two species that occur in aquatic environments with distinct variations in the oxygen concentration, *M. festivus* from várzea lakes, and *A. pallidus* from forest streams. The geographic distribution presented by both species provides a rich source of natural variable responses to hypoxia, which can be used to explore distinct strategies when facing low dissolved oxygen concentration. In order to find out which strategies are used by those fish species found in such different environments, we evaluated the oxygen use, the liver metabolism shift, and liver mitochondrial respiration from both species after exposure to hypoxia. Based

on its environment, we hypothesized that *A. pallidus* would be less tolerant to hypoxia, with a lower anaerobic capacity and few changes to mitochondrial respiration.

Material and methods

Experimental fish

The fish sampling areas were selected according to natural dissolved oxygen, daily and seasonal, variation. *Aequidens pallidus* specimens were collected in streams at Reserva Nacional Adolpho Ducke (02°53'S, 59°58'W), a protected area located near Manaus, Amazonas, Brazil. Fish were sampled according to Mendonça et al. (2005) by hand and seine nets. *Mesonauta festivus* specimens were collected using monofilament gillnets of standardized dimension in Catalão Lake (3°10'S, 59°54'W), another protected area located near Solimões River, close to Manaus, Amazonas, Brazil.

After sampling, fishes were transported to the Laboratory of Ecophysiology and Molecular Evolution (LEEM), located in the Brazilian National Institute for Research of the Amazon (INPA), where it was kept for 1 week, to acclimate from handling, before the following experiments. While in the laboratory, the animals were fed daily and kept in circulating water.

Respirometry and loss of equilibrium

These experiments were conducted in the Laboratory of Ecophysiology and Molecular Evolution (INPA). The critical oxygen tension (PO_{2crit}) was determined prior to the hypoxia exposition experiment to decide the oxygen concentration for hypoxia experiments. The PO_{2crit} methodology is the same used in previous works (Heinrichs-Caldas et al. 2019) as first described by Steffensen (1989). Eight *A. pallidus* specimens (9.2 ± 1.3 g) and eight *M. festivus* (13.7 ± 2.2 g) were initially kept for 3 h in respirometry chambers to recover from handling with continuously water flush and controlled temperature ($28^\circ\text{C} \pm 0.5$) inside a bath aquarium. An automated apparatus DAQ-M (Loligo System, Tjele, Denmark), which works in recirculating

cycles, was used to measure oxygen consumption in the following steps: wait, flush, and measurement, with time between them to be determined; with this, it is determined the duration of a “loop.” The recirculating cycle is controlled by AutoResp software (Loligo System, Tjele, Denmark). During the flush phase, peristaltic pumps were used to exchange the water of the chambers with the aquarium. The oxygen measurement of the chambers occurred through optical cables connected to OXY-4 or Witrox-4 (Loligo System, Tjele, Denmark) and to sensors spot attached inside the chambers. The determination of PO_{2crit} was obtained by suppressing the flush phase, so the PO_2 decreased as the oxygen was consumed inside the chambers. The oxygen consumption rate was calculated, and PO_{2crit} was determined as the point where the PO_2 regression line of the oxygen regulation intersected the oxygen conforming, initiating the suppressed metabolic rate by segmented linear regression using the software SegReg (www.waterlog.info) (De Boeck et al. 2013). The PO_{2crit} experiment lasted around one and a half hour. In the following experiments, the species were exposed to an oxygen concentration equivalent to around 70% of its PO_{2crit} .

After determining the PO_{2crit} , the routine metabolic rate, MO_2 ($\text{mg O}_2 \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$), was measured. Eight *A. pallidus* specimens (8.9 ± 2.7 g) and eight *M. festivus* (13.2 ± 1.9 g) were kept in the same situation as described above, except this time fish was exposed to a full “loop” in the respirometry chamber (180 s flush + 90 s wait + 360 s measurement). The animals were kept overnight with regular oxygen concentration ($6.79 \text{ mg O}_2 \cdot \text{L}^{-1} \pm 0.15$ for both species), followed by 3 h in hypoxia ($0.70 \text{ mg O}_2 \cdot \text{L}^{-1} \pm 0.9$ for *M. festivus*, and $1.23 \text{ mg O}_2 \cdot \text{L}^{-1} \pm 0.87$ for *A. pallidus*), where the oxygen was decreased slowly by displacing O_2 with nitrogen gas directly into the water. The MO_2 was calculated as $MO_2 = -\Delta O \cdot V_{resp} \cdot B^{-1}$, where ΔO is the rate of change in oxygen concentration ($\text{mg O}_2 \cdot \text{h}^{-1}$), V_{resp} is the volume of the respirometry chamber, and B is the mass of the individual (kg).

To achieve the time of loss of equilibrium in hypoxia, $LOE_{(hyp)}$, eight animals of each species were kept in the chambers described above. Then, the oxygen concentration was set at $0.79 \text{ mg O}_2 \cdot \text{L}^{-1} \pm 0.8$ for *M. festivus* and $1.25 \text{ mg O}_2 \cdot \text{L}^{-1} \pm 1.3$ for *A. pallidus*, until the animal was not able to maintain the

equilibrium. If the animal did not respond after 3 gentle touches to the chamber, the time to loss of equilibrium was determined (McBryan et al. 2016).

Hypoxia exposure

For the experiment, both species were divided into two groups, normoxia and hypoxia ($n = 8$). Each animal was randomly placed in a 1.5 L individual plastic container 1 day before the experiment to acclimate from handling. During the acclimation, the animals were kept at $28 \text{ }^\circ\text{C} \pm 0.3$ with constant aeration (O_2 concentration: $6.82 \text{ mg O}_2 \cdot \text{L}^{-1} \pm 0.12$). For the hypoxia group, the oxygen concentration was slowly decreased by N_2 pump directly in the water for around 1:30 h, and after the oxygen concentration reached $0.73 \text{ mg O}_2 \cdot \text{L}^{-1} \pm 0.9$ for *M. festivus* and $1.23 \text{ mg O}_2 \cdot \text{L}^{-1} \pm 0.5$ for *A. pallidus*, the animals in the hypoxia group were exposed to 3 h of hypoxia. Meanwhile, the normoxic group was kept under constant aeration $6.89 \text{ mg O}_2 \cdot \text{L}^{-1} \pm 0.27$. Dissolved oxygen concentration was monitored with an Oxymeter 5512-Ft (YSI, USA, Ohio, Yellow Springs). The 3-h exposition to hypoxia was chosen based on a preview exposition, for both species, in the respirometry, to assure that this was enough for metabolic depression and opercular movements increase, which ensure that the animals were in hypoxia. For each group and each species, eight animals were sampled and euthanized by head concussion followed by a cut in the spine cord. Then, the liver was excised and stored at $-80 \text{ }^\circ\text{C}$ until the following assays. For the mitochondrial respiration, a small piece of the liver was immediately put in BIOPS buffer ($2.77 \text{ CaK}_2\text{EGTA}$, $7.23 \text{ K}_2\text{EGTA}$, $5.77 \text{ Na}_2\text{ATP}$, $6.56 \text{ MgCl}_2 \cdot 6\text{H}_2\text{O}$, 20 taurine, 20 imidazole, 0.5 dithiothreitol, 50 K-MES, 15 sodium phosphocreatine, and 50 fructose, all measured in mM). This experiment was performed in the morning and is in accordance with CONCEA Brazilian Guide for Animals Use and Care under INPA’s authorization (CEUA: protocol #054/2017).

Liver mitochondria respiration and ROS production

Respiratory flux through complexes I, II, III, and IV of the electron transport chain was measured in liver permeabilized ant. After the tissues were immersed in BIOPS, $10 \text{ } \mu\text{g/L}$ of Saponin was added to 2 mL BIOPS with the tissue and left to react for

30 min. After this, the livers were dried, weighed, and immersed in 0.5 mL of MiR05 buffer (pH 7.24) at 20 °C: 0.5 EGTA, 3.0 MgCl₂·6H₂O, 60 potassium lactobionate, 20 taurine, 10 KH₂PO₄, 20 Hepes, 160 sucrose, and 1.0 BSA 1.0 (all in mM), essentially fatty acid free, and homogenized by hand. This homogenate was then diluted in 2.5 mL MiR05 in order to measure the oxygen consumption in an Oxygraph 2K Oroboros (Innsbruck, Austria) (Gnaiger et al. 2000).

For the mitochondrial respiration, we used two protocols, a SUIIT protocol and P_{50} /catalytic efficiency protocol. Before the addition of the following substrates, oxygen was added to the chambers so the oxygen would not run out during the experiment. In order to measure complex I (CI) state II respiration, 2 mM malate and 10 mM pyruvate were added to the chambers in order to measure CI without ADP. Later, 2.5 mM ADP was added to stimulate oxidative phosphorylation (OXPHOS), and 10 mM glutamate was added to saturate CI. Cytochrome *c* of 10 μM was added in order to access mitochondrial membrane integrity. Phosphorylating respiration with CI and complex II (CII) substrates (CI+II) was measured after the addition of 10 mM succinate. The ETS, respiratory electron transfer-pathway capacity, was measured by the addition of carbonyl cyanide *p*-(trifluoromethoxy) phenyl-hydrazone (0.5 mM FCCP). The activity of CI, II, and III complexes was then inhibited by the addition of rotenone (0.5 μM), malonate (15 mM), and antimycin (1 μM), respectively. RCR was calculated as ETS/leak state ratio. In order to activate the complex IV (CIV) respiration, the terminal oxidase of the mitochondrial ET-pathway, it was added 2 mM ascorbate and 0.5 mM TMPD (N,N,N',N'-tetramethyl-*p*-phenylenediamine dihydrochloride) to the mitochondria medium.

In order to access the P_{50} and the catalytic efficiency, the liver was prepared as described above, followed by the activation of CI and CII, by adding malate, pyruvate, glutamate, ADP, and succinate, in the same concentrations as described above. After this, the oxygen in the chamber was left to be consumed until full depletion, which took around one and a half hour for each sample. Then, the P_{50} and catalytic efficiency (J_{\max}/P_{50}) were calculated. For both measurements, the oxygen consumption was calculated when the oxygen pressure reached the half of its maximum.

Total ROS production was measured alongside the mitochondrial respiration. SOD of 22.5 U·mL⁻¹ was added to catalyze the reaction of the superoxide produced by the mitochondria and 3 U·mL⁻¹ horseradish peroxidase was added to catalyze the reaction of hydrogen peroxide with 15 μM Amplex UltraRed which will result in the release of resorufin, a fluorescent substance. The equipment will detect the resorufin using a wavelength of 525 nm and amplio-metric filter set (AmR); from Oroboros Instruments. The resorufin signal was calibrated with additions of exogenous hydrogen peroxide.

Enzyme activities and lipoperoxidative damage

Lactate dehydrogenase (LDH; E.C. 1.1.1.27) and citrate synthase (CS; E.C. 4.1.3.7) were measured in the liver following a well-established protocol for Amazonian fish tissues (Driedzic and Almeida-Val 1996) modified for plate spectrophotometer reader. For the assay, 0.01 g of tissue was homogenized in a 4× imidazole buffer (50 mM imidazole, 1 mM EDTA, and 1% Triton x-100 at pH 7.4) and centrifuged at 10,000×g in a Refrigerate Centrifuge 5430 R (Eppendorf, Hamburg, GE) for 15 min at 4 °C. LDH assays were performed in 0.2 mL 96-well plate containing 0.15 mM NADH, 1 mM KCN, and 50 mM imidazole, pH 7.4 at 25 °C. The LDH reactions started with 1 mM pyruvate. CS assays were performed in a 0.2 mL 96-well plate with 0.25 mM DTNB and 75 mM Tris base, pH 8.0 at 25 °C, and the reactions were initiated with 0.4 mM acetyl Co-A and 0.5 mM oxaloacetate.

To measure SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6) activities, and lipid peroxidation (LPO) levels, 0.01 g of tissue was homogenized in a buffer containing Tris base 20 mM, EDTA 1.0 mM, dithiothreitol 1.0 nM, sucrose 50 nM, and KCl 150 nM. Then, the homogenates were centrifuged at 15,000 g for 20 min at 4°C and were used in the following proportions: 1:10 w/v for SOD, 1:4 w/v for CAT and LPO. SOD activity was determined according to Turrens (1997) based on the cytochrome *c* reduction rate inhibition by superoxide radical at 550 nm and 25 °C. SOD enzyme activity is expressed as U SOD mg protein⁻¹, where 1 U of SOD corresponds to the quantity of enzyme that promoted the inhibition of 50% of cytochrome *c*. To measure CAT activity, the H₂O₂ decomposition inhibition rate was measured at 240 nm, and the results were expressed as μmol H₂O₂

$\text{min}^{-1} \text{mg protein}^{-1}$. The LPO was measured based on the Fe^{+2} to Fe^{+3} oxidation by hydroperoxides in acid medium, in the presence of ferrous oxidation-xylenol orange, at 560 nm, as determined by Jiang et al. (1991).

All the enzyme activities, the LPO, and the total protein were determined in triplicates at 28 °C using the plate spectrophotometer SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA, USA). Total protein was quantified following the Bradford assay (Bradford 1976).

Data analysis

Data are expressed as mean \pm S.E.M. The normality and homogeneity of variances were checked before parametric testing. The PO_{2crit} and $LOE_{(hyp)}$ were examined by a *T*-test, testing for differences between the two species, *M. festivus* and *A. pallidus*. A two-way analysis of variance (two-way ANOVA) was used to test if there were differences between the two species, normoxia and hypoxia exposition. The statistical analyses were performed in SigmaStat (v. 3.5) and graphics were made in SigmaPlot software (v. 11.0).

Results

Environment description

The fish for this experiment were sampled in the wild, in very distinct environments, such as flooded areas, which continually go through daily and seasonal oxygen and temperature variation as in the case of fishes sampled in the Catalão Lake, and the streams' forest species, as those occurring at Reserva Ducke, where normoxic water is constant year-round. Figure 1 shows the daily oxygen concentration and temperature during the sampling expedition for 24 h. These expeditions were performed in October, and the oxygen concentration and temperature were measured using an Oximeter 5512-Ft (YSI, USA, Ohio, Yellow Springs) every 2 h.

Respirometry and loss of equilibrium

There was difference in the PO_{2crit} in these two species. *M. festivus* showed a lower ($P=0.006$) PO_{2crit} ,

$1.01 \pm 0.07 \text{ mg O}_2 \cdot \text{L}^{-1}$, while *A. pallidus* PO_{2crit} was $1.80 \pm 0.2 \text{ mg O}_2 \cdot \text{L}^{-1}$.

Comparing both species, *M. festivus* and *A. pallidus*, the oxygen consumption rates were different under hypoxia, with the *M. festivus* showing a MO_2 lower than *A. pallidus* ($P=0.003$, $F=4.74$) (Fig. 2). These animals also showed different rates of suppression in the MO_2 . While *M. festivus* was able to depress the MO_2 around 41%, *A. pallidus* was able to decrease its MO_2 only approximately 13%.

Regarding the $LOE_{(hyp)}$ values, *M. festivus* presented a higher ($P=0.001$) $LOE_{(hyp)}$ value, needing $121.14 \pm 12 \text{ min}$ to lose equilibrium when oxygen concentration was below $1 \text{ mg O}_2 \cdot \text{L}^{-1}$, while for *A. pallidus* species, the loss of equilibrium was with $62.12 \pm 4.4 \text{ min}$.

Mitochondrial respiration and ROS production

The mitochondrial respiration (Fig. 3) showed no difference between species and treatments for complex I ($P=0.213$, $F=1.657$), complex IV ($P=0.407$, $F=0.718$), and H+ Leak ($P=0.317$, $F=1.055$). Complex I+II was higher for *M. festivus* in normoxia ($P=0.015$, $F=3.772$) when comparing both species. *A. pallidus* decreased ETS in hypoxia ($P=0.006$, $F=4.323$). CIII was higher for *M. festivus* in normoxia and hypoxia compared to *A. pallidus* ($P=0.008$, $F=4.15$); the same happened to RCR, which was higher for *M. festivus* in both treatments ($P=0.012$, $F=3.93$).

For mitochondrial P_{50} (Fig. 4), *M. festivus* showed no differences between treatments, while *A. pallidus* showed a lower P_{50} under hypoxia ($P=0.014$, $F=3.89$). The inverse relation was found for *A. pallidus* when measuring the catalytic efficiency, which was higher during hypoxia ($P<0.001$, $F=6.62$), while for *M. festivus*, there was no difference ($P=0.477$, $F=1.028$).

The comparison between *A. pallidus* and *M. festivus* regarding total ROS production (Fig. 4) was higher for *A. pallidus* in normoxia ($P=0.004$, $F=4.652$) and showed no difference between both species in hypoxia ($P=0.23$, $F=1.87$). *A. pallidus* showed a decrease in total ROS production in hypoxia ($P=0.033$, $F=3.22$).

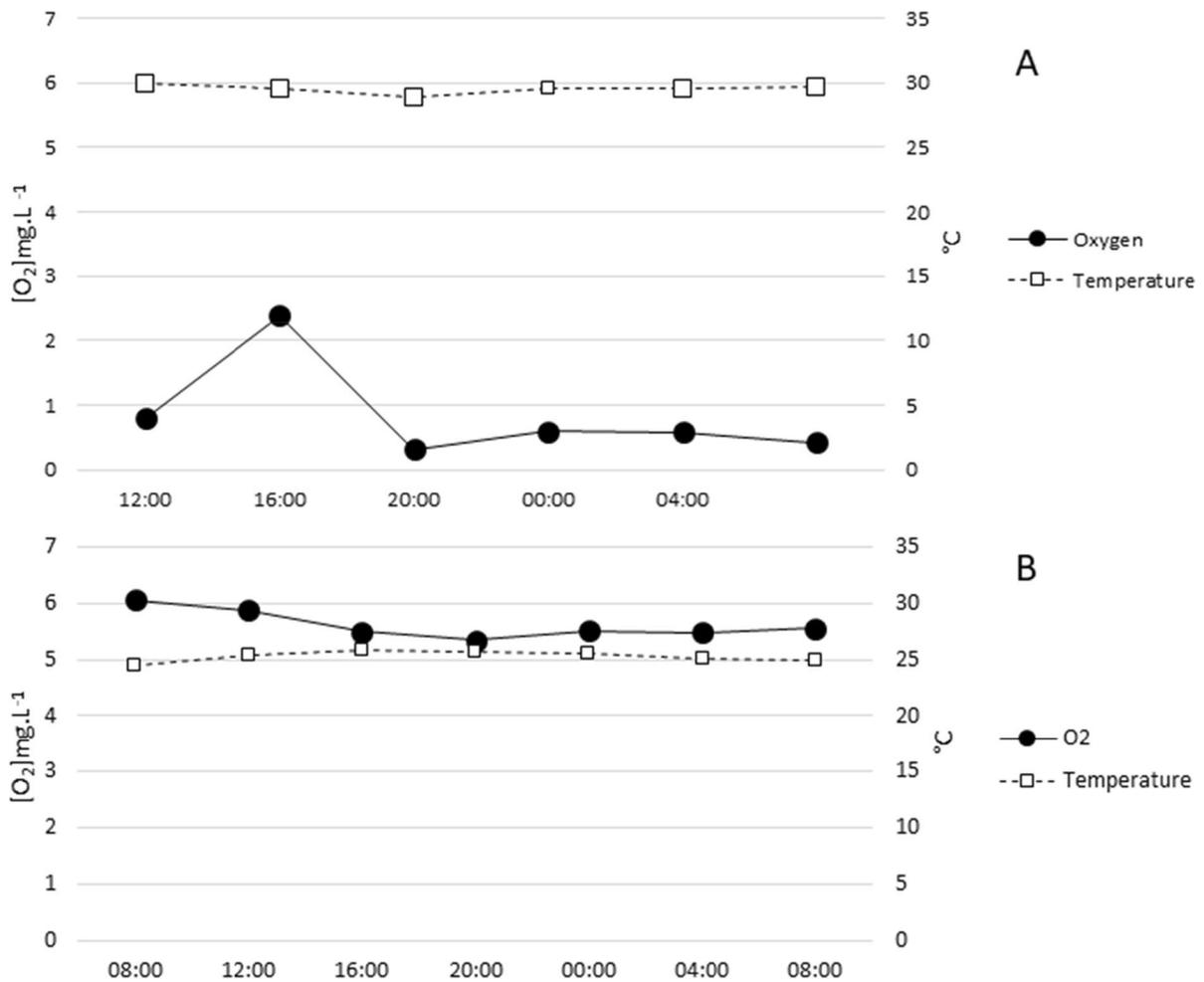


Fig. 1 Oxygen concentration and temperature measurements in both fish sampling sites (Catalão Lake, 3°10'S, 59°54'W, and Reserva Florestal Adolpho Ducke forest streams, 02°53'S,

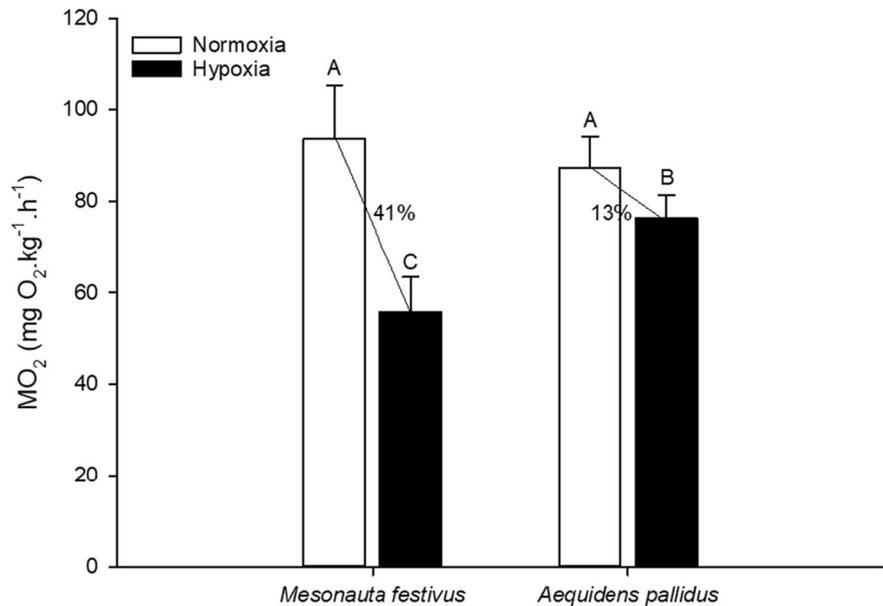
59°58'W) during 24 h. These data were collected during the fishes sampling.

Enzyme activities and lipoperoxidative damage

Enzyme activities showed different patterns for both species. LDH activity was higher ($P=0.021$, $F=15.02$) for *M. festivus* during hypoxia exposure ($P=0.048$, $F=2.32$), while *A. pallidus* showed no difference between treatments ($P=0.774$, $F=0.41$). CS showed no difference ($P=0.913$, $F=0.174$) in hypoxia or normoxia in both species. LDH/CS was higher ($P=0.006$, $F=6.89$) for *M. festivus* under hypoxia, and *A. pallidus* showed no difference between treatments ($P=0.156$, $F=2.04$). SOD activity showed no difference between species ($P=0.290$,

$F=1.52$), even though *A. pallidus* increased SOD activity during hypoxia ($P=0.021$, $F=3.48$). CAT activity was lower in *M. festivus* in both treatments when comparing to *A. pallidus*. ($P<0.001$, $F=7.46$). *A. pallidus* increased CAT activity under hypoxia ($P<0.001$, $F=9.53$), while *M. festivus* showed no differences between treatments ($P=0.605$, $F=0.741$). LPO increased in hypoxia for *M. festivus* ($P<0.001$, $F=12.031$) and *A. pallidus* ($P<0.001$, $F=8.046$), and, in hypoxia, it was higher ($P=0.001$, $F=5.21$) for *M. festivus*. All data are shown in Table 1.

Fig. 2 *Mesonauta festivus* ($n=8$) and *Aequidens pallidus* ($n=8$) MO_2 during 3 h of normoxia and hypoxia. The decrease in MO_2 is indicated by the % symbol. The statistical significance was analyzed using a two-way ANOVA. Letters indicate the differences between species and treatments ($P<0.05$). Error bars indicate standard error of the means.



Discussion

Hypoxia tolerance and metabolic strategies

In general, different environmental features affect the adaptative and evolutionary responses of species. It is extensively reported that the oxygen concentration affects the physiological, biochemical, and life cycle activities of fishes, bringing several different responses and adjustments. Furthermore, oxygen concentration also has profound effects on both phenotypes and geographical distribution of aquatic organisms. The fish's responses to hypoxia we found in the present work are related to their environment conditions and life history, which may interfere in species survival and adaptation to a new oxygen regime. In the next paragraph, we will detail such argument.

The species studied in this article showed different strategies to cope with hypoxia, which are related to their life history. By exhibiting a lower PO_{2crit} , a higher depression of the MO_2 under hypoxia and a longer $LOE_{(hyp)}$ time, *M. festivus* showed to be more tolerant to this stressor than its related species, *A. pallidus*. A lower PO_{2crit} and a higher MO_2 depression indicate a higher capacity to extract O_2 from the environment and to maintain cellular function, which is reflected by the $LOE_{(hyp)}$, a direct measure that shows how long an animal can keep its ecological function under hypoxic pressure. Even though the

use of PO_{2crit} to determine hypoxia tolerance is under debate (Wood 2018; Regan et al. 2019), in the present work, it showed to be a reliable measurement, since it corresponded to the higher metabolic depression and higher $LOE_{(hyp)}$ time for *M. festivus*. A behavioral response observed in this work is the increase in *A. pallidus* movements in the aquaria when the animal is in hypoxia, other than that, during recovery phase, after hypoxia exposure, half of the *A. pallidus* died, while *M. festivus* showed no death after PO_{2crit} and $LOE_{(hyp)}$ trials.

The glycolytic responses for both species were also different. While *A. pallidus* showed no modulation in LDH and CS enzyme activities under hypoxia, *M. festivus* increased LDH activity and LDH/CS, indicating a metabolic switch in liver respiration from aerobic to anaerobic. The LDH/CS enzyme activities ratio determines which type of metabolism is preferred by the animal, indicating, or not, a metabolic shift (Almeida-Val and Hochachka 1995). Working with two cichlids species, *A. crassipinnis* and *S. aequifasciatus*, Chippari-Gomes et al. (2005) observed that the CS/LDH ratio decreased when both animals were exposed to hypoxia, indicating the activation of an anaerobic metabolism, corresponding to other features, revealing that these animals are considered good anaerobes.

Hochachka and Somero (2002) explained that Amazonian fishes could rely in two strategies when facing hypoxia: (1) the animals can depress the

aerobic metabolism through the reduction of oxygen consumption and (2) increase anaerobic glycolysis, suggesting that both strategies could occur separately or concomitantly. *M. festivus* showed both strategies concomitantly, which makes this species a hypoxia-tolerant fish. The same strategy was previously reported for *Cichlasoma* sp. (Almeida-Val et al. 1993) and *Astronotus crassipinnis* (Heinrichs-Caldas et al. 2019), both hypoxia-tolerant species that inhabit flooded forests and lakes. These are species that belong to the Cichlid family, the same family of the species studied in the present work. Considering such results, we can suggest that the high hypoxia tolerance presented by *M. festivus* is related to the fluctuation of oxygen concentration in its environment. Instead, *A. pallidus*, which also belongs to the cichlid family, may be considered hypoxia sensitive and, based on the results presented in this work, this sensitivity might be molded through its preferential habitat, the highly oxygenated waters from the forest streams.

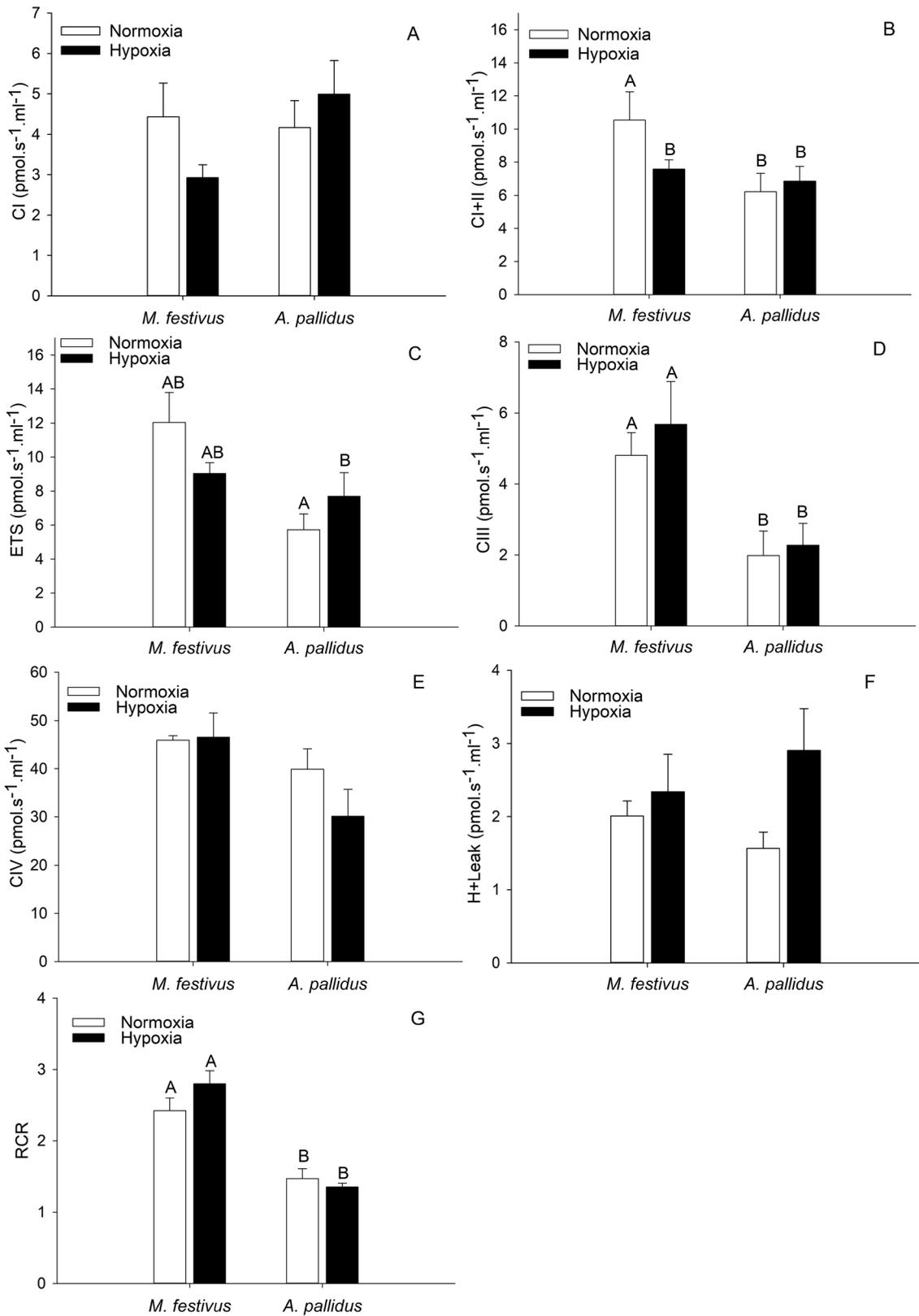
Oxidative stress and defenses

Changes in temperature, pH, oxygen, and salinity are common stressors in aquatic environment and then impact most aquatic species under natural and artificial conditions. These stressors bring a disruption between ROS production and elimination of oxidative products by antioxidant defenses, characterizing an oxidative stress (Chowdhury and Saikia 2020). Several works showed that different species show different oxidative responses and that these responses are related to hypoxia, both through long-term exposure and/or exposure to quick changes in oxygen availability. As a consequence, these hypoxia exposures cause the activation of antioxidant defenses, leading or not to an oxidative stress (Heise et al. 2007; Leveelahti et al. 2014; Johannsson et al. 2018; Castro et al. 2020).

In the present work, the oxidative damages and the activation of antioxidant defenses are determined by the oxygen regime of species natural environment. While *M. festivus* showed no difference in CAT and SOD activities, *A. pallidus* increased the activity of both enzymes when exposed to hypoxia, even though both species increased LPO levels. Otherwise, exposing *A. pallidus* to hypoxia increased the catalase activity, which catalyzes H_2O_2 decomposition to

H_2O . Thus, we hypothesize that the decrease in total mitochondrial ROS accumulation in this species aims to avoid the appearance of oxidative stress in the liver. The increase in SOD activity for *A. pallidus* also indicates an oxidative stress as well, since this enzyme is responsible for general defenses against superoxide. The activation of both enzymes, CAT and SOD, in hypoxia was also observed in killifish (*Fundulus heteroclitus*), when exposed to different hypoxia patterns, in order to mitigate the ROS imbalance (Du et al. 2016). Changes in these and other antioxidant enzymes were also observed in *Perccottus glenii* when exposed to hypoxia (Lushchak and Bagnyukova 2007). Instead, after hypoxia exposure, *Cyprinus carpio*'s liver did not change CAT or SOD activities (Lushchak et al. 2005). Herein, the increase in LPO levels for both species indicates the occurrence of oxidative lipid damage, resulting in an oxidative stress for both species under hypoxia, which suggests that, although in different intensities, none of the studied species went through hypoxia free of oxidative damage. LPO is an example of ROS-induced damage with the polyunsaturated fatty acids reacting with free radicals. An increase in LPO levels was already observed for *Mytilus galloprovincialis* and *Mugil cephalus* when exposed to hypoxia (Ekambaram et al. 2016; Woo et al. 2013). The increase in these enzyme activities and LPO levels in *A. pallidus* liver depicted the loss of cellular homeostasis in hypoxia. We can also hypothesize that the increase in LPO for both species is related to the lipid utilization by the animal during hypoxia. As observed by De Boeck et al. (2013), in Oscar (*A. ocellatus*) exposed to hypoxia, the main energy source comes from lipids, which might reflect in the LPO levels.

In general, antioxidant responses to hypoxia appears to be species- and tissue-specific, and as our results suggest, the responses are related to the animal's hypoxia tolerance. For some species like the deer mice (*Peromyscus maniculatus*) (Mahalingam et al. 2017), a higher capacity in decreasing ROS production and increasing antioxidants activities seems to be a feature of high tolerant species, some other like the marine sculpins (*O. maculosos*), a less-tolerant animal, showed a higher response in liver oxidative stress than the other species studied, the *S. marmoratus*, a higher hypoxia-tolerant animal (Lau et al. 2019). Herein, our study showed two different strategies of tolerance to hypoxia; *A. pallidus*



- ◀ **Fig. 3** Complexes I (A), I+II (B), ETS (C), III (D), and IV (E), H+Leak (F), and RCR (G) in both species (*Mesonauta festivus* and *Aequidens pallidus*) liver mitochondria when the animals were exposed to 3 h of normoxia and hypoxia. The statistical significance was analyzed using a two-way ANOVA. Letters indicate the differences between species and treatments ($P < 0.05$). Error bars indicate standard error of the means.

is less tolerant than *M. festivus*, presenting a higher activation of liver antioxidant defenses, revealed by the response to total mitochondrial ROS production and LPO. Thus, we are confident that these species, *M. festivus* and *A. pallidus*, are acclimatized to their specific environments, which results in differential hypoxia tolerance. The adaptation to their environment during their evolutionary time has driven them to develop specific and different adjustments in their metabolism.

In addition to these results, total mitochondrial ROS production decreased in *A. pallidus* liver during hypoxia, while *M. festivus* showed no difference between treatments. The increase in mitochondrial ROS can lead to a depletion in ATP production and damage the ETS (Dröse et al. 2016). As a defense from antioxidant damage, the increase observed in CAT and SOD activity by *A. pallidus* is directly related to mitochondrial ROS production, since these enzymes act as the first oxidative defense in the cell (van der Oost et al. 2003). Other than that, the decrease in total ROS observed in *A. pallidus*' liver seems to be linked to the increase in the ETS during hypoxia, as discussed below. With the increase in the antioxidant defenses and the following decrease in total ROS production, we can affirm that this species maintains the regular mitochondrial function during hypoxia, presenting no strategies to afford hypoxia periods. *M. festivus*, on the other hand, presents lower levels of ROS production and no changes in antioxidant defense mechanisms, although the only difference between treatments is the increase in LPO.

Mitochondrial respiration

The mitochondrial liver respiration responses observed in our results showed to be species-specific and related to the hypoxia tolerance (Figure 3). While *M. festivus* showed no acute regulation in most of its mitochondrial respiration complexes, except in CI+II, *A. pallidus*, which is sensitive to hypoxia, increased the ETS in order to endure hypoxia. Mitochondrial

oxidative phosphorylation (OXPHOS) plays a central role regulating cell energy production. In the OXPHOS, mitochondrial complexes I, III, and IV generate proton-motive force, while complex II transfers electrons to CIII, which by proton-motive force will generate ATP along with the electron transport system (ETS), making the OXPHOS a key component in oxygen usage in hypoxia (Paradis et al. 2016). However, protons can migrate to the matrix independent of this process, a mechanism named “proton leak,” which uncouples substrate oxygen from ATP generation. The OXPHOS showed few differences for both species in this study. While *M. festivus* decreased the CI+II, *A. pallidus* showed no change, even though *A. pallidus* CIII was lower in hypoxia in comparison to *M. festivus*.

Distinct hypoxia acclimation (intermittent and constant) in killifish mitochondria showed no difference in OXPHOS capacity and ETS (Du et al. 2016), while after 2 weeks of anoxia at 5 °C, *T. scripta*, an anoxia-tolerant turtle, exhibited a reduction in maximal state II, III, and IV respiration rates in cardiac permeabilized fibers and isolated mitochondria (Galli et al. 2013). In the present work, the increase in ETS, as observed in *A. pallidus* liver, is one of the factors that regulates the decrease in total ROS production, since there is a decrease in electron slip, which is related to the unchanged CI (Cadenas 2018; Solaini et al. 2010). Even though there was a tendency in the increase for both species, none of them changed their H+ leak, showing that the use of uncoupling mechanism is not used by these species when facing hypoxia, at least for this period of exposure. Our results also show that, even though both species do not regulate the CIII in hypoxia, *M. festivus* CIII was higher, both in normoxia and hypoxia, when compared to *A. pallidus*.

Mitochondrial P_{50} and its catalytic efficiency is a great measure to relate mitochondrial function to hypoxia tolerance because it shows to what extent the respiration can be sustained under low oxygen pressure. However, in general, when the mitochondrial P_{50} is analyzed across different species with different rates of hypoxia tolerance, there seems to be no consistent association between those characteristics (Sokolova et al. 2019). In the present work, *A. pallidus* decreased the mitochondrial P_{50} and increased the catalytic efficiency (J_{\max}/P_{50}) during hypoxia, indicating an effort to increase capacity to maintain

Figure 4 Catalytic efficiency (A), mitochondrial P_{50} (B) and total mitochondrial ROS production (C) in liver mitochondria from both species (*Mesonauta festivus* and *Aequidens pallidus*) when the animals were exposed to 3 h of normoxia and hypoxia. The statistical significance was analyzed using a two-way ANOVA. Letters indicate the differences between species and treatments ($P < 0.05$). Error bars indicate standard error of the means.

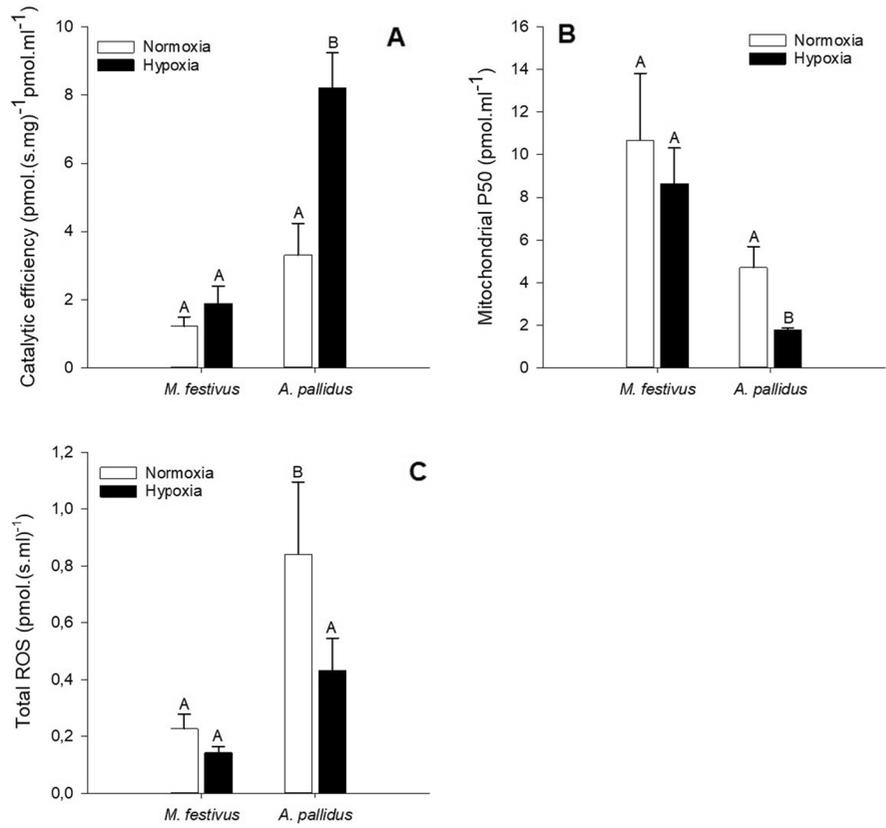


Table 1 Enzyme activity of lactate dehydrogenase (LDH), citrate synthase (CS), and the ratio between LDH and CS (LDH/CS), superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (LPO) in liver of *M. festivus* (n=8) and *A. pallidus* (n=8) exposed to three hours of normoxia and hypoxia. The statistical significance was analyzed using

a two-way ANOVA. LDH, CS and LDH/CS enzyme activity were expressed as $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}\cdot\text{prot}^{-1}$. SOD activity was expressed as $\text{U}\cdot\text{min}^{-1}\cdot\text{mg}\cdot\text{prot}^{-1}$. CAT activity was expressed as $\text{mM}^{-1}\cdot\text{mg}\cdot\text{prot}^{-1}$. LPO was expressed as $\mu\text{M}\cdot\text{cumene}\cdot\text{hydroperoxide}\cdot\text{mg}\cdot\text{prot}^{-1}$. Letters indicate differences between species and treatments ($P < 0.05$).

| | | LDH | CS | LDH/CS | SOD | CAT | LPO |
|--------------------|----------|--------------------------|---------------|---------------------------|--------------------------|--------------------------|-----------------------------|
| <i>M. festivus</i> | Normoxia | 1.10 ± 0.11 ^A | 0.065 ± 0.004 | 17.32 ± 1.76 ^A | 3.87 ± 0.65 ^A | 0.17 ± 0.01 ^A | 22.97 ± 3.13 ^A |
| | Hypoxia | 1.79 ± 0.22 ^B | 0.062 ± 0.003 | 24.51 ± 2.47 ^B | 5.14 ± 0.63 ^A | 0.22 ± 0.02 ^A | 119.43 ± 15.69 ^B |
| <i>A. pallidus</i> | Normoxia | 1.13 ± 0.36 ^A | 0.06 ± 0.003 | 17.43 ± 2.5 ^A | 4.04 ± 0.47 ^A | 0.74 ± 0.05 ^B | 22.53 ± 2.5 ^A |
| | Hypoxia | 1.15 ± 0.32 ^A | 0.06 ± 0.003 | 18.69 ± 2.94 ^A | 5.89 ± 0.46 ^B | 1.51 ± 0.15 ^C | 80.51 ± 4.55 ^C |

its function with low O_2 . These results indicate that the increase in the mitochondria capacity to sustain the respiration in low O_2 is a tentative to compensate for its low anaerobic ability, as observed in *A. pallidus* under hypoxia exposure.

The respiratory control ratio (RCR) for both species showed no difference between treatments (Fig. 1) but was higher, both in hypoxia and normoxia, for *M. festivus* mitochondria. RCR is usually

measured to indicate the leak rate or ETS coupling efficiency of mitochondria (Rolf and Brown 1997). These results suggest that *M. festivus* mitochondria have a higher respiratory control ratio than *A. pallidus* mitochondria, and this feature might be related to its higher tolerance, same as observed by Hickey et al. (2012) when comparing a higher tolerant species (Epaulet shark) to a lower tolerant one (Shovel-nose ray).

When the mitochondrial responses are compared to the hypoxia tolerance, our results show some contrasting patterns compared to other species. As observed in *M. festivus*, some tolerant species show no regulation in ETS under hypoxia (Sappal 2016). In other cases, hypoxia can decrease ETS activity in hypoxia in low-tolerant species (Ivanina et al. 2016), which contrasts with the results obtained for both species studied here, particularly for *A. pallidus*, that increased the ETS activity. The decrease in CI+II observed in *M. festivus* liver also shows no relation to hypoxia tolerance, since contrasting results show that this decrease can happen in both tolerant and sensitive species (Sokolova et al. 2019).

As several works have shown, it is difficult to relate the mitochondrial respiration to the ability to tolerate hypoxia. In the present work, we compared species that are phylogenetically close related and showed that the differences in the hypoxia tolerance are induced by the history of environmental oxygen fluctuation, which seems to be related to specific changes in mitochondrial respiration, along the evolutionary divergence between them. As suggested by Sokolova et al. (2019), the studies with phylogenetically related species may unveil the relations between the hypoxia tolerance and mitochondrial respiration patterns like mitochondrial P_{50} .

A brief discussion about Cichlidae phenotypic plasticity

The phenotypic plasticity is the ability of an animal to respond to different environments using more than one phenotype resulted from a single genotype (Price et al. 2003). The Amazonian cichlids are able to cope with hypoxia for weeks or months, due to behavioral, physiological, and biochemical modifications (Almeida-Val et al. 1993). The studies conducted with cichlids and different hypoxia levels and duration have shown a trend when it comes to metabolic responses; they either rely on the anaerobic metabolism or/and suppress their energy demand and keeping low rates of oxidative stress (Muusze et al. 1998, Chippari-Gomes et al. 2005, Heinrichs-Caldas et al. 2019).

The pressure exercised by some environments, like *várzea* lakes, where there is a great variation in the dissolved oxygen levels, leads to a higher phenotypic plasticity induced by the gene expression. In this work,

Almeida-Val et al. (1995) reported that the sensitivity of 15 species to hypoxia was related to its *ldh* tissue distribution in different tissues, for example, specimens from hypoxic environments showed a restriction of *ldh-b* expression in the heart, while specimens from normoxic waters showed a regular *ldh* distribution. Also, in this study, *Cichlasoma amazonarum* was a special case, showing both patterns, with changes after 51 days of hypoxia exposure, resulting in a metabolic shutdown in this species, showing that the *ldh* gene expression is regulated according to environmental conditions (Almeida-Val et al. 1995). To go directly to the point, the phenotypic plasticity observed in Amazonian cichlids may be considered the key that allows the animals to survive chronic and periodic hypoxia.

What comes to our attention is that all these studies were conducted with animals from *várzea*, *igapós*, or lakes, showing an influence of the dissolved oxygen variation in the plasticity of these animals to endure hypoxia. As far as we know, this is the first work accessing the hypoxia responses and tolerance of a cichlid fish from forest streams, a stable environment when it comes to dissolved oxygen concentration. Our results with *A. pallidus*, the species from forest stream, showed us a different trend from the one discussed previously; this animal does not change liver oxidative metabolism, with no change in the LDH or CS activity, presenting a small decrease in the oxygen consumption rate, and some changes in the mitochondrial metabolism in order to keep the aerobic respiration, which has shown to be an ineffective strategy, making this animal sensitive to hypoxia, and, apparently, presenting no or low phenotypic plasticity when it comes to this stressor, which is different from any other Amazonian cichlid species ever studied. Our results with this species showed us the need to understand how these species from such a stable environment may or may not develop its plasticity to endure different stressors that might come as a result of global warming and deforestation. Other than that, we also suggest that, in this case, the life history seems to be more important than the natural plasticity exhibited by the Amazonian Cichlids group.

Conclusion

In conclusion, analyzing the results obtained in the present work, we confirmed that the hypothesis that

natural variation in dissolved oxygen brings a higher hypoxia tolerance to the species that live in that environment. Furthermore, comparing closely related species showed that different responses reflect in their ability to endure hypoxia. *M. festivus* higher hypoxia tolerance is related to this species ability in modulating the metabolic rate and anaerobic metabolism, by decreasing the oxygen consumption rate and increasing the activity of the LDH, while keeping low levels of oxidative stress, a response intrinsically related to its environment natural oxygen variation. On the other hand, *A. pallidus*, a species less tolerant to hypoxia, is not able to activate the anaerobic metabolism in the liver and cannot decrease the oxygen consumption rate effectively, relying on the mitochondrial modulation through the increase of its catalytic efficiency and decrease of its P_{50} , which enhance mitochondrial affinity by oxygen, meaning that less oxygen is necessary to fulfill half of mitochondrial performance. At last, but not least, we can affirm that both species use different strategies to face hypoxia and that those strategies might contribute to their specific tolerance to low oxygen, which is clearly related to their natural environment variation of oxygen levels. The present work suggests that *A. pallidus* will be more threatened by a sudden hypoxia appearance in its environment, which is certainly predictable as a result of global warming and deforestation, leading to a loss of fish diversity.

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Author contribution Both authors, Waldir Heinrichs-Caldas and Vera Maria Fonseca de Almeida-Val, contributed equally for the manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable

Declarations

Ethics approval Animal use and experiments are in accordance with CONCEA Brazilian Guide for Animals Use and Care under INPA's authorization (CEUA: protocol #054/2017).

Competing interest The authors declare no competing interests.

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