**INSTITUTO NACIONAL DE PESQUISA DA AMAZÔNIA – INPA**
**CURSO DE PÓS-GRADUAÇÃO EM BIOLOGIA DE ÁGUA DOCE E PESCA INTERIOR – BADPI**

# Sobrevivendo ao ambiente amazônico: mecanismos mitocondriais de resposta à hipóxia e à reoxigenação em peixes da Amazônia

Manaus- Amazonas

Março, 2022

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# Sobrevivendo ao ambiente amazônico: mecanismos mitocondriais de resposta à hipóxia e reoxigenação em peixes da Amazônia

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Agências Financiadoras: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (n° 465540/2014-7) e Fundação de Amparo à Pesquisa do Amazonas (FAPEAM) (n° 062.01187/2017) financiaram o INCT-ADAPTA II. Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (n° 001).

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Tese apresentada ao Programa de Pós-Graduação em Biologia de Água Doce e Pesca Interior (BADPI), como parte dos requisitos para obtenção do título de Doutora em Ciências Biológicas.

Manaus- Amazonas

Março, 2022

**SINOPSE**

Este estudo teve como objetivo avaliar o efeito da hipóxia constante, bem como de oscilação na concentração de oxigênio dissolvido, sobre os ajustes metabólicos de espécies amazônicas, avaliando desde as taxas de absorção de oxigênio até os mecanismos bioquímicos e celulares de consumo.

Palavras-chave: Hipóxia. Reoxigenação. Peixe. Metabolismo. Mitocôndria. Estresse Celular.

*Dedico esta tese às mulheres da minha vida:*

*à minha mãe, Mila Braga, à minha irmã, Ariel Braz, e à minha avó, Imaritinha.*

## AGRADECIMENTOS

À minha mãe, Mila Braga, por acreditar, respeitar e incentivar todas as minhas escolhas, por me motivar a ser um ser humano melhor e por ser meu exemplo de bondade, amor, cumplicidade e responsabilidade. Te agradeço por sempre significar alegria para mim, por toda motivação e incentivo. Também à minha irmã, Ariel Braz, por sonhar junto comigo os meus sonhos, pelas conversas valiosas sobre os nossos valores comuns, por ser minha melhor amiga e meu exemplo diário de generosidade. Nada disso seria possível sem vocês duas na minha vida.

À minha querida orientadora, Dra. Vera Maria Fonseca de Almeida e Val, por ter me acolhido no laboratório desde 2010 quando eu ainda era iniciante no mundo da pesquisa. Por sempre ter me motivado, acreditado no meu potencial, confiado no meu trabalho e por todas as oportunidades que me foram oferecidas. Por ser uma inspiração como mulher na ciência e pelo compromisso e tempo dedicado à minha formação. Também pelo amor e carinho comigo durante esses anos de convivência, pelas conversas acolhedoras, pelo respeito mútuo e por sempre me fazer sentir como sua filha científica!

Ao professor Adalberto Luis Val, por todas as discussões científicas que fizeram com que a realização dessa tese fosse possível, por estar sempre disponível e acessível a solucionar minhas dúvidas, por confiar em mim e me motivar a seguir caminho da ciência e por ser minha referência de dedicação à pesquisa. Em especial, pelo carinho e amizade durante esses 11 anos e pelos encorajamentos repetidos semanalmente durante as reuniões de sexta-feira. Eles funcionam!

Ao meu coorientador, Tyson MacCormack, pelo suporte na discussão dos experimentos, pela disponibilidade em solucionar minhas dúvidas, pela preocupação em acompanhar o andamento do meu trabalho e por todos os *insights* e tempo dedicado para a conclusão desta tese. Também pela amizade, ensinamentos e por me acolher com tanto carinho no seu laboratório durante meu estágio doutoral na Mount Allison University.

À Nazaré Paula Silva, minha querida "Nazinha", pelo suporte e dedicação para a conclusão deste trabalho, sem medir esforços para que todas as nossas demandas fossem atendidas. Obrigada também pelo carinho comigo durante todos esses anos, pela amizade e por todas as comidas deliciosas que fazes, especialmente o teu maravilhoso cuscuz.

Aos amigos do LEEM, Dona Valzinha, Bruna Osterno, Melina Rizzato, Mayara Cristina, Talita Lauren, Beatriz Enola, Giovana Bataglion, Guilherme Azambuja e Jaqueline Custódio, pela ajuda direta e indireta na realização desta tese, pela convivência, momentos de descontração e discussões científicas. Particularmente, agradeço ao Derek Campos, pelo suporte durante a realização dos experimentos e tempo dedicado à realização de algumas análises junto comigo.

Às minhas amigas do peito, Yasmin Dantas e Júlia Mesquita, pela ajuda com as análises no laboratório, dias de coleta e, em especial, pela amizade e por todas as palavras de acolhimento, incentivo e amor. Também à minha dona Valzinha, pelos momentos de descontração no laboratório, cafezinhos e pela amizade.

Ao meu querido amigo, Lucas Castanhola Dias, pelo apoio de sempre nas análises de microscopia e pelos conhecimentos compartilhados. Também à equipe do Laboratório Temático de Microscopia e Nanotecnologia do INPA pelo auxílio no processamento das minhas amostras e pela amizade, principalmente à Maria Fabiele (Fabi) e Marcela Magalhães.

À equipe técnica do Centro Multiusuário para Análise de Fenômenos Biomédicos da Universidade do Estado do Amazonas (CMABio), especialmente ao Jander Matos pelo suporte no imageamento das minhas amostras no microscópio eletrônico de transmissão.

Agradeço também a secretaria do LEEM, especialmente à Dona Rai, Claudinha e Raquel, pela dedicação em atender nossas requisições acadêmicas com agilidade, seriedade e eficiência. Também ao técnico Rogério, pelo auxilio nas demandas dos experimentos e análises.

Ao programa de Pós-Graduação em Biologia de Água Doce e Pesca Interior (PPG BADPI) do Instituto Nacional de Pesquisas da Amazônia, pela oportunidade de cursar o doutorado.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e à Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM) pelo apoio financeiro destinado ao PPG BADPI para a manutenção da infraestrutura e atividades do curso.

Agradeço também à CAPES pela concessão da bolsa durante todo o doutorado, bem como pela extensão de prazo remunerada oferecida devido ao atraso no desenvolvimento regular das atividades, afetadas devido às restrições do isolamento social necessário ao combate à pandemia da CoViD-19.

Ao projeto INCT-ADAPTA, fomentado pelo CNPq e pela Fundação de Amparo à Pesquisa da Amazônia (FAPEAM), pelo recurso que permitiu com que a elaboração deste trabalho fosse possível.

## RESUMO

Os ambientes de áreas alagadas da Amazônia apresentam condições frequentemente hipóxicas, onde os de gradientes de oxigênio são conhecidos por atingirem concentrações muito próximas de zero, principalmente à noite, afetando diretamente a capacidade de sobrevivência dos peixes. O presente trabalho de tese teve como objetivo avaliar o efeito da hipóxia, bem como o da reoxigenação, sobre as respostas metabólicas de peixes amazônicos avaliando desde as taxas respiratórias de consumo de oxigênio, até mecanismos celulares de regulação do status redox e controle de qualidade mitocondrial. Tanto a hipóxia quanto a reoxigenação frequentemente produzem alterações significativas nos processos metabólicos como um todo, o que inclui a produção de espécies reativas de oxigênio (ROS) que, quando em grande quantidade, são altamente prejudiciais para os processos celulares. As ROS podem oxidar e danificar as membranas mitocondriais, bem como as enzimas do sistema de transporte de elétrons (ETS) e do ácido tricarboxílico (TCA), levando à supressão da fosforilação oxidativa (OXPHOS) e superprodução de mais moléculas de ROS. Nossos resultados mostraram que os peixes amazônicos apresentam uma alta tolerância cardíaca à hipóxia em condições ambientais naturais. Porém, outros órgãos altamente metabólicos, tais como o fígado, são mais comprometidos quanto à sua capacidade de geração de energia. Apesar disso, peixes altamente tolerantes à hipóxia, tal como o *A. ocellatus*, apresentam capacidades mitocôndrias para neutralizar o conteúdo de ROS tanto durante a hipóxia, como após a reoxigenação. Esse trabalho apresenta, pela primeira vez, mecanismos de respiração mitocondrial nunca antes descritos para as espécies aqui estudadas, relacionando essas respostas aos padrões de taxa de consumo de oxigênio e lançando luz sobre a tolerância celular dos peixes amazônicos em seus ambientes naturais.

## ABSTRACT

The floodplain areas of the Amazon often present hypoxic conditions, in addition to oxygen gradients that are known to reach concentrations close to zero, especially at night, directly affecting the survival capacity of fish. The present thesis aims to evaluate the effects of hypoxia, as well as reoxygenation on the metabolic responses of Amazonian fish, ranging from respiratory rates of oxygen consumption to cellular mechanisms of redox status regulation and mitochondrial quality control. Both hypoxia and reoxygenation often produce significant changes in metabolic processes as a whole, which includes the production of reactive oxygen species (ROS) in large amounts, resulting in harmful damage to cellular processes. ROS can oxidize and damage mitochondrial membranes as well as ETS and tricarboxylic acid (TCA) enzymes, leading to suppression of OXPHOS and overproduction of more ROS molecules. Our results showed that Amazonian fish have a high cardiac tolerance to hypoxia under natural environmental conditions. However, important metabolic organs such as the liver are more compromised in terms of their catalytic ability to generate energy. Despite this, highly hypoxia-tolerant fish, such as *A. ocellatus*, have mitochondrial skills to neutralize ROS content both during hypoxia and after reoxygenation. As far as we know, this work presents, for the first time, mechanisms of mitochondrial respiration never before described for the Amazon fish species living in hypoxia, relating these responses to patterns of oxygen consumption rate, and shedding light on the cellular tolerance of Amazonian fish to their natural environments.

**SUMÁRIO**

## LISTA DE TABELAS

### CAPÍTULO 1



### CAPÍTULO 2



### CAPÍTULO 3



### CAPÍTULO 4

## LISTA DE FIGURAS

### INTRODUÇÃO GERAL



### CAPÍTULO 1

## CAPÍTULO 2



## CAPÍTULO 3

## LISTA DE ABREVIAÇÕES E SIGLAS

| | |
|---|---|
| ATP | adenosina trifosfato |
| DO | concentração de oxigênio dissolvido |
| mt$\dot{M}O_2$ | consumo de oxigênio mitocondrial |
| CCCP | carbonilcianeto m-clorofenil-hidrazona |
| CI | complexo mitocondrial I |
| CII | complexo mitocondrial II (ou succinato desidrogenase, SHD) |
| COX | citocromo c oxidase |
| ER | retículo endoplasmático |
| ETS | capacidade máxima do sistema de transferência de elétrons |
| $FADH_2$ | dinucleótido de flavina e adenina |
| $\dot{M}O_2$ | consumo de oxigênio |
| MRC | células ricas em mitocôndrias |
| NADH | dinucleótido de nicotinamida e adenina |
| OXPHOS | fosforilação oxidativa |
| $P_{LOE}$ | ponto de perda de equilíbrio |
| $PO_2$ | pressão de oxigênio |
| $PO_{2crit}$ | pressão crítica de oxigênio |
| PVC | células pavimentosas |
| RCR | razão de controle respiratório |
| RMR | taxa metabólica de rotina |
| ROS | espécies reativas de oxigênio |
| $\Delta p$ | força próton-motriz |
| TCA | ciclo do ácido tricarboxílico |

## 1. INTRODUÇÃO GERAL

A bacia hidrográfica do Amazonas é a mais extensa rede hidrográfica do globo terrestre, ramificando-se por todos os países do norte da América do Sul, desde os Andes até o Oceano Atlântico (Eva e Huber, 2005). Isso permite diversos habitats aquáticos, incluindo cursos d'água com diferentes tamanhos (praias, lagos, florestas inundadas e áreas inundáveis), além de peculiares características físicas e químicas da água, as quais dão origem a três tipos: água branca, preta e clara. Estas vão diferir na quantidade e natureza de seus sedimentos e em seus níveis de carbono orgânico dissolvido, íons dissolvidos, pH, densidade e temperatura (Wallace, 1889; Sioli, 1984; Val et al., 2016). Essa diversidade ambiental é adicionada ao ciclo regular de variações no nível da água nos diferentes períodos do ano (Figura 1), decorrentes dos pulsos de inundação da região como descrito por Junk et al. (1989). Como resultado disso, variações nos parâmetros físicos e químicos da água também são esperados, particularmente a concentração de oxigênio dissolvido na água. Essa variação na concentração de oxigênio é decorrente de processos que alteram o ciclo destes compostos, tais como fotossíntese e respiração, difusão de luz, tamanho, profundidade, intensidade de vento, cobertura de macrófitas, vegetação de borda, decomposição orgânica e difusão molecular do oxigênio, que interagem de maneira complexa determinando a quantidade de oxigênio disponível nesse ambiente amazônico (Junk et al., 1983; Val, 1993; Crampton, 1996).

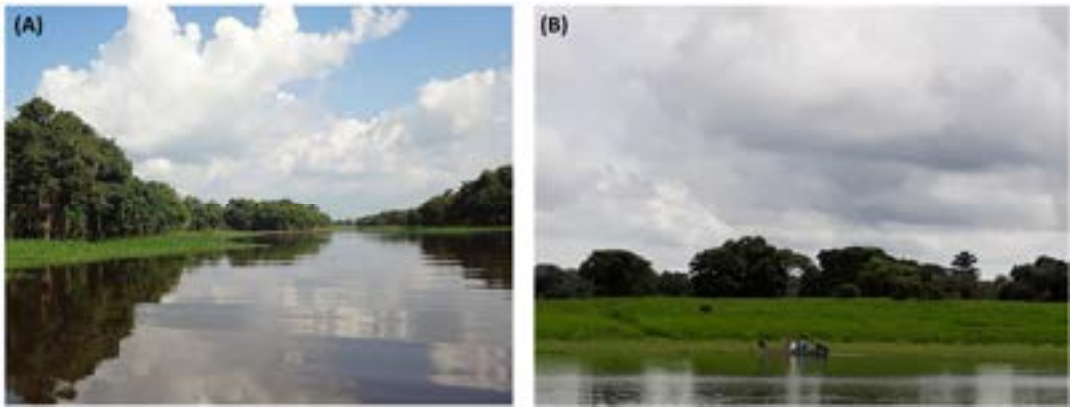



**Figura 1**. Fotografia do Lago Catalão (Amazonas-Brasil), um típico ambiente de área alagada da Amazônia, durante (A) a estação seca (novembro de 2018) e (B) a estação das cheias (abril de 2018). Note as alterações da paisagem associadas à variação do nível da água durante as diferentes estações.

Assim, considerando todas essas particularidades inerentes aos ambientes amazônicos, em particular, suas peculiares características de heterogeneidade das águas e parâmetros físicos e químicos, especialmente a interação destes com as concentrações do oxigênio na água, torna-se importante considerar estudos investigando as adaptações das espécies que neste ambiente vivem frente às diversas variações deste elemento fundamental para a sobrevivência. Os peixes amazônicos, por exemplo, são capazes de enfrentar drásticas mudanças, diárias e sazonais, na disponibilidade de oxigênio que ocorre naturalmente, onde o mesmo local pode apresentar normóxia (≅6 $mgO_2$ $L^{-1}$), hipóxia e até mesmo anoxia (0 $mgO_2$ $L^{-1}$) em um mesmo dia. Tal fato permitiu que várias espécies desenvolvessem diferentes estratégias durante o processo evolutivo para suportar essas variações de oxigênio e sobreviver nesses ambientes. Uma delas pode ser demonstrada pelos diferentes mecanismos de respirações acessórias. Esse tipo de adaptação pode ser encontrada em espécies de diferentes posições da história evolutiva (convergência evolutiva), como ocorre com espécies evolutivamente menos derivadas, tal como o teleósteo *Pterygoplichthys pardalis* (bodó), que usa o estômago como um órgão respiratório acessório, subindo à superfície e engolindo ar quando o oxigênio na água é escasso (Brito, 1981; Val 1995). Até mesmo teleósteos mais recentes, tal como o *Astronotus ocellatus*, que desenvolveram modificações metabólicas para se tornarem mais tolerantes às baixas concentrações de $O_2$, exibindo um conjunto de adaptações que vão desde de comportamentais até moleculares, passando por ajustes fisiológicos e bioquímicos.

Essas distintas estratégias evolutivas para lidar com as condições de hipóxia ocorrem devido ao oxigênio ser um elemento fundamental para a sobrevivência, uma vez que é utilizado para obtenção de energia, a qual é realizada principalmente pelas mitocôndrias no processo de respiração celular. Nesse processo, o $O_2$ serve como o principal aceptor de elétrons no sistema de transporte de elétrons (ETS) mitocondrial. A ETS transfere elétrons de substratos ricos em energia (NADH e $FADH_2$) para $O_2$ gerando uma força próton-motriz (Δp) (que inclui os gradientes eletroquímicos e de concentração de prótons) através da membrana interna da mitocôndria. A força motriz que direciona a atividade da ETS é dada pelo consumo de $O_2$ pela citocromo c oxidase (COX) (Blombberg e Siegbahn 2014). Os Complexos mitocondriais I, III e IV atuam como bombas de prótons gerando Δp, enquanto o Complexo II transfere elétrons de substratos ligados ao $FADH_2$para o Complexo III (Figura 2). A energia de Δp gerada pela ETS é

conservada na forma de ATP pelo Complexo mitocondrial V ($F_O$, $F_1$-ATP sintase) no processo de OXPHOS. A dependência da COX pelo $O_2$ (e, portanto, da ETS) torna a OXPHOS mitocondrial uma ferramenta chave para entender os mecanismos mitocondriais de estresse induzido pela hipóxia e pela reoxigenação (Paradis et al. 2016).

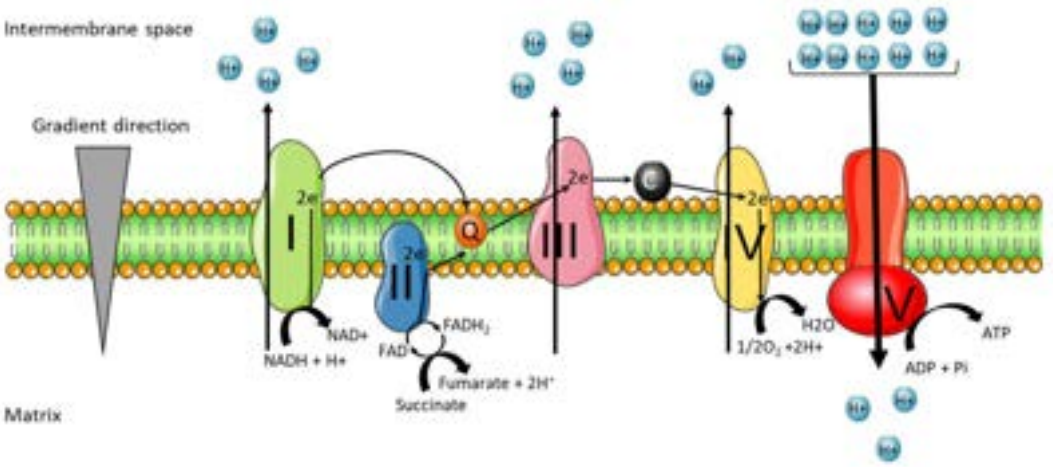



**Figura 2**. Esquema representativo do complexo da cadeia respiratória mitocondrial. A cadeia respiratória mitocondrial está localizada na membrana interna da mitocôndria. Composto por quatro complexos e duas coenzimas, permite a produção de ATP através da fosforilação oxidativa (OXPHOS). Os complexos I (NADH: coenzima Q oxidorredutase) e II (succinato desidrogenase) transferirão, cada um, dois elétrons para a coenzima Q10 (CoQ10). Os dois elétrons transferidos do complexo I vêm da oxidação do NADH, e os do complexo II vêm da oxidação do succinato a fumarato. CoQ10 permitirá a transferência de elétrons para o complexo III (CoQ10-citocromo C oxidorredutase). O complexo III passará então esses elétrons para o citocromo C, que faz a ligação com o complexo IV (citocromo C oxidase). O complexo IV reduz $O_2$ em uma molécula de $H_2O$. Os complexos I, III e IV são bombas de prótons, que permitem a passagem de prótons da matriz para o espaço intermembranar, na direção oposta ao gradiente. Os complexos I e III permitem a passagem de quatro prótons e o complexo IV de dois prótons. Uma vez que o espaço intermembranar é enriquecido com prótons, o último complexo da cadeia, a ATP sintase permitirá a passagem dos prótons na direção do gradiente. Este fluxo de prótons permitirá a síntese de ATP a partir de ADP. (figura consultada em Andrieux et al., 2021. doi: 10.3390/ijms222111338).

Nesse sentido, uma vez que a hipóxia leva à limitação do oxigênio nas mitocôndrias, o qual é substrato da COX, o que geralmente é esperado é uma diminuição da taxa de transferência de elétrons no ETS e, assim, diminuição na taxa de bombeamento de prótons que gera Δp (Kalogeris et al. 2012). Isso diminui a atividade de OXPHOS e pode resultar em deficiência de ATP na ausência de uma diminuição concomitante no consumo de ATP. Além disso, um declínio na atividade da COX resulta no acúmulo de

transportadores de elétrons parcialmente reduzidos no ETS mitocondrial, o que leva à produção excessiva de espécies reativas de oxigênio (ROS - do inglês, *Reactive Oxygen Species*) tanto durante a hipóxia, quanto depois da reoxigenação (Dröse et al. 2016). A produção elevada de ROS pode resultar em uma cascata viciosa de liberação de ROS danificando ETS, amplificando a deficiência de ATP e, eventualmente, resultando em colapso mitocondrial e apoptose (Zorov et al. 2014). Assim, mecanismos relacionados à regulação da respiração mitocondrial e status redox são essenciais para a sobrevivência à baixas concentrações de oxigênio. Apesar disso, pouco se sabe até o momento sobre os mecanismos estratégicos que regem as funções mitocondriais em peixes amazônicos, tampouco se existem mecanismos alternativos mitocondriais que podem atuar como um agente antioxidante no combate à formação de ROS para esse grupo de organismos.

O que se sabe até o momento, é que muitos peixes amazônicos deprimem suas taxas metabólicas durante a privação de oxigênio (Almeida-Val et al., 2000; Val, 1993.). Isso provoca redução da frequência cardíaca e, consequentemente, menor eficiência na entrega de oxigênio aos tecidos, resultando em taxas de produção de ATP mais baixas por meio de vias anaeróbicas. Essa capacidade de diminuir severamente muitas vias que produzem energia pode ser o ponto chave para a sobrevivência de muitas espécies. Tendo isso em mente, o que se sugere é que a redução do metabolismo leva ao aumento da tolerância à hipóxia por promover maior atividade antioxidante a fim de neutralizar as ROS e evitar o estresse oxidativo (Du et al., 2016). Apesar disso, essas suposições devem ser cautelosas, uma vez que a fisiologia mitocondrial pode diferir entre as espécies tolerantes e não tolerantes. Assim, a evolução de distintas estratégias respiratórias em diferentes posições no ramo evolutivo, nos leva a pensar que estes mecanismos podem ter sido resultado de respostas adaptativas para lidar com o estresse oxidativo promovido pelas alterações na concentração de oxigênio na água encontrada nos ambientes amazônicos.

## 2. OBJETIVOS

### *2.1 Objetivo Geral*

Avaliar o efeito da hipóxia, bem como de flutuações na concentração de oxigênio dissolvido, sobre os mecanismos metabólicos e de tolerância mitocondrial em peixes amazônicos.

### *2.2 Objetivos Específicos*

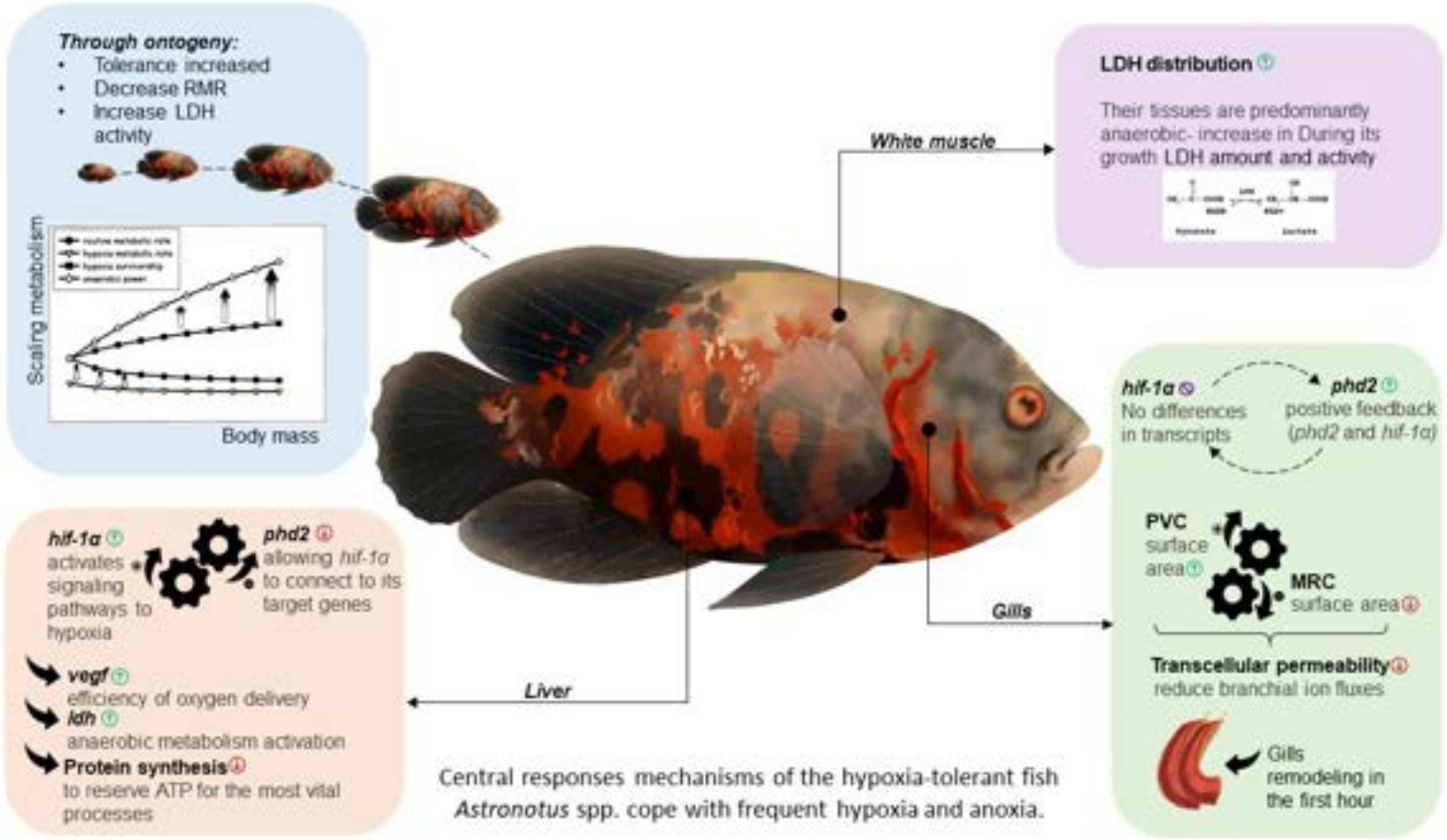
2.2.1. Capítulo 1. Apresentar uma visão geral dos principais mecanismos conhecidos envolvidos na tolerância dos peixes amazônicos à hipóxia;

2.2.2. Capítulo 2. Avaliar o efeito de diferentes estações (cheia e seca) sobre a fisiologia mitocondrial cardíaca de peixes amazônicos em seu ambiente hipóxico natural, bem como compreender tais respostas são espécie-específica;

2.2.3. Capítulo 3. Avaliar o efeito da exposição prolongada à hipóxia, bem como à reoxigenação, sobre os mecanismos mitocondriais de consumo de oxigênio e produção de ROS hepáticos através da determinação do perfil metabólico de espécies Amazônicas tolerantes à hipóxia;

2.2.4. Capítulo 4. Investigar se a maior tolerância à hipóxia de *Astronotus ocellatus* ao longo da ontogenia está associada a uma refinada relação entre ajustes mitocondriais, metabólicos, regulação do status redox e controle de qualidade mitocondrial em indivíduos adultos e juvenis expostos à hipóxia e os efeitos dessa tolerância no processo de recuperação.

# 3. CAPÍTULO 1




Central responses mechanisms of the hypoxia-tolerant fish *Astronotus* spp. cope with frequent hypoxia and anoxia.

Received: 22 February 2021 | Revised: 25 June 2021 | Accepted: 12 July 2021

DOI: 10.1002/jez.2531

REVIEW

JEZ-A ECOLOGICAL AND INTEGRATIVE PHYSIOLOGY WILEY

# Ecological adaptations of Amazonian fishes acquired during evolution under environmental variations in dissolved oxygen: A review of responses to hypoxia in fishes, featuring the hypoxia-tolerant *Astronotus* spp.

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**Funding information**
Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Grant/Award Number: 465540/2014-7; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Grant/Award Number: Finance Code 001; Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEAM), Grant/Award Number: 062.01187/2017

## Abstract

The Amazon Basin presents a dynamic regime of dissolved oxygen (DO) oscillations, which varies among habitats within the basin, including spatially, daily, and seasonally. Fish species inhabiting these environments have developed many physiological adaptations to deal with the frequent and periodic events of low (hypoxia), or no (anoxia) DO in the water. Cichlid fishes, especially the genus *Astronotus* (*A. ocellatus* and *A. crassipinnis*), are hypoxic-tolerant species that can survive in very low DO levels for long periods, while adults often inhabit places where DO is close to zero. The present review will focus on some metabolic adjustments that Amazonian fish use in response to hypoxic conditions, which include many strategies from behavioral, morphological, physiological, and biochemical strategies. These strategies include ASR (aerial surface respiration), lip expansion, branchial tissue remodeling, increases in glycolytic metabolism with the increase of blood glucose levels, and increases in anaerobic metabolism with increases of plasma lactate levels. Other groups over evolutionary time developed obligate aerial respiration with changes in pharyngeal and swim bladder vascularization as well as the development of a true lung. However, most species are water-breathing species, such as *A. ocellatus* and *A. crassipinnis*, which are detailed in this study because they are used as hypoxia-tolerant model fish. Herein, we draw together the literature data of the physiological mechanisms by which these species decrease aerobic metabolism and increase anaerobic metabolism to survive hypoxia. This is the first attempt to synthesize the physiological mechanisms of the hypoxia-tolerant *Astronotus* species.

**KEYWORDS**
anaerobic survival, fish, gill remodeling, hypoxia, signaling pathways, tolerance

  

## 1 | THE AMAZONIAN ENVIRONMENT: SHAPING THE EVOLUTION OF HYPOXIA-TOLERANCE IN AMAZONIAN FISH SPECIES

In Amazonian waters, dissolved oxygen (DO) patterns have varied over evolutionary time and could be postulated as drivers of selective evolutionary pressure. During the Cambrian, hypoxic and anoxic waters occurred in the entire Amazon Basin due to low atmospheric oxygen concentrations (Almeida-Val et al., 2006; Randall et al., 1981). Since the Cambrian, the Amazon Basin has gone through evolutionary changes, including increased complexity and the appearance of new aquatic environments that were dynamic and challenging to fish adaptation (Randall et al., 1981). Currently, some challenges still occur such as low and variable oxygen distribution in the waters. Today, the DO is not determined by oxygen concentration in the air, as occurred during the evolutionary ages, but rather by many different phenomena intrinsic to the entire basin (Almeida-Val & Farias, 1996; Val & Almeida-Val, 2006). The current hypoxic waters of the Amazon reflect complex interactions among physical, chemical, and biological parameters. Some of these phenomena include the photosynthetic rates, the respiration of bacteria, interactions between animals and plants, light penetration, organic matter decomposition, wind incidence, the amounts of minerals, and soil composition.

The annual flood pulses, which are the main driving forces in Amazonian rivers, result in water level increases of up to 13 m every year, and climate change is affecting these levels even more; in the current year, the level reached its highest value (30 m). The flood regime promotes seasonal hypoxia in some habitats, especially in flooded places such as *igapós* (forests inundated in the acidic blackwaters) and *várzeas* lakes (forests inundated in the whitewater; Val et al., 1998; see Figure 1 for oxygen variation in a particular flooded area). The DO concentration in the water column in Amazon lakes, such as Lago Catalão (Figure 1), is generally higher near the surface, reflecting the oxygen additions from photosynthesis and atmospheric exchange, and the DO becomes depleted as the water column is deeper. However, there are differences between the dry and flood seasons. During the flood season (represented by March in the Figure 1), the DO is very close to zero at the surface and in the entire water column all day long, and there is no consistent difference in concentrations between day and night. However, in other Amazon lakes such as Lago Tupé, the DO can reach up to 2.5 mg $O_2$ $L^{-1}$ during the flood season (Aprile & Darwich, 2009). The surface waters in Amazon floodplain lakes are generally subsaturated in oxygen during the flood season due to an abundance of allochthonous organic inputs and the predominance of aerial photosynthesis in aquatic plant communities (Melack & Forsberg, 2001). On the other hand, the opposite scenario is observed during the dry season (represented by October in the Figure 1), where the DO concentration is comparatively higher, and the period of the day has an important effect on the DO concentration. Moreover, during the dry season, many parts of the lakes have only few centimeters, and the maximum depth has been reduced to 6 m (Caraballo et al., 2014). Thus, oxygen remains stratified throughout most of the diel cycle, with concentrations peaking during the day and declining at night. Levels of oxygen sufficient for wildlife are generally restricted to the top 5 m of the water column, and a predominance of bacterial consumption, combined with an abundance of organic substrates, results in anoxic conditions throughout most of the year in waters below this depth.

Thus, understanding how fish can adapt to such variable conditions has been a question of many scientists (Junk, 1984; Val & Almeida-Val, 1999). The Amazonian fish species have developed a plethora of mechanisms to deal with the DO variation and hypoxic conditions in Amazonian aquatic environments (see Table 1; see also Val & Almeida-Val, 1995, 1999). Understanding these different adaptations that allowed many species to cope with these oxygen

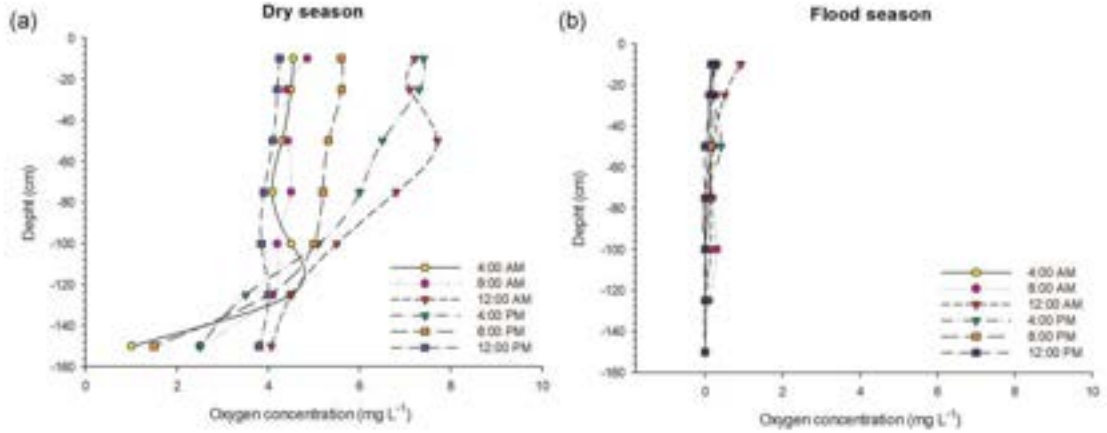


**FIGURE 1** Variation of the dissolved oxygen concentrations (mg $L^{-1}$) during 24 h at different depths (cm) in the Catalão Lake (Amazonas-Brazil) at October 2000 and March 2001. Note that the oxygen concentration varies depending on the period of the year, time of day, and measurement depth (Chippari-Gomes et al., 2003, unpublished data) [Color figure can be viewed at wileyonlinelibrary.com]

**TABLE 1** The present table describes the main mechanisms Amazon fish present to survive hypoxic environments. It is described the respiration mode, morphological "adaptation strategies" acquired during evolutionary time, and behavioral/physiological/biochemical adjustments. All of them provide phenotypic plasticity to each species

| | | Strategies to survive in Amazon hypoxic environments | | |
|---|---|---|---|---|
| **Family** | **Species** | **Respiration mode** | **Main morphological and physiological/biochemical strategies** | **Source** |
| **Arapaimidae** | *Arapaima gigas* | OAB | This species has a modified swim bladder, which is used as an air-breathing organ. It drowns in ~10 min without access to air, despite the presence of gills. During the growth from juveniles to adults, its branchial arches change, losing the lamellae, so that branchial arches (without lamellae in the adults) are used only to release $CO_2$ to water. Due to the routine exposure to air, it has a high antioxidant defense capacity. | (Brauner et al., 2004; Pelster et al., 2020; Val & Almeida-Val, 1995) |
| **Serrasalmidae** | *Colossoma macropomum* | WB | Resistant to hypoxia through depletion of metabolism and has morphological modifications—increases the lower lip allowing the animal to capture more oxygen while swimming near the surface (ASR). | (Val et al., 1998) |
| **Bryconidae** | *Brycon amazonicus* | WB | Metabolic suppression and development of a dermal extension of the inferior lip to perform aquatic surface respiration (ASR) | (Braum & Junk, 1982) |
| **Erythrinidae** | *Hoplerythrinus unitaeniatus* | FAB | Their swim bladder is modified to function as an accessory-breathing organ, which is ventilated by a buccal force pump air, which is drawn into the mouth and forced into the swim bladder with the mouth and opercula closed. | (Fange, 1976; Singh, 1976) |
| **Gymnotidae** | *Electrophorus electricus* | OAB | Its mouth is structurally adapted for air breathing by an extensively diverticulated and richly vascularized oral mucosa. Air is regularly taken into the mouth and later expelled at the opercula opening. | (Johansen et al., 1968) |
| **Rhamphichthyidae** | *Rhamphichthys marmoratus* | FAB | It has an aerial respiratory system that consists mainly of the esophagus, the pneumatic duct, and the posterior chamber of the gas bladder, where the diffusion of gases occurs. | (Liem et al., 1984) |
| **Callichthyidae** | *Hoplosternum littorale* | FAB | Their posterior intestine works as an accessory air-breathing organ, where approximately 63% of the gut is modified for oxygen extraction. This is also one of the few fish species with bisphosphoglycerate (BPG) in its red blood cells, which improve the capacity for oxygen extraction from the air. | (Persaud et al., 2006; Val, 1993) |
| **Loricariidae** | *Pterygoplichthys pardalis* | FAB | They use the stomach as an accessory respiratory organ, which functions with optional air-breathing when oxygen in the water is scarce. | (Brito, 1981; Val 1995) |
| **Synbranchidae** | *Synbranchus marmoratus* | FAB | They can extract oxygen from the air a few times, but at high rates and sufficiently. This mechanism allows the species to handle the oxygen captured by transporting the oxygen to the blood through low internal oxygen tensions. Although it was considered an oxiconformer for a long time, it was recently demonstrated that this species could regulate oxygen consumption in hypoxia. | (Graham, 1997; Svendsen et al., 2018) |
| **Cichlidae** | *Acaronia nassa* | WB | LDH B (adapted to recovering periods of anaerobiosis, as it has the ability to convert lactate to pyruvate during recovery from periods of hypoxia) reduced in cardiac muscle. | (Almeida-Val et al., 2006) |

(Continues)

**TABLE 1** (Continued)

| Family | Species | Strategies to survive in Amazon hypoxic environments: Respiration mode | Main morphological and physiological/biochemical strategies | Source |
|---|---|---|---|---|
| | *Cichlassoma amazonarum* | WB | This species can decrease aerobic metabolic rate and change the predominance of isoform B or A in the heart, depending on the availability of oxygen in their habitat. In hypoxia isozyme $A_4$ expression increases in heart and brain, while isozyme $B_4$ increases in the liver and "disappears" in the skeletal muscle. | (Almeida-Val et al., 1995) |
| | *Heros severum* | WB | Reduction in cardiac muscle LDH-B. | (Almeida-Val et al., 2006) |
| | *Pterophylum sp.* | WB | Predominance of isoform $A_4$ in skeletal muscle and predominance of isozyme $B_4$ in the heart. | (Almeida-Val et al., 2006) |
| | *Hypselecara sp.* | WB | Predominance of isozyme $A_4$ in skeletal muscle and predominance of isozyme $B_4$ in the heart. | (Almeida-Val et al., 2006) |
| | *Astronotus crassipinnis* | WB | Their energy requirement is strongly supported by anaerobic metabolism; this species shows LDH B (adapted to recovering periods of anaerobiosis, as it can convert lactate to pyruvate during recovery from periods of hypoxia) reduced in heart, and shows higher concentrations of liver and muscle glycogen, increase in the HIF1 expression has been observed in liver. | (Almeida-Val et al., 2006) |
| | *Astronotus ocellatus* | WB | Their energy requirement is strongly supported by aerobic metabolism, increased isoform LDH A (specialized in anaerobic metabolism), shows higher concentrations of glycogen in liver and muscle tissues, and increases the HIF1 expression as its congeneric species. | (Almeida-Val et al., 2006; Muusze et al., 1998) |
| | *Satanoperca jurupari* | WB | They can decrease aerobic metabolic rate and change the predominance of isoenzyme B or A in the heart, depending on the availability of oxygen in their habitat.<br>Some studies point this species as a non-tolerant fish.[a] | (Almeida-Val et al., 1995, 1999; Chippari-Gomes et al., 2003) |
| | *Geophagus cff. harreri* | WB | Reduction in cardiac muscle LDH-B | (Almeida-Val et al., 2006) |
| | *Geophagus sp.* | WB | Predominance of isozyme $A_4$ in skeletal muscle and predominance of isozyme $B_4$ in the heart | (Almeida-Val et al., 2006) |
| | *Acarichthys heckelli* | WB | They have a predominance of isoform LDH A (predominant in anaerobic metabolism) in their skeletal white muscle, and the LDH B, which predominates in heart tissue is adapted to recovering from periods of anaerobiosis. | (Almeida-Val et al., 2006) |
| | *Cremicichla sp* | WB | Predominance of isozyme $A_4$ in skeletal muscle and predominance of isozyme $B_4$ in the heart. | (Almeida-Val et al., 2006) |
| **Lepidosirenidae** | *Lepidosiren paradoxa* | OAB | This species possesses well-developed lungs and reduced gills. The lungs play a dominant role in $O_2$ uptake and $CO_2$ excretion, suggesting that gas exchange regulation resembles amphibians. | (Abe & Steffensen, 1996; Almeida-Val et al., 2010) |

[a]OAB, obligatory air-breathing; FAB, Facultative air-breathing; WB, water breathing.

oscillation regimes included studies on behavioral, morphological, physiological, biochemical, and molecular mechanisms. However, these have never been subject of a single study that puts together the main findings of the adaptions to hypoxia in Amazonian fish species. Herein, we reviewed most of the adaptations in a variety of fishes of the Amazon Basin that can be seen in Table 1. These strategies, which may occur separately or concomitantly, allow the fish of the Amazon Basin to present significant phenotypic plasticity, as they can quickly turn on or off several biochemical, physiological, and morphological mechanisms relying on fast gene up or down-regulation (Almeida-Val et al., 2006).

Most Amazonian species that can survive in hypoxic environments used coupled mechanisms of behavioral and morphological changes that allowed a better and more efficient oxygen uptake. Some water-breathing fish (such as *Colossoma macropomum* and *Brycon amazonicus*) explore the water surface, which is the most oxygen-rich water layer, during low DO conditions and expand their lower lips to improve water flows through the gills during aquatic surface respiration (ASR; Braum & Junk, 1982). Meanwhile, facultative air-breathing fish go swimming up the surface and gulp air, developing breathing mechanisms to respond to the lack of water DO through specialized organs, such as the stomach, intestine, and swim bladder (*Pterygoplichthys pardalis*, *Hoplosternum littorale*, and *Hoplerythrinus unitaeniatus*, respectively; Godoy, 1975; Graham, 1997; Hochachka & Lutz, 2001; Stevens & Holeton, 1978). In addition, some species are obligatory air-breathers, like *Arapaima gigas* and *Lepidosiren paradoxa*, which are specialized to breathe atmospheric air and present many adaptive traits, including morphological, physiological, and molecular changes (Stevens & Holeton, 1978). In contrast to the mechanisms previously described, which promote alternatives to minimize the effects of hypoxia, the *Astronotus* spp. use physiological, biochemical, and molecular strategies to survive in hypoxic waters. The genus *Astronotus* is the most well-known hypoxia-tolerant Amazonian water-breathing fish as seen in Table 1.

*Astronotus ocellatus* and *Astronotus crassipinnis* (Oscars) belong to the Cichlid family, which has many species considered tolerant to hypoxia (Almeida-Val et al., 1995). However, the *Astronotus* species stand out for their surprisingly high tolerance, being able to resist 5% oxygen saturation for 20 h and up to 6 h of complete anoxia at 28°C (Muusze et al., 1998). Thus, *Astronotus* spp. have been an important biological model for studies investigating the physiological mechanisms of tolerance to hypoxia (Almeida-Val et al., 1999, 2000, 2011; Bailey et al., 1999; Baptista et al., 2016; Cassidy et al., 2018; Chippari-Gomes et al., 2005; De Boeck et al., 2013; Heinrichs-Caldas et al., 2019; Jung et al., 2020; Lewis et al., 2007; Matey et al., 2011; Muusze et al., 1998; Richards et al., 2007; Robertson, Kochhann, et al., 2015; Scott et al., 2008; Sloman et al., 2006; Wood et al., 2007, 2009). This review seeks to synthesize mechanisms of hypoxia and anoxia tolerance observed in the Oscar species. Herein, we discuss the mechanisms related to the efficient metabolic rate ($\dot{M}O_2$) of this animal, the molecular mechanisms involved in regulating response to hypoxia, the role of the oxidative process related to aerobic and anaerobic metabolism, the mechanism of gill remodeling during hypoxia, and the importance of antioxidant defenses during hypoxia exposure. The present work discusses some physiological mechanisms to better understand the high resilience of *Astronotus* spp. in response to an extreme environment, as demonstrated by the high abundance of this group just after the severe drought of 2005, which dried out about 70% of aquatic habitats in the Amazon floodplains (Röpke et al., 2017).

## 2 | INCREASE OF ANAEROBIC SURVIVAL THROUGHOUT ONTOGENY

*Astronotus* species exposed to low DO levels may seek out better-oxygenated habitats, or respond with mechanisms that save energy or extract oxygen from the environment more efficiently, resulting in physiological and behavioral changes (Muusze et al., 1998). Other Amazonian fish species increase hypoxia tolerance while growing, which is a pattern that has been studied in *Astronotus ocellatus* (Almeida-Val et al., 1999) and observed in other species, including *C. macropomum* (Saint-Paul, 1984) and *Crenuchus spilurus* (Braz-Mota, unpublished data). However, this is not a general trend, because some species can decrease hypoxia tolerance during growth, and others show no differences in their tolerance (Nilsson & Östlund-Nilsson, 2008). In nature, *Astronotus* is much more active in the juvenile phase and generally lives closer to the water surface, where oxygen availability is higher. In contrast, adults are often found in hypoxic or even anoxic environments, showing that the ability to cope with hypoxia increases as the fish grows (Almeida-Val et al., 1999; Sloman et al., 2006).

The oxygen consumption rate has been extensively used as a proxy of the animal's metabolic rate ($\dot{M}O_2$), and the energy expended by an unfed organism to maintain spontaneous movements is known as the routine metabolic rate (RMR; Chabot et al., 2016). Hypoxia tolerant species have been known to show lower RMRs (Killen et al., 2014). Indeed, the improvement of hypoxia tolerance in *A. ocellatus* has been associated with a decrease in RMR (obtained through the oxygen consumption rate) along with the animal's ontogeny (Almeida-Val et al., 1999, 2000; Sloman et al., 2006). This is evident in the average intraspecific scale exponents (*b* value) of the logarithmic relationship between the oxygen consumption rate (metabolism) and body mass. The average *b* value determined for *A. ocellatus* is 0.52, an index lower than other tropical species such as *Oreochromis niloticus* (0.81) and *C. macropomum* (0.83) (Farmer & Beamish, 1969; Saint-Paul, 1984). These results indicate the decreased aerobic dependence of *A. ocellatus* as it grows, a pattern also supported by an increase in LDH (lactate dehydrogenase) activity in several organs of *A. ocellatus* as the fish grows (Almeida-Val et al., 2000). This suggests that larger *A. ocellatus* individuals are more tolerant to hypoxia because they have greater anaerobic potential. So far, both species have responded similarly in every experimental hypoxia or anoxia in the laboratory, although we cannot affirm that *A. ocellatus* and *A. crassipinis* will respond to environmental challenges in similar ways.

An important tissue in this process is the white muscle, since most of the time, it relies on anaerobic glycolysis, and during its growth, it increases the activity not only of the carbohydrate pathway enzymes but also the amount of the LDH enzyme (Almeida-Val et al., 1999, 2000). Under normoxia, LDH levels in the white muscle of *Astronotus* are very similar to several Amazonian fish species (Almeida-Val et al., 2006), but different patterns are observed under hypoxia. For instance, *A. crassipinnis* increases LDH levels (405 μmol $min^{-1}$ $g^{-1}$) after exposure to 0.70 mg $O_2$ $L^{-1}$, while an inverse pattern is observed in the cichlid *Symphysodon aequifasciatus*, which decreases the activity of LDH enzyme levels (60 μmol $min^{-1}$ $g\,wt^{-1}$) until death at 0.6 mg $O_2$ $L^{-1}$ (Chippari-Gomes et al., 2005). Such results reveal the important contribution of the white muscle to hypoxia survival. However, highly aerobic tissues like brain and heart muscle also increase their LDH activities during hypoxic exposure (Almeida-Val et al., 1999, 2011), indicating integrated anaerobic recruitment due to the lack of oxygen. Furthermore, the positive correlation between the LDH and body mass is observed for *A. ocellatus* in the white muscle, heart, brain, and liver tissues ($r \geq 0.8$) (Almeida-Val et al., 1999), indicating a metabolic strategy for sustaining hypoxia that becomes increasingly important among individuals with larger body sizes. Thus, the increase of both the time of loss of equilibrium (LOE) and the LDH activity, which is higher with a higher body mass (Almeida-Val et al., 1999), can be explained by the scaling effects of the reduction in metabolic rate and an increase in the anaerobic pathway for compensation with lower ATP demands.

## 3 | THE ADVANTAGES OF MAINTAINING A LOW ROUTINE METABOLIC RATE

As mentioned above, RMR is an important measurement of oxygen consumption that provides an idea of the energy expenses by the animal to maintain essential movements, such as swimming and routine activity (Chabot et al., 2016). Below the RMR value, there is the standard metabolic rate (SMR) value, which corresponds to the rate of oxygen consumption. The SMR parallels the minimum energy requirement of a fish at rest in an unfed state. Above the RMR, there is the maximum metabolic rate (MMR) value, which reflects the maximum aerobic metabolic rate that a fish can reach at a certain temperature under an ecologically relevant situation (Chabot et al., 2016; Norin & Clark, 2016).

Evidence obtained over the past several decades shows that the key adaptation to long-term hypoxia is a simultaneous reduction in metabolic rate and metabolic demands, that is, a reduction or near-suspension of many bioenergetic processes (Bickler & Buck, 2007). For this reason, fish that have a low metabolic rate often show low activity, and this seems to be related to less dependence on aerobic metabolism (Stoffels, 2015). During hypoxic events, the increase of the ATP supply via anaerobiosis is limited. Thus, the reduction in aerobic demand by decreasing the ATP demand is the only viable long-term strategy for a vertebrate to survive without oxygen, and this characteristic of a decreased metabolic rate can give fish a remarkable tolerance to hypoxia, although it implies a lower activity due to lower oxygen consumption.

Such tolerance is usually, accompanied by other physiological and morphological characteristics, such as changing to a low critical oxygen tension value, branchial remodeling capacity, blood with high oxygen affinity, a stronger ability of metabolic depression, and high-energy reserves to feed the anaerobic metabolism (Bickler & Buck, 2007; Wells et al., 2005). Instead, active species show another set of physiological responses, including high gill surface areas and higher mitochondrial densities (Moyes et al., 1992). These responses, respectively, support high rates of respiration and energy demand, and a low oxygen affinity for hemoglobin that promotes the offloading of oxygen to muscle supports an active lifestyle and high dispersion capacity (Stoffels, 2015), which means that active species are highly oxygen-dependent.

On the other hand, low-activity species are less oxygen-dependent, as observed in some benthic and benthic-pelagic Amazonian fish species (Table 2). In general, benthic and benthic-pelagic species are less metabolically active than pelagic fish species (Chabot et al., 2016). Furthermore, Table 2 also shows that the pelagic Cichlids *Astronotus* spp., *Chaetobranchopsis orbicularis*, and *Geophagus proximus* are the fish with the lowest RMR that can be justified by the low aerobic activity of these species. However, such RMR is not a rule in cichlids and may be more related to lifestyle than phylogeny, since the cichlid *Cichla monoculus*, an active pelagic predator, holds three times the mass-specific RMR of *A. ocellatus* (Table 2), indicating *Astronotus* that metabolism is similar to the species that live at the bottoms of aquatic environments, which usually hypoxic or anoxic (Anjos et al., 2008).

The characteristically low metabolic rate that Oscars show under normoxia may be important to their ability to adjust to hypoxia. *A. ocellatus* decrease their metabolic rate 55% at water oxygen saturation levels below 4%, while *S. aequifasciatus*, another cichlid, decreases its metabolic rate around 67% before losing the equilibrium at 10% oxygen saturation (Chippari-Gomes et al., 2005). The average RMR under hypoxia is similar for both cichlid species, but the RMR of *A. crassipinnis* in normoxia is considerably lower (Chippari-Gomes et al., 2005). Thus, since the Oscars present a lower metabolic depression, we can assume that their physiological processes are less compromised than *S. aequifasciatus*. Considering the depression in metabolic rate among Amazonian cichlid species, the genus *A. ocellatus* is the only one that has a lower reduction rate at a lower oxygen concentration (Figure 2). The metabolic depression in the *Astronotus* genus, although similar to some of the other cichlids, occurs in extremely low concentrations of oxygen, indicating a greater resistance of *Astronotus* spp. in maintaining their metabolic activities during hypoxia. In addition, the *Astronotus* reduction rate is lower than in *Apistogramma agassizii*, *C. orbicularis*, *S. aequifasciatus*, and *Geophagus altifrons* (Figure 2), indicating a lower impairment of its metabolic functions during hypoxia. In addition, *A. crassipinnis* can survive almost 3 h of anoxia, while *S. aequifasciatus* loses equilibrium (LOE index) 3.5 h after exposure to concentrations below 0.60 mg $O_2$ $L^{-1}$ (Chippari-Gomes et al., 2005). These responses reinforce the idea

**TABLE 2** Interspecific variation in routine metabolic rate of the pelagic and benthic/bento-pelagic Amazonian fish species

| Water-column position | Species | Metabolic rate ($mgO_2\ kg^{-1}\ h^{-1}$) | Body mass (g) | Temperature (°C) | Source |
|---|---|---|---|---|---|
| Pelagic species | *Aequidens pallidus* | 156.5 ± 22.5 | 1.22 ± 0.97 | 25 ± 0.5 | (Campos et al., 2018) |
| | *Apistogramma agassizii* | 220 ± 9.2 | 1.0 ± 0.07 | 29 ± 0.6 | (Braz-Mota et al., 2018) |
| | *A. agassizii* | 410 ± 80 | 0.59 ± 0.08 | 26 ± 0.5 | (Kochhann et al., 2015) |
| | *Apistogramma hippolytae* | 165.1 ± 17.4 | 2.02 ± 1.16 | 25 ± 0.5 | (Campos et al., 2018) |
| | *Arapaima gigas* | 357.76 ± 16.9 | 4.7 ± 0.38 | 28 ± 1.0 | (Pelster et al., 2020) |
| | *A. gigas* | 117 ± 12.8 | 687 ± 52.0 | 28 ± 1.0 | (Pelster et al., 2020 |
| | *Astronotus ocellatus* | 108.4 ± 0.6 | 107.7 ± 33.3 | 28 ± 3.0 | (Muusze et al., 1998) |
| | *A. ocellatus* | 64 ± 5.7 | 230 ± 11.0 | 28 ± 1.5 | (Sloman et al., 2006) |
| | *A.ocellatus* | 128 ± 7.6 | 16.2 ± 1.9 | 28 ± 1.5 | (Sloman et al., 2006) |
| | *A. ocellatus* | 138.7 ± 16.5 | 186 ± 10.0 | 28 ± 1.0 | (Lewis et al., 2007) |
| | *A. ocellatus* | 82.56 ± 5.7 | 258.5 ± 15.5 | 28 ± 1.5 | (Scott et al., 2008) |
| | *A. ocellatus* | 89.6 ± 16.0 | 98 ± 3.0 | 28 ± 1.5 | (De Boeck et al., 2013) |
| | *A. ocellatus* | 85 ± 12.0 | 67.3 ± 4.1 | 27.5 ± 0.8 | (Duncan, 2020) |
| | *Astronotus crassinipinis* | 110 ± 8.3 | 45.3 ± 3.5 | 28 ± 0.5 | (Heinrichs-Caldas et al., 2019) |
| | *Brycon amazonicus* | 294 ± 49.0 | 7.1 ± 0.2 | 28 | (Lapointe et al. 2018) |
| | *Chaetobranchopsis orbicularis* | 70.06 ± 3.1 | 46.6 ± 3.8 | 27 | (Souza et al., 2021) |
| | *Characidium pteroides* | 198.3 ± 31.0 | 1.12 ± 0.22 | 25 ± 0.5 | (Campos et al., 2018) |
| | *Cichla monoculus* | 320 ± 8.0 | 571.4 ± 13.8 | 27.5 ± 0.8 | (Duncan, 2020) |
| | *Colossoma macropomum* | 160 ± 20.0 | 55.41 ± 2.58 | 26 ± 1.0 | (Barroso et al., 2020) |
| | *Crenuchus spilurus* | 211.8 ± 17.0 | 0.99 ± 0.26 | 25 ± 0.5 | (Campos et al., 2018) |
| | *Cyphocharax abramoides* | 169.84 ± 9.9 | 71.5 ± 38 | 31 | (Johansson et al., 2018) |
| | *Hemigramus cf. geisleri* | 331.1 ± 27.0 | 0.35 ± 0.15 | 25 ± 0.5 | (Campos et al., 2018) |
| | *Geophagus proximus* | 69 ± 2.8 | 55.26 ± 2.49 | 27 | (Souza et al., 2021) |
| | *Hyphessobrycon melazonatus* | 285.1 ± 42.0 | 0.64 ± 0.12 | 25 ± 0.5 | (Campos et al., 2018) |
| | *Iguanodectis geisleri* | 308.7 ± 28.0 | 1.55 ± 0.2 | 25 ± 0.5 | (Campos et al., 2018) |
| | *Microcharacidium eleotrioides* | 236.5 ± 18 | 1.12 ± 0.20 | 25 ± 0.5 | (Campos et al., 2018) |
| | *Mylossoma duriventre* | 240 ± 15 | 149.5 ± 9.5 | 27.5 ± 0.8 | (Duncan, 2020) |
| | *Nannostomus beckfordi* | 226.2 ± 22 | 0.55 ± 0.05 | 25 ± 0.5 | (Campos et al., 2018) |
| | *Nannostomus marginatus* | 207.1 ± 28 | 0.49 ± 0.05 | 25 ± 0.5 | (Campos et al., 2018) |
| | *Paracheirodon axelrodi* | 300 ± 10.3 | 0.78 ± 0.08 | 29 ± 0.6 | (Braz-Mota et al., 2018) |
| | *Paracheirodon simulans* | 247 ± 70.01 | 0.06 ± 0.02 | 25 | Campos et al., 2016 |
| | *Paracheirodon innesi* | 260 ± 10.06 | 0.16 ± 0.02 | 26.2 ± 0.16 | (Cooper et al., 2019) |
| | *Psectrogaster amazonica* | 190 ± 2.5 | 196 ± 11.7 | 27.5 ± 0.8 | (Duncan, 2020) |
| | *Pygocentrus nattereri* | 210 ± 0 | 167.5 ± 10.4 | 27.5 ± 0.8 | (Duncan, 2020) |
| | *Pyrrhulina aff brevis* | 188.5 ± 24 | 2.16 ± 0.7 | 25 ± 0.5 | (Campos et al., 2018) |
| Benthic/Bentho-pelagic | *Ageneiosus ucayalensis* | 45 ± 1.8 | 241.3 ± 19.4 | 27.5 ± 0.8 | (Duncan, 2020) |
| | *Anablepsoides micropus* | 129.57 ± 23 | 1.07 ± 0.44 | 25 ± 0.5 | (Campos et al., 2018) |
| | *Hemisorubim platyrhynchos* | 39 ± 0.5 | 198.8 ± 10.0 | 27.5 ± 0.8 | (Duncan, 2020) |

(Continues)

TABLE 2 (Continued)

| Water-column position | Species | Metabolic rate ($mgO_2\ kg^{-1}\ h^{-1}$) | Body mass (g) | Temperature (°C) | Source |
|---|---|---|---|---|---|
| | *Pinirampus pirinampu* | 60 ± 15 | 312.9 ± 13.8 | 27.5 ± 0.8 | (Duncan, 2020) |
| | *Pterygoplichthys pardalis* | 33 ± 2.1 | 151.7 ± 12.2 | 27.5 ± 0.8 | (Duncan, 2020) |
| | *P. pardalis* | 60 ± 5.1 | 43.40 ± 2.07 | 26.0 ± 0.6 | (Braz-Mota, unpublished data) |
| | *Sorubim lima* | 35 ± 0.4 | 237.0 ± 17.7 | 27.5 ± 0.8 | (Duncan, 2020) |
| | *Synbranchus marmoratus* | 37.7 ± 4.4 | 224 ± 4.7 | 26.0 ± 0.1 | (Svendsen et al., 2018) |

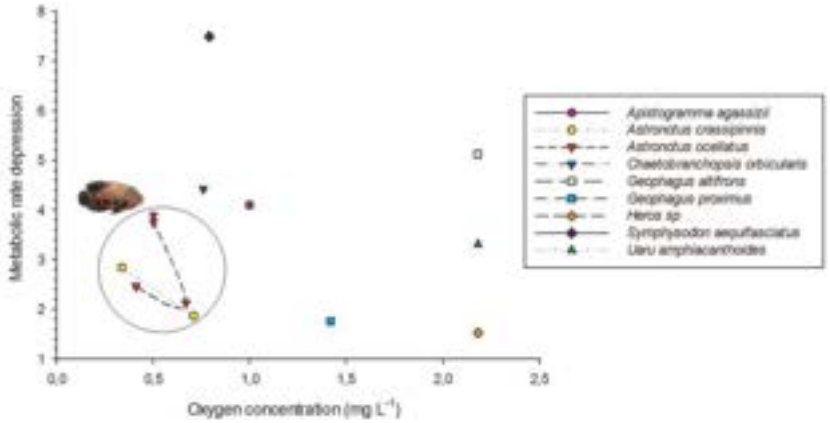


FIGURE 2 Metabolic rate depression (RMR normoxia/RMR hypoxia) in different hypoxia exposure concentrations of nine different Amazon Cichlids species. The plots into the circle represent the two *Astronotus* species from the different experiments, showing that the hypoxia exposure condition is directly related to the metabolic rate depression. Continuous lines were plotted to show the similarities between the responses of the *Astronotus* species. Figure generated from data extracted from Chippari-Gomes et al. (2000, 2005), Heinrichs-Caldas et al. (2019), Kochhann et al. (2015), Lewis et al. (2007), Muusze et al. (1998), Sloman et al. (2006), and Souza et al. (2021) [Color figure can be viewed at wileyonlinelibrary.com]

that the maintenance of a low resting metabolic rate can be the key for survival after exposure to stressors involving energy limitation, such as hypoxia. Further studies in genomics or metabolomics could help to understand the machinery behind the ability of *Astronotus* spp. to survive at so low an RMR compared to other ectotherms.

Likewise, aerobic scope (i.e., the difference between RMR and MMR) of *A. ocellatus* adults is ≥49 mg $O_2$ $kg^{-1}$ $h^{-1}$ (Muusze et al., 1998). This value is extremely low compared to other fish species (Campos et al., 2018; Slesinger et al., 2019; Zhang et al., 2018), even when compared with those fish species known to be hypoxia-tolerant, such as *Carassius auratus* (≥72 mg $O_2$ $kg^{-1}$ $h^{-1}$; Ferreira et al., 2014; Fu et al., 2011) and *Fundulus heteroclitus* (≥144 mg $O_2$ $kg^{-1}$ $h^{-1}$; Healy & Schulte, 2012). Thus, to survive an environment with long hypoxia periods, the strategy adopted by Oscar is the maintenance of a lower dependence on aerobic metabolism. Indeed, hypoxia-tolerant fish seem to have developed lower oxygen dependencies, with lower RMR and MMR values (Killen et al., 2016). The benefits of a reduced RMR could include tolerance to food deprivation and an ability to occupy habitats or niches with low or sporadic food availability. Thus, although a low aerobic scope in Oscars may limit aerobic performance, these fish also have the advantage of colonizing sparsely inhabited hypoxic environments, which may offer some protection from predators, or reduce competition for food or other resources.

## 4 | THE CRITICAL $O_2$ TENSION ($PO_{2\ CRIT}$) VERSUS TOLERANCE TO HYPOXIA IN AMAZONIAN SPECIES

When exposed to hypoxia, fish have different mechanisms to maintain ATP production via aerobic pathways. Such mechanisms allow a stable oxygen uptake rate, where fish are independent of environmental $PO_2$ (oxyregulator). When oxygen uptake declines with declining environmental $PO_2$, fish are characterized as oxyconformers. Below the transition point, known as the critical oxygen pressure ($PO_{2\ crit}$), the supply cannot be sustained, and oxygen uptake declines linearly with a decrease in ambient $PO_2$, a response known as

oxyconforming (Rogers et al., 2016). The high tolerance to hypoxia is usually related to a low $PO_{2\ crit}$ (Mandic et al., 2009) once aerobic metabolism becomes compromised.

For Amazonian fish species, the $PO_{2\ crit}$ values described in the literature are quite variable. For example, $PO_{2\ crit}$ values for the Serrasalmidae *C. macropomum* and *A. ocellatus* are variable (see Table 3). Since the Oscar shows a positive relationship between the physiological tolerance of hypoxia and size, we would expect a lower $PO_{2\ crit}$ for adults compared with juveniles. Although this pattern was verified by Scott et al. (2008; see Table 3), the opposite was verified when evaluating the data set obtained so far in all experiments with *A. ocellatus* (Table 3). In general, there is no relationship between the $PO_{2\ crit}$ and the body mass of the species *A. ocellatus*, indicating that this parameter does not explain the tolerance of this species to hypoxia. This lack of relationship between $PO_{2\ crit}$ and body mass was also observed in the Amazonian fish *C. macropomum* (Table 3), since this is also a species that increases in its tolerance with growth (Saint-Paul, 1984).

Despite several authors using the $PO_{2\ crit}$ as an indicator of hypoxia tolerance in fish (Burggren et al., 2019; Chabot et al., 2016; Claireaux & Chabot, 2016; Regan et al., 2019; Rogers et al., 2016), this hypoxia tolerance indicator has recently been criticized on several grounds. One of the criticisms is because the $PO_{2\ crit}$ values are extremely variable for both hypoxia-sensitive and hypoxia-tolerant species (Wood, 2018). In addition, the $PO_{2\ crit}$ has been criticized as a tolerance index because of the lack of agreement between $PO_{2\ crit}$ and $LOE_{crit}$ (Dhillon et al., 2013; Mandic et al., 2013). Moreover, for some species, there is no well-defined breakpoint that describes the $PO_{2\ crit}$, with the rate of oxygen consumption declining over a wide range of oxygen partial pressure (Urbina

**TABLE 3** Critical oxygen pressure ($PO_{2\ crit}$) values of genus *Astronotus* spp. in comparison with the other Amazon fish species

| Species | $PO_{2\ crit}$ (mg $O_2$ $L^{-1}$) | Temperature (°C) | Body mass (g) | Source |
|---|---|---|---|---|
| *Astronotus ocellatus* | 1.01 | 26 ± 0.1 | 9.6 ± 2.2 | (Braz-Mota, unpublished data) |
| *A. ocellatus* | 1.41 | 27 ± 1.0 | 10.9 ± 0.4 | (Cardoso et al., 2020, unpublished data) |
| *A. ocellatus* | 1.13 | 28 ± 3.0 | 16.2 ± 1.9 | (Sloman et al., 2006) |
| *Astronotus crassipinnis* | 1.26 | 28 ± 0.1 | 45.3 ± 3.5 | (Heinrichs-Caldas et al., 2019) |
| *A. ocellatus* | 1.70 | 27 ± 1.0 | 53.6 ± 4.8 | (Cardoso et al., 2020, unpublished data) |
| *A. ocellatus* | 2.73 | 28 ± 1.0 | 98.0 ± 3.0 | (De Boeck et al., 2013) |
| *A. ocellatus* | 2.47 | 27 ± 1.0 | 98.8 ± 5.9 | (Cardoso et al., 2020, unpublished data) |
| *A. ocellatus* | 3.78 | 28 ± 3.0 | 129.0 ± 11 | (Scott et al., 2008) |
| *A. ocellatus* | 1.19 | 27 ± 2.0 | 158.7 ± 31.2 | (Baptista, unpublished data) |
| *A. ocellatus* | 2.52 | 28 ± 3.0 | 230 ± 11 | (Sloman et al., 2006) |
| *A. ocellatus* | 1.74 | 28 ± 3.0 | 388 ± 2 | (Scott et al., 2008) |
| *A. ocellatus* | 1.02 | 27 ± 1.0 | 582.5 ± 22.3 | (Cardoso et al., 2020, unpublished data) |
| *Cyphocharax abramoides* | 1.56 | 31 | 71.5 ± 13.9 | (Johannsson et al., 2018) |
| *Colossoma macropomum* | 1.24 | 26 ± 0.1 | 23.0 ± 4.1 | (Braz-Mota, unpublished data) |
| *C. macropomum* | 1.49 | 26 ± 0.1 | 81.1 ± 11.8 | (Silva et al., 2019) |
| *C. macropomum* | 1.99 | 30 ± 1.0 | 183 | (Saint-Paul, 1984) |
| *C. macropomum* | 2.08 | 28 | 51 | (Giacomin et al., 2018) |
| *C. macropomum* | 1.47 | 28 | 10–25 | (Robertson, Val, et al., 2015) |
| *Chaetobranchopsis orbicularis* | 0.76 | 27 | 46.6 ± 3.8 | (Souza et al., 2021) |
| *Geophagus proximus* | 1.42 | 27 | 55 ± 26 | (Souza et al., 2021) |
| *Hoplerythrinus unitaeniatus* | 2.14 | 25 ± 1.0 | 250 ± 50 | (Oliveira et al., 2004) |
| *Pterygoplichthys pardalis* | 1.37 | 26 ± 0.1 | 46.1 ± 2.0 | (Braz-Mota, unpublished data) |
| *Paracheirodon axelrodi* | 1.63 | 26 ± 1.0 | 0.20 ± 0.05 | (Campos et al., 2017) |
| *Paracheirodon simulans* | 0.82 | 26 ± 1.0 | 0.06 ± 0.02 | (Campos et al., 2017) |
| *Symbranchus marmoratus* | 1.83 | 26 ± 0.1 | 224 ± 4.7 | (Svendsen et al., 2018) |

et al., 2012; Wood, 2018). Thus, despite being an important parameter to understand the regulation of metabolism during hypoxia, we believe that $PO_{2\ crit}$ does not express the high ability of *Astronotus* to survive during hypoxia, but it is an important measure associated with other variables. Thus, the combination of RMR, LDH level, time to LOE, and $PO_{2\ crit}$ could establish a fish species' oxygen dependence.

## 5 | MECHANISMS OF HYPOXIA SIGNALING PATHWAYS AND GENE EXPRESSION

Adapting to long and short-term environmental changes is a pivotal factor for organisms during evolutionary processes (Hochachka & Somero, 2002). The reduction in aerobic metabolism observed in *A. ocellatus* in response to hypoxia is due to its effective gene machinery and its regulation of this animal's metabolism, i.e., the species presents a high level of gene regulation and enzyme regulation, which results in phenotypic plasticity. Under low oxygen concentrations, HIF (hypoxia-inducible factor), a protein regularly synthesized and degraded when oxygen is present in the cellular milieu, is one of the first proteins to enter the nucleus and start activating or downregulating target genes. Associated with its general transcription factor and other possible accessory factors, HIF-1 triggers the transcription of more than 100 genes of interest (Bracken et al., 2003; Nikinmaa & Rees, 2005). Among them, one may recognize the genes involved in the regulation of glycolysis, erythropoiesis, catecholamine metabolism, angiogenesis, transposons, iron metabolism, and inhibition of protein synthesis, as well as several other genes directly linked to the absence of oxygen (Bracken et al., 2003; Cassidy et al., 2018; Egg et al., 2013; Semenza, 2014).

Significant increases in *hif-1α* expression have been observed in the livers of *A. ocellatus* (Baptista et al., 2016) and *A. crassipinnis* (Heinrichs-Caldas et al., 2019) after exposure to hypoxia, suggesting that both species can regulate their glycolytic metabolisms. This gene regulation response is highly efficient, with *hif-1α* mRNA returning to normal levels after 3 h of reoxygenation (Baptista et al., 2016; Heinrichs-Caldas et al., 2019), demonstrating an adaptive modulation in the regulation of gene expression. The HIF liver upregulation under hypoxia observed in both *Astronotus* species appears to be a variable response for Amazonian fish. For instance, the Amazonian characid fish *C. macropomum*, another hypoxia-tolerant species, decreases liver *hif-1α* expression under hypoxia (Silva et al., 2019). This shows a distinct arrangement of the molecular machinery, since this species has behavioral and morphological (lip expansion) adaptations (Table 1) and is more dependent on aerobic metabolism than *Astronotus* spp. are, as is observable by differences in the metabolic rate (Table 2).

With *hif-1α* activation due to exposure to hypoxia, *Astronotus* spp. also change the expression of genes responsible for vascular endothelial growth factor (VEGF; Baptista et al., 2016) and molecular mechanisms involved in regulating protein synthesis (Cassidy et al., 2018), the activity of prolyl hydroxylase 2 (phd2; Vasconcelos-Lima, unpublished data), and an increase in the expression of the lactate dehydrogenase-a gene (Almeida-Val et al., 1999, 2011). Hypoxia exposure increases *VEGF* expression in the liver of *A. ocellatus* (Baptista et al., 2016), increasing the efficiency of oxygen delivery to tissues through the formation of new blood vessels and allowing physiological functions to be maintained during hypoxia. Furthermore, the signaling molecules 4EBP1 and eIF2-α indicate a downregulation of protein synthesis to reserve ATP for the most vital processes (Cassidy et al., 2018). This regulatory strategy allows *A. ocellatus* to maintain ATP levels with enough stability to prolong fish survival under hypoxic conditions (Lewis et al., 2007), reducing protein synthesis and, consequently, decreasing the metabolic rate by 20%–36% (Cassidy et al., 2018). In addition, hypoxia decreases the phd2 expression in the liver of *A. ocellattus*, suggesting that a lower level of PHD2 allows the HIF-1 protein to connect to its target genes (Vasconcelos-Lima, unpublished data). In contrast, the gills of *A. ocellattus* show an increase in phd2 transcription response to hypoxia, which seems to be induced by the *HIF* gene itself; which, once stabilized, can act as a transcription factor binding to the PHRE2 at the *HRE* gene region, thus establishing positive feedback between the PHD2 protein and HIF-1 (Vasconcelos-Lima, unpublished data).

Furthermore, in *A. ocellatus*, increases in the expression of *ldh* genes were observed in the skeletal and cardiac muscles after hypoxia and during acute anoxia (Almeida-Val et al., 2011), demonstrating the high dependence on anaerobic metabolism in this fish species. To date, there is one single transcriptome analysis of *A. ocellatus* under hypoxia determining differentially expressed mRNA through RNAseq methodology. As part of the PhD thesis from Baptista (unpublished data), under the supervision of one of the authors, Vera Almeida-Val (unpublished data), we found a positive regulation of genes related to the glycolytic pathway (MDH, AK, GAPDH), heat shock proteins (HSPs), oxygen transport (Hb), osmoregulation (NKA), lipid solubilization (Apo), branchial motility (Actb), and redox homeostasis (txnip) during hypoxia and recovery. This indicates the complex regulatory ability of the branchial tissues of *Astronotus* spp. This indicates the existence of molecular machinery to activate the mechanisms of responses to deal with the hypoxia challenge, or even during the recovery processes after reoxygenation, when high oxygen levels may induce the increase of reactive oxygen species (ROS). Thus, it is clear that HIF plays a fundamental role in the signaling pathway of metabolic responses to hypoxia, controlling its own activation through *phd2*, increasing the capacity of producing new blood vessels, as seen by the increase of *VGEF* expression, and activating the anaerobic metabolism, as seen by the increase in LDH. A reduction in protein synthesis is also regulated by HIF, which would save energy for other essential functions. Thus, the results demonstrate that the transcription of the genes, mainly those controlled by HIF, is a response immediately after exposure to hypoxia, and the intensity of this response is tissue-specific and varies according to the demand, the period of acclimatization, and the degree of hypoxia. These regulatory mechanisms are essential to support the survival of *Astronotus* spp. under hypoxia conditions, and to

explore this issue, further studies on the ability of fish to acclimate to different DO regimes leading to different $PO_{2\ crit}$ values should take place. These regulatory mechanisms are some of the properties fish may have to acclimate and respond throughout phenotypic plasticity to sudden changes in environmental oxygen availability.

## 6 | OSMORESPIRATORY COMPROMISE UNDER HYPOXIA

Since most Amazonian fish live in waters that are not only frequently hypoxic but also ion poor (Duncan & Fernandes, 2010; Val & Almeida-Val 1995), the trade-off between respiratory gas exchange as well as osmoregulation and ionoregulation is particularly important for these species. Fish can alter branchial function, reorganizing blood flow pathways and reversibly remodeling the gills (Wood & Eom, 2021). In general, larger gill surface areas and thin water–blood barriers are the main factors that increase oxygen uptake capacity (Crampton et al., 2008, Wegner et al. 2010). While evaluating the gill morphology of Amazonian fish in normoxic conditions, Duncan (2020) observed that the species living in hypoxic environments, such as *A. ocellatus* and *P. pardalis*, in addition to having a low RMR (Table 2), also have low total gill surface area. Besides, the blood–water barrier, essential for facilitating gas transport through the branchial epithelium is also reduced in these species (Duncan, 2020). Thus, these species can maintain osmorespiratory control without altering the delicate balance between gas and ion exchange by the gills.

During severe hypoxia, the oxygen transfer factor (an index of the diffusion capacity of branchial $O_2$) decreases in *A. ocellatus*, probably due to the thickening of the water–blood barrier and reduction in lamellar recruitment to prevent ion loss as well as active ion absorption (Matey et al., 2011; Wood et al., 2007). However, the decrease in ionic fluxes does not occur due to the lack of cellular $O_2$ in the ionocytes (Scott et al., 2008), but rather seems to be an adjustment to reduce the metabolic demands of the whole animal, since the energy cost for ionic regulation is high (Figure 3). The ability to have a reduction in gill fluxes during hypoxia is commonly associated with tolerant fish species (Wood & Eom, 2021), but different responses are observed in Amazonian fish, where *Hyphessobrycon bentosi* and *Moenkhausia diktyota* increase their $Na^+$ flux patterns under hypoxia, while others (*Paracheirodon axelrodi* and *Hemigrammus rhodostomus*) exhibit unchanged fluxes when exposed to hypoxia (Robertson, Val, et al., 2015).

Branchial remodeling in response to hypoxia occurs during the first hour of exposure to hypoxia in *A. ocellatus* and is characterized by changes in the composition, density, and organization of the branchial epithelium cells (De Boeck et al., 2013; Matey et al., 2011; Wood et al., 2009). The mitochondria-rich cells (MRCs) are drastically affected by hypoxia, showing a reduction in number and area as well as changes in their morphology including a "deep-hole" in apical crypts and reduction in subapical microvesicles (Matey et al., 2011).

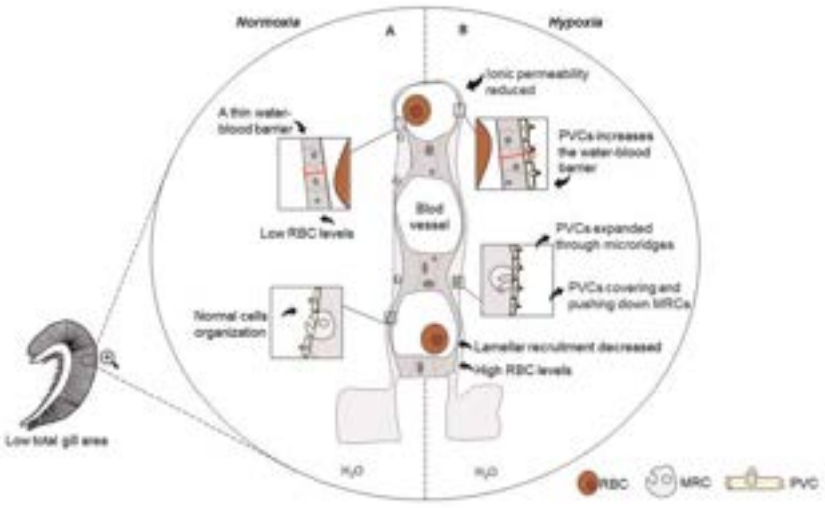


**FIGURE 3** Schematic figure showing the osmoregulatory compromise of *Astronotus* spp. during normoxia and hypoxia. In normoxia (side A), it compensates the low total gill area with the thin water–blood barrier, facilitating gas exchange considering the reduced number of red blood cells (RBCs). The apical crypt of the mitochondria-rich cells (MRCs) is shallow-basin and subapical microvesicles are well defined. Pavement cells (PVCs) have few microridges. In hypoxia (side B), the uptake of oxygen by the gills is reduced and MRCs are covered and pushed down by the PVCs, decreasing the ionic fluxes and the energetic costs to perform this function. PVCs have a larger area due to increased microridges, which promotes a thickening of the blood–water barrier. As a strategy, oscar increases the amount of RBCs in hypoxia and the affinity of hemoglobin for oxygen. *Astronotus* spp. has a refined osmorespiratory compromise under hypoxia saving energy mechanism by the reduction of ionic fluxes and adjustments in respiratory control [Color figure can be viewed at wileyonlinelibrary.com]

Since MRCs play a central role in ion regulation, a reduction in their number and changes in morphological characteristics of the surface, including deeper apical crypts, promote a reduction in the area available for ion leakage through the apical membrane, thus explaining the reduction in gill fluxes already mentioned. In addition, the pavement cells (PVCs) extend in area, thereby covering the MRCs, which also contributes to the reduction of their apical exposure (Matey et al., 2011). This new cellular reorganization can explain the reduction in the intercellular permeability observed during hypoxic events (Wood et al., 2007, 2009). Due to the reduction in the number and area of MRCs and the decrease in energy expenditure on ionic regulation, the Oscar fish can activate metabolic depression when environmental oxygen levels fall below their SMR (Almeida-Val et al., 2000; De Boeck et al., 2013; Muusze et al., 1998). Thus, *Astronotus* spp. can reduce branchial ion and water fluxes through the cellular and functional restructuring of the branchial epithelium cells.

## 7 | CONCLUSION AND PERSPECTIVES

Herein, we highlight important knowledge gaps that need to be filled, and we encourage new studies to understand how evolution has induced this group of species to adapt and live in variable oxygen conditions. The two species of the genus *Astonotus* have unique mechanisms to cope with frequent periods of hypoxia and anoxia. Based on the hypoxic survival strategies established by Mandic & Regan (2018), the hypoxic survival strategy of *Astronotus* spp. is the total hypoxic response (THR; Mandic & Regan 2018). The THR of *Astronotus* spp. is supported by the ability of these species to sustain a combination of a wide array of mechanisms that enhance $O_2$ uptake, transport, and delivery, as well as increased anaerobic metabolism, and metabolic rate depression. *Astronotus* spp. have lower RMR values than average among Amazonian fish species. In addition, the expression of key genes in response to hypoxia linked to HIF shows that the Oscar has a quick trade-off between normoxia and hypoxia by using a refined and precise gene regulation mechanism, compared to other teleosts. The lower dependence of aerobic metabolism is an important strategy in tolerating hypoxia in this genus and includes reducing cellular functions such as protein synthesis and ionic regulation, which are highly energy-demanding processes. Metabolic changes in the Oscar deserve further investigations, such as the mitochondrial mechanism that allows these fish to survive in challenging conditions and how the hypoxia affects the generation of the ROS, since it could promote the redox imbalance into mitochondria. Considering that hypoxia leads to alterations in pathways such as HIF1, which controls complex IV subunits, changes in glucose transport, and glycolytic enzymes, it may be possible that the Oscar presents unique mechanisms for maintaining mitochondrial function. The surprising physiological, biochemical, and morphological changes of Oscars in tolerating hypoxia inspire further studies to understand the different strategies of these particular species to thrive in the Amazon's often extreme conditions.


### ACKNOWLEDGMENTS

The authors are grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Amazonas (FAPEAM), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) by supporting INCT/ADAPTA (CAPES – Finance Code 001; CNPQ process #465540/2014-7; FAPEAM process #062.01187/2017). Susana Braz-Mota is the recipient of a doctoral fellowship from CAPES and Vera M. F. Almeida-Val is the recipient of a Research Fellowship from CNPq.


### CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

### DATA AVAILABILITY STATEMENT

Data are available on request from the authors.


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**How to cite this article:** Braz-Mota, S., Almeida-Val, V. M. F. (2021). Ecological adaptations of Amazonian fishes acquired during evolution under environmental variations in dissolved oxygen: A review of responses to hypoxia in fishes, featuring the hypoxia-tolerant Astronotus spp. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, 1–16. https://doi.org/10.1002/jez.2531

## 4. CAPÍTULO 2

*Artigo formatado de acordo com as normas da revista *Journal of Experimental Biology.*

# Is the low oxygen concentration a challenge to wild Amazonian fish? The mitochondrial heart physiology under environmental hypoxia

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| **List of abbreviations** | |
|---|---|
| ASR | aquatic surface respiration |
| ATP | adenosine triphosphate |
| CI | complex I |
| CII | complex II |
| CIV | complex IV |
| DO | concentration of dissolved oxygen in the water |
| ETS | respiratory electron transfer-pathway capacity |
| $FAD^+$ | flavin adenine dinucleotide |
| $NAD^+$ | nicotinamide adenine dinucleotide |
| $PO_2$ | partial pressure of oxygen |
| RCR | respiratory control ratio |
| ROS | reactive oxygen species |

**Abstract**

The Amazon floodplain areas commonly show daily and seasonal oxygen variations. Living in such conditions imposes many challenges, including combining an effective oxygen delivery and energy production maintenance in the face of low environmental $PO_2$. However, Amazonian fish seems to have acquired physiological mechanisms to thrive with these environments, making heart function crucial in this process. Herein, we study heart mitochondrial physiology mechanisms of Amazonian fishes aiming to understand how different fish hearts deal with their natural hypoxic environment, also aiming to identify the species-specific characters of hypoxia tolerance in those species. Seven fish species were collected in two seasons; the dry season, where the concentration of dissolved oxygen in the water (DO) is higher (≅ 4 mg $L^{-1}$ $O_2$), and the flood season, where the DO is substantially lower (≅ 1 mg $L^{-1}$ $O_2$). We examined the effects of the different seasons on mitochondrial phosphorylation components ($H^+$ leak, CI+CII, ETS, CII, CIV, and RCR) and the emission of reactive oxygen species (ROS). Heart mitochondria respiration was lower in the dry season than in the flood season for most species, suggesting an enhanced respiratory capacity during the low DO season, indicating greater heart respiratory capacity. $H^+$ leak increased in all species and RCR was unaffected in several other species that showed larger increases in ROS. Our findings showed that the Amazon fish species present refined mechanisms to increase the mitochondria respiration during the flood season, indicating that the mitochondrial responses are more dependent on the seasonal condition than on the intrinsic characteristics of the species. As far as we know, this is the first study to show the mitochondrial physiological responses of wild Amazonian fishes, and brings a perspective to propose future studies to investigate the physiological consequences of the water levels variation in Amazon fish species.

**Key-words:** Amazon Environment. Fish. Mitochondria. Hypoxia. Heart. Respiration. Oxygen Reactive Species.

## 1. Introduction

Daily and seasonal oxygen variations characterize the Amazon flooded areas, and the fish species that inhabit these environments can tolerate prolonged and severe hypoxia (Junk et al., 1984; Val & Almeida-Val 1995; Val et al., 1998; Braz-Mota & Almeida-Val, 2021). One of the biggest challenges faced by fish in hypoxia is controlling the balance between oxygen consumption, the efficiency of its distribution to tissues, and cardiorespiratory adjustments to preserve cardiac aerobic metabolism (Farrell & Richards, 2009; Richards, 2009). Mitochondria are central to many of the cellular effects of hypoxia, and mitochondrial physiology appears to differ between hypoxia-tolerant and -intolerant species. For instance, the capacity for oxidative phosphorylation in permeabilized heart fibres is reduced by acute hypoxia in the hypoxia-intolerant shovelnose ray (*Aptychotrema rostrata*) and it is unaffected by acute hypoxia exposure in the hypoxia-tolerant epaulette shark (*Hemiscyllum ocellatum*) (Hickey et al. 2012).

To date, there are some well-established ideas about how Amazonian fish survive the severe hypoxia conditions observed in flooded environments, including a decrease in routine metabolic rate (Kochhann et al., 2015; Heinrichs-Caldas et al., 2019), an concomitant increase in anaerobic metabolism (Almeida-Val et al., 1999; Almeida-Val et al., 2000), a requirement for hepatic glycogenolysis to increase plasma glucose to supply energy via anaerobic glycolysis (Chippari-Gomes et al., 2005; Vasconcelos-Lima et al 2021), and turning on or off a series of genetic pathways to control these responses (Baptista et al., 2016; Vasconcelos-Lima et al., 2021). Nevertheless, information about the mitochondrial functioning of Amazonian fish under natural environmental conditions is still lacking. It could be a critical factor in providing a better understanding of the regulation of metabolism in hypoxic conditions.

The Amazon River Basin contains the world's highest fish species diversity, with a hydrologic cycle that creates a patchy distribution of floodplain lakes at low water and affords dispersal and colonization opportunities through reconnected lakes, rivers and flooded forests during high water. With threats to connectivity by dam construction (Fearnside et al., 2021) and ongoing climate change, extreme drought and flood events are predicted to become much more frequent. For example, in June of 2021, the Rio Negro

reached the highest flood mark in 119 years, at more than 30 meters. The high temperatures of Amazonian waters, combined with the significant increases predicted by climate change, limit dissolved oxygen solubility, so the frequency and duration of hypoxic and/or anoxic episodes are elevated in floodplain areas, which can further intensify oxygen stratification in these environments (Braz-Mota & Almeida-Val, 2021). Reduced oxygen availability can damage essential organs like the heart (Pörtner et al., 2004; Pörtner & Knust, 2007) because rising temperatures reduces blood oxygen solubility concurrent with increases in metabolic rate. Moreover, oxygen diffusion rates are low at elevated temperatures and, then, Amazonian species already live very close to their thermal limits (Campos et al., 2019) and may have little capacity to cope with additional stressors. As hypoxic events become even more drastic in Amazonian environments, limitations on cardiac function may impact fish survival.

Catalão Lake, the place studied in this work, is a floodplain lake with spatially and temporally heterogeneous water chemistry due to the direct influence of both the Solimões and Negro rivers during flood pulse cycles. This lake has two extreme periods: the dry season (low water) and flood season (high water). In the dry season, the lake may or may not communicate with the main river because the water volume is smaller. The opposite is observed in the flood season, where the contribution of water from the rivers is outstanding, making their limnological characteristics (e.g. DO) comparatively more homogeneous (Thomaz et al., 2007). The dynamics of the DO in this habitat follows the variation in water level. In November, where the water levels are the lowest of the year, oxygen levels reach the highest concentrations. In contrast, the lowest oxygen concentration is found in June, where the water levels are at the peak of the flood.

According to Randall *et al*. (1981) hypoxia and anoxia have been common events in the evolutionary history of Amazon species and, consequently, the species have evolved many different strategies to face such environmental constraints. Adaptations have resulted from the evolution of many different respiration strategies, such as facultative air breathing, aquatic surface respiration (Braum & Junk 1982; Saint-Paul 1984; Brauner & Val 2006) and metabolic depression (Almeida-Val *et al*., 2000). For instance, *Mylossoma aureum* and *Thiporteus albus,* when facing hypoxia, develop a

dermal extension on their lower jaw and rise to the water surface, in order to access the oxygen rich water layer. Under the same conditions, *Pterygoplichthys pardallis* (bodó) show bimodal respiration in order to extract oxygen from the air when water oxygen concentrations are limiting (Stevens & Holeton, 1978). Furthermore, *Astronotus ocellatus* exhibits a profound metabolic depression when exposed to hypoxia and tolerates six hours in complete anoxia (Muusze et al., 1998). The life history and behavior of many species are associated with significant aerobic metabolic demands; for example, *Osteoglossum bicirrhosum* performs explosive swimming bursts to eat insects out of water column (Goulding, 1989) and *Cichla monoculus* and *Hoplias malabaricus* are highly active carnivores (Val & Almeida-Val, 1995; Duncan 2020).

How, then, are amazonian fish able to cope with severe hypoxia at high temperatures for months and still maintain physiological function? To answer this question, we must consider two main challenges related with variable oxygen availability in aerobic biological systems; (i) how can the cardiomyocytes maintain and regulate energy production (as ATP) under low oxygen stress; and (ii) how to counteract the oxidative stress in heart that is accompanied by re-oxygenation? The re-oxygenation process can generate a large production of reactive oxygen species (ROS), which can cause injuries, irreparable cell damage, and death (e.g., Sanderson et al., 2013). ROS are continuously produced by mitochondrial respiration and act as signaling molecules under normal conditions (D'Autréaux & Toledano, 2007). However, changes in the steady-state concentration of ROS elicit the activation of antioxidant enzymes, which quench these highly unstable free radicals by transforming them into non-toxic products (Kehrer & Klotz, 2015). In the present study, we present comprehensive research on the mitochondrial physiology of wild Amazonian fish to determine if there are patterns in how species deal with seasonal hypoxia. We hypothesize that during flood hypoxic periods, Amazon floodplain fish will decrease rates of mitochondrial respiration and ROS production. We further hypothesize that specific responses to this challenge will depend on the position in the water column where these species inhabit.

## 2. Material and methods

### *2.1 Ethical statement*

The permit for the collection and fish samplings were authorized by Instituto Chico Mendes de Conservação da Biodiversidade—ICMBio/Brazil under licenses number 29837-13. All the experimental procedures were approved by the Animal Use Ethics Committee of the Brazilian National Institute for Research of the Amazon (CEUA/INPA) (protocol number: 018/2017).

### *2.2 Collection site characterization*

The collections were performed at Catalão Lake located near the confluence of the rivers Solimões and Negro, in the city of Iranduba, state of Amazonas, Brazil (03º09'47"S and 059º 54'29"W). Due to its proximity to both rivers, the sediment-filled Solimões and the blackwater Negro rivers periodically reach and flood Catalão Lake, which receives large quantities of water and solutes. The connection with these different fluvial systems varies annually, alternating between permanent connectivity with at least one of the rivers (mainly the Negro River) and periodic isolation from both systems. The annual variation in water level, combined with changes in the landscape, directly affects the chemical and physical composition of the water, including changes in the dissolved oxygen concentration. During June, the waters are at their highest level, marking the flood's peak, where dissolved oxygen concentrations are lower due to the abundance of allochthonous organic inputs and the predominance of aerial photosynthesis in aquatic plant communities (Melack & Forsberg, 2001). On the other hand, the opposite scenario is observed during the dry season, when the lowest water level is observed in November, where the DO concentration is comparatively higher. The collections performed in this study occurred on April 1-8, 2018 (flood season) and November 27-30, 2018 (dry season).

### *2.3 Collection of species*

Adult individuals of *Osteoglossum bicirrhosum*, *Hoplias malabaricus*, *Thiporteus albus, Mylossoma aureum*, *Pterygoplichthys pardalis, Cichla monoculus* and *Astronotus ocellatus*, were collected using floating gillnets 15 m long and 2 m high with varying stretched mesh sizes (30, 40, 50, 60, 70, 80, 90 and 100 mm). Immediately after being removed from gillnets, animals were euthanized by a blow to their head, followed by severing the spinal cord. The fish were weighed and the heart was immediately removed and immersed in 2 mL of ice-cold relaxing BIOPS buffer (pH 7.1), containing (in mM):

$CaK_2EGTA$ 2.77, $K_2EGTA$ 7.23, $Na_2ATP$ 5.77, $MgCl_2 \cdot 6H_2O$ 6.56, taurine 20, imidazole 20, dithiothreitol 0.5, K-MES 50, sodium phosphocreatine 15, and sucrose 50. Hearts (≅ 3 mg) were kept on ice and transported to the Laboratory of Ecophysiology and Molecular Evolution at Brazilian National Institute for Research of the Amazon (INPA), Manaus, Brazil. BIOPS buffer solution was renewed every 4 hours and mitochondrial respiration was analyzed within 24 h period after sampling.

### *2.4 Mitochondrial physiology in the permeabilized heart muscle fiber*

Tissue was removed from the BIOPS buffer, teased into fiber blocks using a dissecting microscope, and immersed in 1 ml ice-cold BIOPS along with 50 µg/ml saponin. After 30 min, fibers were washed thrice for 10min in 2 mL of modified mitochondrial respiratory medium MiRO5 buffer (pH 7.2), containing (in mM): EGTA 0.5, $MgCl_2 \cdot 6H_2O$ 3, K-lactobionate 60, taurine 20, $KH_2PO_4$ 10, HEPES 20, sucrose 160 and 1 g/l BSA, essentially fatty acid free, at 25 °C (Gnaiger et al., 2000). The fibers were blotted dry on filter paper and weighed into bundles for respiration assays in 2 mL of MiRO5.

Oxygen was added into the gas phase above media prior to closing chambers to supersaturate oxygen concentration. The Complex I (CI) substrates (5 mM malate, 10 mM glutamate and 5 mM pyruvate) were added to measure state II respiration through CI in the absence of ADP (denoted 'Leak I'). Excess ADP (2.5 mM) stimulated oxidative phosphorylation (OXPHOS -I, state III respiration). Cytochrome c (10 µM) was added to test outer membrane integrity. Phosphorylating respiration with CI and complex II (CI+II) substrates (OXPHOS -I, II) was measured by the addition of succinate (10 mM). The respiratory electron transfer-pathway capacity (denoted 'ETS') was assessed by repeated titrations of carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone (FCCP, 0.5 µM). The activity of CI, II and III complexes were, then, inhibited by the addition of rotenone (0.5 µM), malonate (15 mM), and antimycin A (1 µM), respectively. Complex IV activity was recorded by addition of 5 mM ascorbic acid and TMPD, passing all electrons to complex IV. RCR was calculated as ETS/leak state ratio.

ROS emission was measured in parallel with mitochondrial respiration. Superoxide dismutase (SOD; at 22.5 U $mL^{-1}$) was added to catalyze the reaction of the superoxide produced by the mitochondria and horseradish peroxidase (3 U $mL^{-1}$) was added to catalyze the reaction of hydrogen peroxide with Ampliflu Red (15 μM) and produce the fluorescent product resorufin (detected using an excitation wavelength of 525 nm and ampliometric filter set (AmR); Oroboros Instruments). The resorufin signal was calibrated with additions of exogenous hydrogen peroxide.

### *2.5 Data analysis*

The effects of the dry and flood season on the heart mitochondria respiration state and ROS content were evaluated using a Student's t-test (means ± SEM). For non-normal data, a Mann-Whitney test was applied. A significance level of 5% ($p \leq 0.05$) was used in all test procedures. Statistical analyses and graphics employed Sigma Stat and Sigma Plot software (Jandel Scientific, San Jose, USA). R Software was used only for the principle component analysis (PCA) using the Agricolae package, and graphs were built using the ggplot2 package.

## 3. Results

### *3.1 Chemical and physical variables during collection periods*

The species were collected in Catalão Lake in two different periods of the hydrological cycle: flood and dry seasons. With alterations in water level and evident changes in the landscape, these two seasons differ greatly, mainly in DO, which present a continuous daily and seasonal variation. The concentration of dissolved oxygen in the water (DO) measured over 24 h at different depths (Fig. 1) indicated notable differences in oxygen concentrations across season, where mean DO was higher in the dry season (≅ 4 mg $L^{-1}$) and substantially lower in the flood season (≅ 1.0 mg $L^{-1}$ $O_2$) (Table 2). At the same time, few variations were observed in temperature or pH (Table 2).

### *3.2 Mitochondrial function in heart fibers during the dry and flood season*

For most species, heart mitochondrial respiration was lower in the dry season when compared to the flood season (F= 80.00, p=0.001) (Fig. 1 and 2). The flood season was associated with increases in the leak state ($H^+$ leak) for all the species analysed (*O. bicirrhosum* (U= 1.0; p=0.003), *H. malabaricus* (t = 5.2; p= <0.001), *T. albus* (U= 72.0; p= <0.001), *M. aureum* (t = 2.2; p= 0.032), *P. pardalis* (U= 108.0; p= <0.001), *C. monoculus* (U= 6.0; p= 0.016) *A. ocellatus* (t = 3.05; p= 0.010)), indicating an increase in the oxygen consumption during leak respiration in the flood season (Fig 2). In the present study, the respiratory control ratio (RCR) was not statistically different between seasons for most of the species (*O. bicirrhosum* (t= 0.61; p=0.55), *H. malabaricus* (t = 0.0782; p= <0.939), *T. albus* (U=; p=), *M. aureum* (t = 1.21; p= 0.239), *P. pardalis* (t= 0.53; p= 0.598), *C. monoculus* (t= 2.085; p= 0.005)). However, a decrease in RCR was observed in the flood season in *A. ocellatus* (t = 2.61; p= 0.020) (Fig 3B).

This uncoupling observed in the flood season stimulated an increase in respiration of CI in four, out of the seven analyzed species (*O. bicirrhosum* (U= 0.00; p= 0.002), *T. albus* (U= 12.00; p= 0.005), *P. pardalis* (U= 102.0; p= <0.006) and *C. monoculus* (t =2.96, p= 0.011). CI respiration did not change across seasons neither in *H. malabaricus* (t = 2.04; p= 0.061), *M. aureum* (U = 87.0; p= 0.385), or *A. ocellatus* (U = 32.0; p= 1.00) (Fig 2). In addition, the combination of CI+CII respiration in the presence of ADP was higher in the flood season for all seven species analysed (*O. bicirrhosum* (U= 0.000; p=0.002), *H. malabaricus* (U = 0.000; p= <0.001), *T. albus* (U= 6.000; p= 0.001), *M. aureum* (U = 143.000; p= 0.001), *P. pardalis* (U= 119.0; p= 0.001), *C. monoculus* (t = 6.398; p= <0.001) *A. ocellatus* (t = 3.00; p= 0.010)), indicating that the natural hypoxic environment increased the state of maximum coupled respiration (Fig 2). The cardiac ETS- OXPHOS capacity also increased during the flood season in *O. bicirrhosum* (U= 0.0, p=0.002), *H. malabaricus* (U=0.0, p=0.001), *M. aureum* (U= 140, p≤0.001), *P. pardalis* (U=11.1, p≤0.001), *C. monoculus* (t= 6.40, p≤0.001) *A. ocellatus* (U= 0.0, p≤0.001), while for *T. albus* the ETS respiration was similar in across seasons (t = 1.98, p= 0.062). With the addition of rotenone to block CI, the respiration in the CII showed no difference between the seasons for CII of *T. albus* (t = 0.0379; p= 0.970). Whereas, increase in CII respiration was observed for all other species studied (*O. bicirrhosum* (U = 0.000; p= 0.002), *H. malabaricus* (U= 0.0; p= <0.001), *M. aureum* (U = 142.0; p= 0.001), *P. pardalis* (U= 0.0; p= <0.001), *C. monoculus* (U= 0.0; p= 0.002) *A. ocellatus*

(U = 0.0; p= 0.010)). Maximal respiration via OXPHOS CIV, achieved with the addition of TMPD and ascorbate, was higher for all species in the flood season ((*O. bicirrhosum* (t= 12.5; p= 0.001), *H. malabaricus* (U = 0.0; p= <0.001), *T. albus* (t= 7.11; p= <0.001), *M. aureum* (t = 7.3; p= 0.001), *P. pardalis* (U= 120.0; p= <0.001), *C. monoculus* (U= 9.0; p= 0.04) *A. ocellatus* (t = 7.80; p= 0.010)).

The flood season had a substantial effect on the rate of ROS emission from heart mitochondria of *O. bicirrhosum* (U= 50; p= 0.008), *H. malabaricus* (U = 2.0; p= <0.006), *M. aureum* (t = 2.71; p= 0.016), *C. monoculus* (t= 3.24; p= 0.08), and *A. ocellatus* (U= 9; p= 0.004), while no difference was observed for *T. albus* and *P. pardalis* (U=25; p=0.5 and t= 0.27; p=0.78, respectively) (Fig. 3A), these last two species having strategic respiration alternatives to capture more oxygen.

### *3.3 Relationships between the effect of seasons on mitochondrial responses*

Overall, the PCA showed a seasonal differentiation for all seven species analysed here, where fish collected in the flood season were consistently grouped and those collected in the dry season were consistently grouped (Fig. 4). The PCA demonstrated that the grouping of the seven species was mainly seasonally dependent, with the interspecific variation of the responses being less significant than the effect of drought and flood. The cumulative explained variance of PC1 and PC2 was 77.3%, demonstrating a strong season-effect.

## 4. Discussion

Due to the daily and seasonal oxygen variations of the Amazon's flooded areas, it is challenging to understand how animals living in these environments can match $O_2$ supply to demand when the concentration of dissolved oxygen in the water (DO) is close to zero. However, this study provides compelling evidence that mitochondrial heart function in Amazonian fish adapts to seasonal hypoxia, becoming an important tool for fish survival in this severe environmental condition. Herein, we observed an enhanced

heart respiratory capacity improving efficiency in the heart delivering $O_2$ to the tissues, such as the increase in mitochondria function during the flood season for nearly all the fish species analysed. Furthermore, our findings indicate that the improvement in oxygen delivery to tissues associated with controlling the ROS production can also improve the cardiac $O_2$ delivery, which was not directly measured here but is closely related to the heart's ability to function using adenosine triphosphate (ATP) generated by the mitochondria.

Herein, most species increased their heart oxidative capacity during the flood season, where the DO was very low (Fig 2), indicates a higher capacity for mitochondrial $O_2$ consumption. Recently, several studies have shown that prolonged periods of low $O_2$ and daily cycles of hypoxia-reoxygenation can improve the tolerance for dealing with hypoxia and may be part of the suite of beneficial adjustments to cope with chronic hypoxia (Williams et al., 2019; Borowiec et al., 2020; Borowiec & Scott, 2020). Furthermore, fish can respond to and cope with hypoxia by acting cardiorespiratory adjustments that help maintain cellular $O_2$ supply and thus preserve the cardiac aerobic metabolism (Driedzic & Gesser 1994; Farrell & Richards, 2009). Such ability to maintain cellular $O_2$ supply for the heart may prevent the need to reduce cellular $O_2$ demands by this organ, and the relative ability of fish to maintain cellular $O_2$ levels should affect their ability to survive in hypoxia. In addition, it is also possible that the hypoxic conditions promote targeted reductions in the perfusion of organs like the liver, skeletal muscle, kidney, and gills, decrease whole-animal $O_2$ demands and critical organs like the heart and brain are given preferential access to $O_2$, rather than blood flow (Axelsson & Fritsche, 1991).

The increase in mitochondrial oxidative capacity may work as a defense mechanism for Amazon fish to increase their tolerance to hypoxia in the flood season. Dawson et al. (2016) observed that the heart mitochondria of torrent ducks increased their oxidative capacity in high altitudes, where $O_2$ delivery is a challenge. When intracellular $O_2$ tension decreases in the myocardium, a higher oxidative capacity should increase the total mitochondrial $O_2$ flux of the muscle and thus help offset the inhibitory effects of hypoxia (Hochachka, 1985; Scott et al., 2009; Lui et al., 2015). The increase observed in

CI, CII, and the combination of both for most species examined here may reflect the preferential use of fuels that maximize the yield of ATP/mol of $O_2$ consumed. Differences in the relative capacities of $NAD^+$ vs. $FAD^+$-linked dehydrogenases to support oxidative catabolism means that the yield of ATP/mole of $O_2$ consumed can differ substantially between different substrates, and the cardiac function may be the result of the preferential use of carbohydrates instead of fat acids since this confers an energy advantage of up to 40% for the animal (Hochachka & Somero 1984; Driedzic & Gesser, 1994; Driedzic et al., 2021). Thus, although hypoxia causes a decrease in cardiac output, this reduction is comparatively much smaller than the values of reduced metabolic rate $\dot{M}O_2$, suggesting an increase in heart anaerobic adenosine ATP production and/or down-regulation of other ATP consuming processes, such as protein synthesis, to balance energy supply and demand and maintain cardiac function. Therefore, it is advantageous for Amazonian fish to increase their oxidative capacities during the flood season, improving the yield of ATP/mole of $O_2$ consumed. Thus, these animals would have the ability to alleviate the limitations imposed by hypoxia and allow these animals to survive in Amazonian environments.

The cardiac mitochondria of all seven fish species here analyzed showed an increase in proton leak, suggesting a possible mechanism to protect cardiac mitochondrial function against the ROS generated in the flood season. This increase in proton leak limits ROS generation, allowing the mitochondria to operate at a lower membrane potential (Cunha et al., 2011). Thus, the increase in proton leak during the flood season may have occurred due to increased ROS observed in this season, increasing the membrane proton conductance. This high proton conductance would be expected for the species *O. bicirrhosum*, *H. malabaricus*, *M. aureum*, *C. monoculus* and *A. ocellatus*, to be associated with elevated leak respiration to prevent the ROS production in the first place. Although, the reason why the species *T. albus* and *P. pardalis* did not show an increase in ROS is still intriguing, *P. pardalis* is the only species studied here that has facultative air respiration, so it can be considered that the effects of hypoxia for this species have been, at least in part, turned down. At the same time, although *T. albus* is regarded as hypoxia-sensitive, this fish species lives very close to the water's surface and can use aquatic surface respiration (ASR), which can confer an advantage. However, although the ASR brings more oxygen for *T. albus*, the hypoxia effects' minimization is significantly lower

than *P. pardalis*, an air-breathing fish. Thus, despite the ASR helping *T. albus* not form ROS, for example, this species' cardiac vulnerability is still observed by the lack of changes in ETS and CII.

The respiratory control ratio (RCR) is a measure of mitochondrial bioenergetics and coupling of the ETS and indicates mitochondrial efficiency. Here, both Cichlidae species decreased the RCR during the flood season (Fig. 3B). The depression in RCR and relative increase in proton leak may indicate an adaptive response of these two amazon Cichlidae species to ROS, as production trended higher in the flood season. However, the significantly higher production of ROS in the flood season can be related to an adaptation response of the mitochondria for this species. The increase in ROS levels and/or redox status could instead modulate various signaling pathways during chronic hypoxia by influencing the reversible oxidation of cysteine thiol groups of proteins (Smith et al., 2017). Gaining a better understanding of oxidative status and potential signaling by ROS during hypoxia and reoxygenation in Amazon waters can provide valuable insight into key mechanisms for coping with life in this variable environment.

The principle component analysis indicated that season has the greatest influence on mitochondria respiration (Fig. 4), leading us to suppose that some parameter intrinsic to season is modulating mitochondrial function and that hypoxia tolerance in Amazon fishes is unrelated to their position in the water column or the degree of phylogenetic proximity between species. In addition, the distribution of data in the Cartesian plane of PCA for the axis of more explanation (PC1 ≅60%) shows a much smaller plot area during the dry season, indicating that cardiac mitochondrial function follows a well-defined response pattern between the species- when oxygen is in higher concentrations. On the other hand, the species present a much more varied set of responses during the flood, indicating that different mitochondrial variables influence the species and that the mitochondrial differences between the species are much more accentuated during the flood period. Furthermore, PCA showed that RCR responses were opposed to ROS content, reinforcing the idea that more significant uncoupling would be a strategy to prevent ROS formation.

Our results show, for the first time, that heart fibres of Amazon fishes present low leak respiration in the dry season and highly coupled respiration in both seasons, indicating high phosphorylation efficiency in their hearts. Our results further suggest that Amazon fish adapt to hypoxia by increasing their heart mitochondrial respiration even when oxygen levels in the water are low. Mitochondrial responses across season are very similar between species, but absolute mitochondrial respiration rates seem to be related to the metabolic demand of each analysed species. For example, *C. monoculus* is a large species with a pelagic and migratory life history strategy, so it displays a higher aerobic demand (Duncan, 2020) and shows the highest mitochondria respiration level here. On the other hand, the lowest respiration level was observed in the most hypoxia-tolerant Amazonian fish species, which is currently known as *A. ocellatus*, indicating that the cardiac demands differ according to the species' lifestyle, since both species belong to the same family – Cichlidae.

## 5. Conclusions

Our data show that mitochondrial respiration is higher during the flood season, when DO are low compared the dry season when DO are higher. This pattern was observed for most of the species analysed herein, contradicting our hypothesis that fish would reduce mitochondrial respiration in the flood season and that responses would depend on their predominant position in the water column. The high temperatures of the Amazon limit the solubility of oxygen in water that combined with stratification of wetlands allows the waters to become increasingly hypoxic. Cardiac function must be maintained to allow species to thrive under these challenging conditions. We demonstrate that Amazonian fish compensate for this metabolic problem by improving ATP yield per mole of oxygen consumed, optimizing the efficiency of energy production in the myocardium. Field studies on seasonal changes in other components of the oxygen transport cascade in these species would be valuable to better understand how changes in mitochondrial respiration are supported. Given the highly dynamic nature of Amazon and its diversity of ichthyofauna, studies across multiple seasons and additional species might shed further light on the importance of this mitochondrial response to hypoxia.

## Competing interests

The authors declare no competing or financial interests.

**Acknowledgments**

The authors are grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Amazonas (FAPEAM), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) by supporting INCT/ADAPTA (CAPES – Finance Code 001; CNPQ proccess #465540/2014-7; FAPEAM proccess #062.01187/2017). SBM is the recipient of a doctoral fellowship from CAPES. TJM is supported by a New Brunswick Environmental Trust Fund grant. ALV and VMFAV are the recipients of Research Fellowship (level 1) from CNPQ. This work was supported by INCT ADAPTA (FAPEAM/CNPq/CAPES).

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## *LIST OF RESULTS*

**Table 1**. Species, environment, family name, sample size and weight of the *A. ocelatus*, *C. monoculus*, *H. malabaricus*, *M. aureum*, *O. bicirrhosum*, *P. pardalis* and *T. albus* collected in the flood and dry season in Catalão Lake.

| ***Species*** | ***Environment*** | ***Family name*** | ***Sample size*** | | ***Body mass (g)*** | |
|---|---|---|---|---|---|---|
| | | | *Flood* | *Dry* | *Flood* | *Dry* |
| *A. ocellatus* | *Benthic-pelagic* | Cichlidae | 9 | *10* | 302.5 ± 34.1 | 235.9 ± 29.1 |
| *C. monoculus* | *Pelagic* | Cichlidae | 7 | *10* | 238.3 ± 55.7 | 270.2 ± 34.1 |
| *H. malabaricus* | *Benthic-pelagic* | Erythrinidae | 7 | *10* | 246.4 ± 36.5 | 371.5 ± 30.5 |
| *M. aureum* | *Pelagic* | Serrasalmidae | 13 | *11* | 98.0 ± 30.5 | 93.5 ± 30.5 |
| *O. bicirrhosum* | *Pelagic* | Osteoglossidae | 7 | *9* | 726.6 ± 55.7 | 232.5 ± 30.5 |
| *P. pardalis* | *Benthic* | Loricariidae | 12 | *10* | 153.8 ± 32.2 | 228.8 ± 32.2 |
| *T. albus* | *Pelagic* | Triportheidae | 10 | *10* | 28.2 ± 48.3 | 33.7 ± 34.1 |

**Table 2**. Physical and chemical parameters (mean±SEM ) measured during the flood and dry season in Catalão Lake.

| ***Season*** | ***Month*** | ***Oxygen (mg/L)*** | ***Temperature (°C)*** | ***pH*** |
|---|---|---|---|---|
| Flood | April | 1.0±0.1 | 30±1.0 | 4.61±0.7 |
| Dry | November | 4.08±0.7 | 30.02±0.6 | 5.06±0.6 |

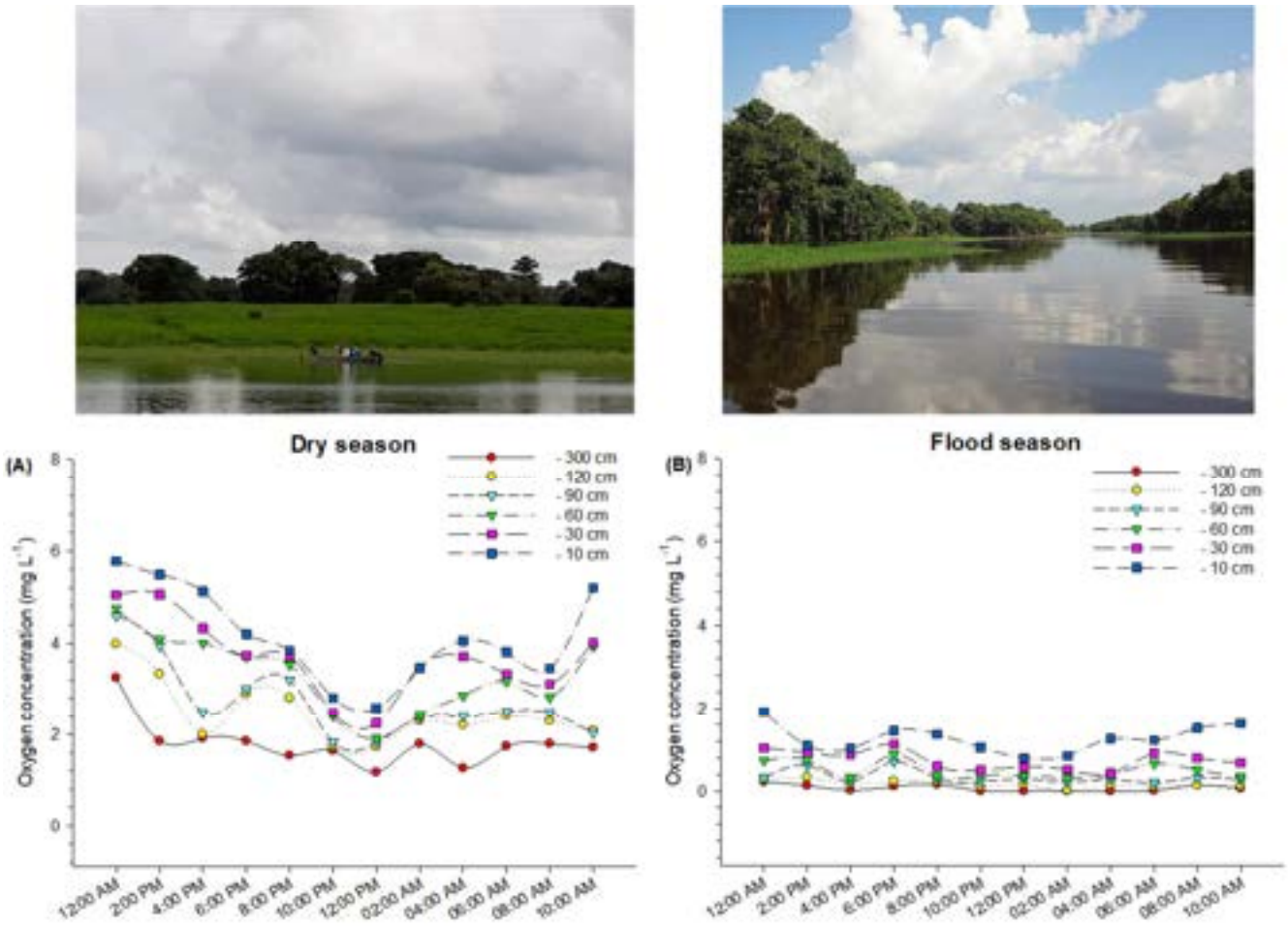


**Figure 1.** Variation of the dissolved oxygen concentrations (mg $L^{-1}$) during 24 h at different depths (cm) in the Catalão Lake (Amazonas- Brazil) during the (A) dry season (November of 2018) and (B) flood season (April of 2018). Note that the oxygen concentration varies depending on the period of the year, time of day, and measurement depth. The images represent the landscape of the seasons in which the animals were collected.

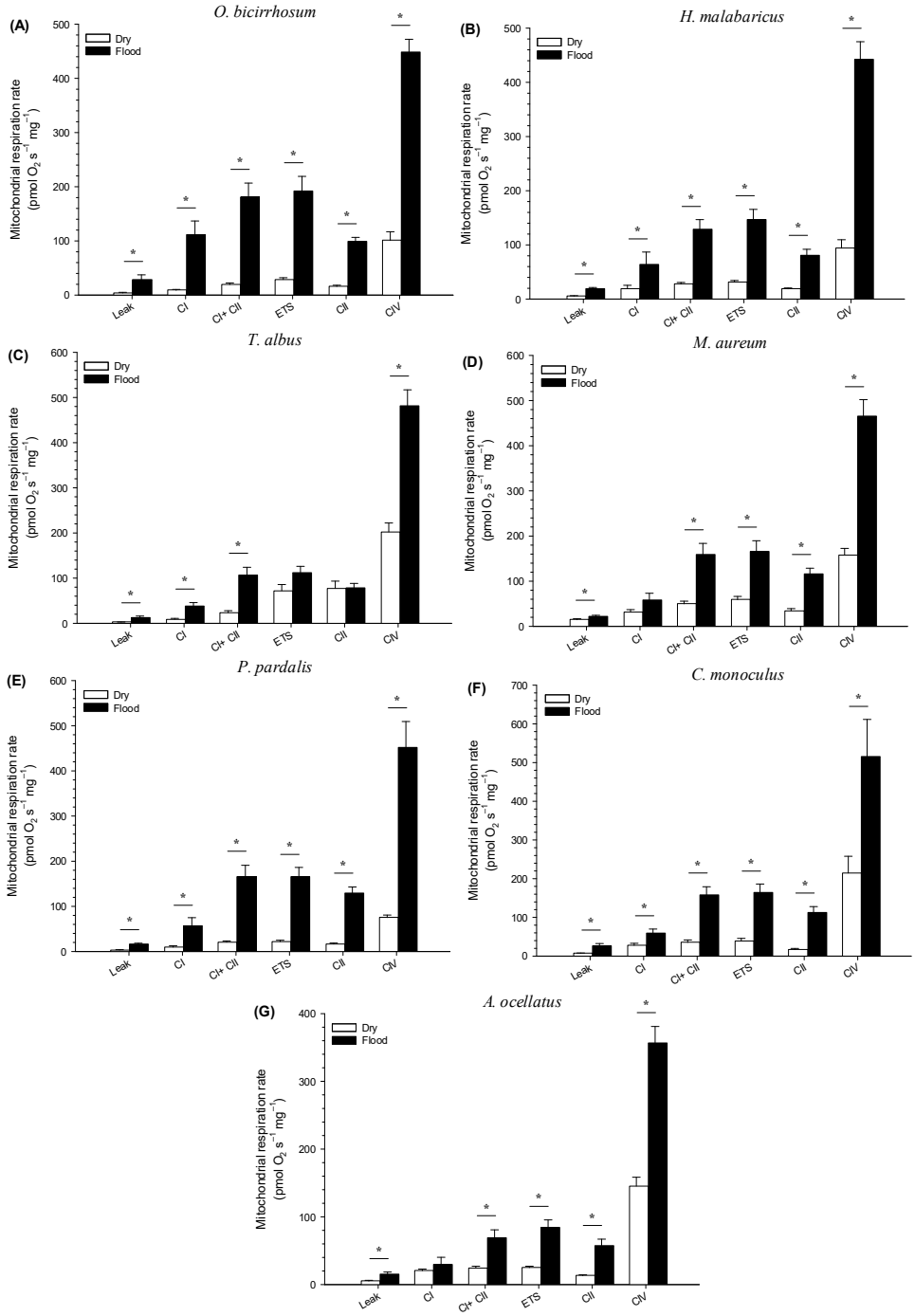


**Figure 2**. Heart mitochondria respiration of the Leak, CI, CI+ CII, ETS, CII and CIV of the (A) *O. bicirrhosum*, (B) *H. malabaricus*, (C) *T. albus*, (D) *M. aureum*, (E) *P. pardalis*, (F) *C. monoculus* and (G) *A. ocellatus* collected in the flood and dry season. Data are presented as mean ± SEM (n = 7-13). Asterisks (*) represent differences between the seasons ($p < 0.05$).

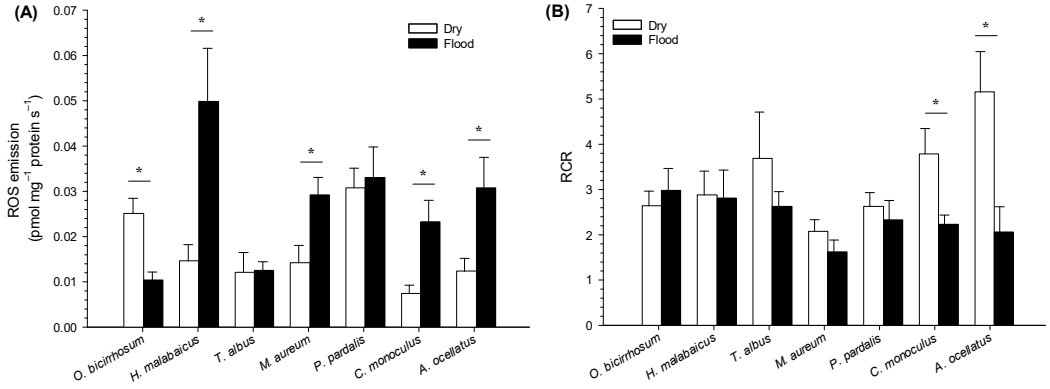


**Figure 3.** (A) Reactive oxygen species (ROS) emission and (B) the respiratory control ratio (RCR) from heart mitochondria in *O. bicirrhosum*, *H. malabaricus*, *T. albus*, *M. aureum*, *P. pardalis*, *C. monoculus* and *A. ocellatus*. Data are presented as mean ± SEM (n = 7-13). Asterisks (*) represent differences between the seasons ($p < 0.05$).

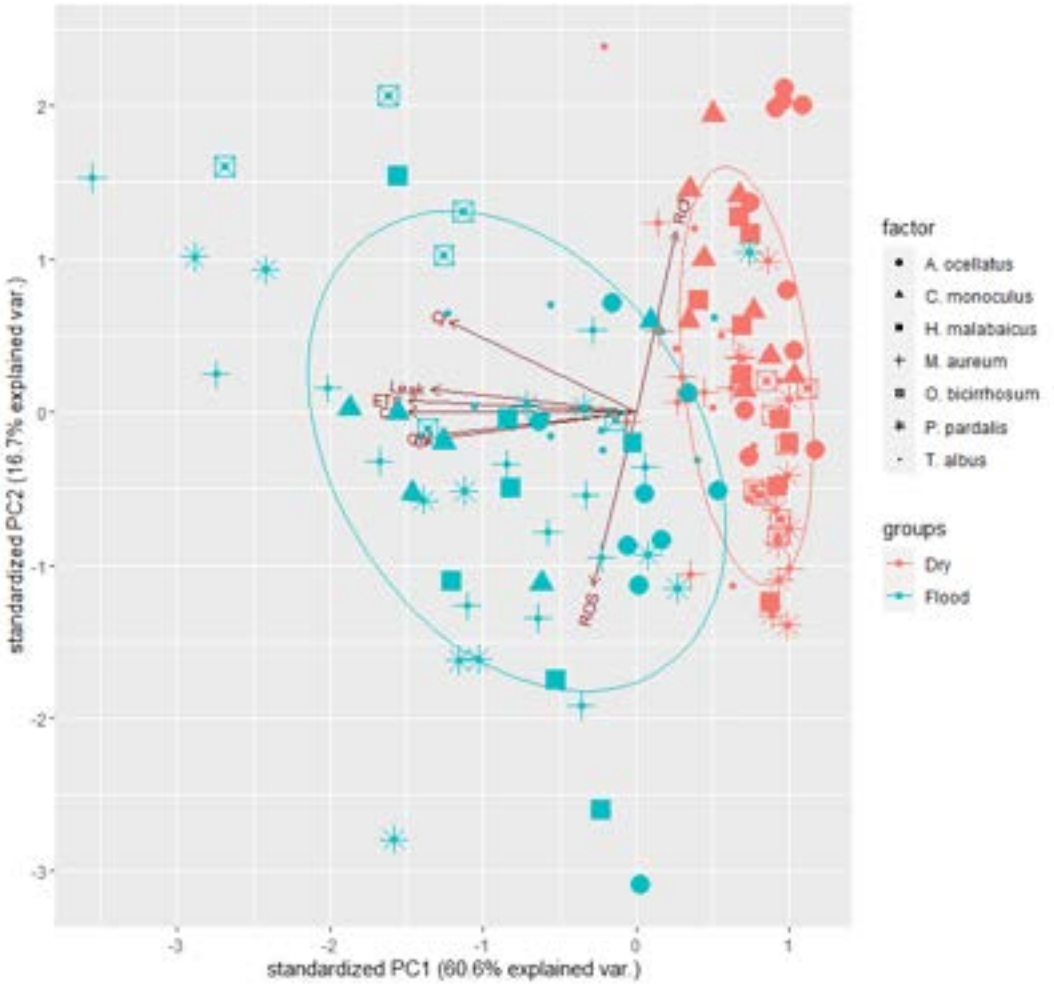


**Figure 4.** Principal components analysis (PCA) was carried out to assess the patterns between the seven amazon fish species studied (*O. bicirrhosum*, *H. malabaricus*, *T. albus*, *M. aureum*, *P. pardalis*, *C. monoculus* and *A. ocellatus*), considering distribution patterns between dry and flood seasons.

## 5. CAPÍTULO 3

*Artigo formatado de acordo com as normas da revista *Journal of Experimental Biology*.

# Metabolic patterns support mitochondrial responses in three Amazonian fish species exposed to continuous and intermittent hypoxia

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| **List of abbreviations** | |
|---|---|
| DO | dissolved oxygen |
| mt$\dot{M}O_2$ | mitochondria oxygen consumption |
| CCCP | carbonyl cyanide m-chlorophyll hydrazine |
| CI | mitochondrial complex I |

| | |
|---|---|
| CII | mitochondrial complex II (or succinate dehydrogenase, SHD) |
| ETS | Maximum capacity of the electron transfer system |
| OXPHOS | oxidative phosphorylation |
| RCR | respiratory control ratio |
| ROS | reactive oxygen species |


**Abstract**

The Amazon waters are characterized by daily and seasonal variations in dissolved oxygen (DO), and fish from these environments developed different tolerance mechanisms to deal with hypoxia throughout the evolutionary process. The present work selected three Amazonian species tolerant to hypoxia to determine their metabolic profile and to evaluate mechanisms of cellular oxygen uptake and ROS production during intermittent and constant hypoxia. First, we assessed, under normoxic conditions, the oxygen consumption profile ($\dot{M}O_2$), in addition to determining tolerance thresholds for each species ($PO_{2crit}$ and $P_{LOE}$). Second, the cellular responses to constant and intermittent hypoxia responses were tested. *A. ocellatus* was considered the species best hypoxia-adapted specie, comparatively, presenting the lowest $PO_{2crit}$ value, mitochondrial adjustments when ROS production is increased, and such tolerance may point to an evolutionary response as it belongs to a more phylogenetically recent group (cichlids) among the teleosts we studied in this work. Both *P. pardalis* and *C. macropomum* reduced the ROS content in hypoxia, although only *C. macropomum* showed mitochondrial uncoupling responses. Our results show that these amazonian species have refined hypoxia tolerance mechanisms in their mitochondria, but the exact physiological level of hypoxia may affect these species differently.


## 1. Introduction

One of the main characteristics of Amazonian waters is the annual and daily variation in the concentration of dissolved oxygen (DO) (Junk, 1984; Val & Almeida-Val, 1999). Such hypoxic waters of the Amazon reflect complex interactions among physical, chemical, and biological parameters. Some of these phenomena include photosynthetic rates, the respiration of bacteria, interactions between animals and plants, light penetration, organic matter decomposition, wind incidence, and mineral and soil

composition (Junk, 1984). The annual variation is marked by the flood season, where the DO is very close to zero at the surface and in the entire water column all day long, and there is no consistent difference in concentrations between day and night. On the other hand, the opposite scenario is observed in the dry season, where the DO is comparatively higher and more variable (Val & Almeida-Val, 1995; Val & Almeida-Val 1999; Val & Almeida-Val, 2006). The daily variation of the DO is marked by the decrease of oxygen, mainly close to noon, due, among other reasons, to the increased respiration of plants (Aprile & Darwich, 2009; Melack & Forsberg, 2001; Randall et al., 1981). Surviving in such particular environments pushed Amazon fish species develop different mechanisms throughout the evolutionary period to deal with hypoxia.

An adequate and continuous oxygen supply is fundamental to effective cell functioning as aerobic metabolism produces more than 90% of the energy in vertebrate mitochondria (Hochachka & Lutz 2001; Somero et al., 2016; Sokolova et al., 2019). Hypoxia can compromise cellular energy supplies in the absence of specialized biochemical and physiological readjustments, triggering signal cascades that result in organ failure and, subsequently, death (Devaux et al., 2019). Some Amazon fish species can survive hypoxia using behavioral and morphological adjustments that allow a more efficient oxygen uptake. For instance, the *Astronotus ocellatus* can tolerate hypoxia by a combination of responses, including enhanced $O_2$ uptake, transport, and delivery, as well as increased anaerobic metabolism and metabolic rate depression (Almeida-Val et al., 2006; Braz-Mota & Almeida-Val 2021; Muusze et al., 1998). At the same time, the Siluriformes *Pterygoplichthys pardalis* is a facultative air-breathing fish that goes to the surface and swallows air, carrying out gas exchange in their stomach (Brito, 1981; Val & Almeida-Val 1995). Another hypoxic-tolerant Amazon fish is *Colossoma macropomum*. Besides decreasing their aerobic metabolic rate, they can expand their lower lip to efficiently collect the oxygen-rich surface water layer during low DO conditions; what helps to improve water flow through the gills during aquatic surface respiration (Val et al., 1998).

Several metrics are used as indices of hypoxia tolerance in aquatic organisms, such as the critical $O_2$ tension ($PO_{2\ crit}$), usually measured as the breakpoint of the two-

segmented linear regression from oxyregulation to oxyconformation, acquired through the oxygen consumption ($\dot{M}O_2$) (Yeager & Ultsch, 1989), and the ability to resist the loss of equilibrium (LOE) in severe hypoxia, measured as the $PO_2$ at LOE ($P_{LOE}$) in the face of progressive hypoxia (Borowiec et al., 2016; Dhillon et al., 2013; Mandic et al., 2013). In addition to these measures, mitochondrial responses to hypoxia can also be considered as an important physiological tool for understanding the hypoxia tolerance mechanism. During fluctuations in DO, for example, mitochondrial alterations can be a potential stressor depending on the frequency and intensity of hypoxia, in addition to the tolerance of the species in question, may present mitochondrial damage, energy deficiency, and cell death (Sokolova et al., 2019). Hypoxia exposure for the Amazon fish species *Astronotus pallidus* increased the mitochondrial electron transport system efficiency by enhancing oxygen affinity and decreasing the total reactive oxygen species (ROS) (Heinrichs-Caldas & Almeida-Val. 2021). Instead, the obligatory air-breathing Amazon fish *Arapaima gigas*, hypoxia caused a significant reduction in mitochondria respiration for small and large fish gills, airbreathing organ, and kidney, despite no changes in the ROS content for both larger and small fish (Pelster et al., 2020).

The elevated ROS production may trigger a vicious cascade of ROS release, damaging the electron transport system (ETS), amplifying ATP deficiency, and eventually resulting in mitochondrial collapse and apoptosis (Zorov et al. 2014). Avoiding these bioenergetics and redox issues is essential for the survival of chronic and intermittent hypoxia, making adjustments of OXPHOS and ETS a key candidate mechanism for hypoxic adaptation. Thus, the present work aims to evaluate the cellular oxygen uptake mechanism and the ROS production in Amazon fish species with different metabolic strategies under low DO during intermittent and constant hypoxia. We, therefore, hypothesized that (i) Amazon fish will have more mitochondrial adaptations for intermittent hypoxia exposure due to the common oxygen variation naturally observed in this environment, (ii) the liver tissue will significantly contribute to metabolic adaptation to hypoxia, and (iii) the three hypoxia-tolerant species will exhibit different mitochondrial strategies to deal with the same physiological level of hypoxia.

## 2. Material and Methods

### *2.1 Animals and housing*

Juveniles of *Colossoma macropomum*, *Astronotus ocellatus* and *Pterygoplichthys pardalis* (n=6) were purchased from a local fish farm (Santo Antônio Farm, Amazon, Brazil), and transferred to the Laboratory of Ecophysiology and Molecular Evolution at the Brazilian National Institute for Research of the Amazon (INPA), where they were maintained indoors for at least 1 month in 4500 L fiberglass tanks, supplied with continuous aeration and flow through well water, vigorously aerated prior aiming the reduction of dissolved $CO_2$ (in $\mu mol\ L^{-1}$; $Na^+$, 43; $Cl^-$,31; $K^+$,10; $Ca^{2+}$,9; $Mg^{2+}$,4; pH 6.0, 6.40 mg $O_2\ l^{-1}$ and 26 °C). Throughout the acclimation period fish were fed ground dry commercial trout pellets once a day and held on a 12 h light/12 h dark photoperiod. Feeding was suspended 24 h before experiments. There was no mortality observed during the acclimation period. Experimental and holding procedures followed the CONCEA (National Council of Animal use in Research and Education) animal care guidelines and were approved by INPA's animal care committee (protocol number: 026/2017).

### *2.2 Respirometry and hypoxia tolerance measurements*

#### 2.2.1 Metabolic rate ($\dot{M}O_2$)

Six animals (n=6) from each species were transferred to a 70 mL glass chamber immersed in a water bath tank with aerated water and held overnight. Routine metabolic rate ($\dot{M}O_2$) was determined using an automated intermittent flow respirometry system (Oxy-4; Loligo Systems, Viborg, DEN). A pump interfaced to a DAQ-M (Loligo systems) data acquisition system controlled the measurement cycle, with a loop consisting of 3 phases: ambient flush (180 s), wait (120 s), and measurement (300 s), and the measurements were collected over 4 h. Metabolic rate was calculated as $\dot{M}O_2 = \Delta O_2 * Vresp * B^{-1}$, where: $\Delta O_2$ is the rate of change in oxygen concentration (mg $O_2\ h^{-1}$), Vresp is the volume of the respirometer chamber, and B is the mass of the individual (kg).

#### 2.2.2 Critical oxygen tension ($PO_{2\ crit}$)

After the 4 hours of the $\dot{M}O_2$ measurements, the same animals were kept in the respirometer chambers without flushing or aeration and the oxygen consumption was measured. The $PO_{2\ crit}$ was calculated as the breakpoint using the $\dot{M}O_2$ data across all $PO_2$

by the BASIC program developed by Yeager and Ultsch (Yeager & Ultsch, 1989), which uses stepwise regression to calculate the two best-fit regression lines, and then determines the breakpoint using the $\dot{M}O_2$ data across all $PO_2$.

### 2.2.3 Loss of Equilibrium ($P_{LOE}$)

Six animals (n=6) from each species were transferred overnight to a tank in normoxic conditions. Then, the $PO_2$ was reduced to 1.5 mg $O_2$ $L^{-1}$. At that time, the water surface was covered with bubble wrap, and nitrogen bubbled into the water to rapidly lower $PO_2$ (rate of decline was 3.16 mg $O_2$ $L^{-1}$ $hour^{-1}$). The animals were kept in the tanks until they individually loose of equilibrium, at which point $PO_2$ was recorded as $P_{LOE}$, and fish were individually euthanized.

## *2.3 Experimental hypoxia acclimations*

All three fish species were individually exposed to one of the three conditions: normoxia, constant hypoxia or intermittent hypoxia for 7 days. Groups of eight individuals of each species were transferred in pairs to each of the four cages immersed in one of the three larger 70 L experimental tanks. Fish were kept in these conditions overnight to allow recovery from handling. All experimental conditions were controlled in each one of the three large tanks, which were fitted with filters to maintain water quality. During the experiments, (see table 2 for oxygen concentration) normoxia conditions were achieved by continuously bubbling the water with air, constant hypoxia was achieved by the bubbling of water with nitrogen gas using a solenoid valve integrated to a DO control system (Loligo Systems, Tjele, Denmark), and intermittent hypoxia was achieved by injecting nitrogen (rate of decline was 2.86 mg $O_2$ $L^{-1}$ $hour^{-1}$) (19:00 to 07:00 h) or air (07:00 to 19:00 h) using the same DO control system. The level of hypoxia in the constant and intermittent hypoxia exposures was set to 80% of the $PO_{2\ crit}$ for each species (0.80±0.05 mg $O_2$ $l^{-1}$ to *A. ocellatus*, 0.99±0.19 mg $O_2$ $l^{-1}$ to *C. macropomum* and 1.09±0.22 mg $O_2$ $l^{-1}$ to *P. pardalis*). The experiments employed a semi-static system where 80% of the water volume was replaced every 24 h. Aqueous ammonia levels were measured using the colorimetric assay developed by Verdouw et al., 1978. After the seven days of the experiment, the fish were weighed, blood samples were taken, and plasma

was separated and frozen at -80 °C for lactate measurements. Then, the liver was removed and weighed for analysis of hepatosomatic index (ratio of body mass:liver mass) and immediately immersed in 2 mL of ice-cold relaxing BIOPS buffer (pH 7.1), containing (in mM): $CaK_2EGTA$ 2.77, $K_2EGTA$ 7.23, $Na_2ATP$ 5.77, $MgCl_2 \cdot 6H_2O$ 6.56, taurine 20, imidazole 20, dithiothreitol 0.5, K-MES 50, sodium phosphocreatine 15, and sucrose 50.

### *2.4 Determination of mitochondria respiration and ROS content*

Fresh liver tissue (≅ 5 mg) was removed from the BIOPS buffer and gently homogenized in 1 ml ice-cold BIOPS along with 50 µg $ml^{-1}$ saponin. After 30 min, the liver was washed thrice for 10 min in 2 mL of modified mitochondrial respiratory medium MiRO5 buffer (pH 7.2), containing (in mM): EGTA 0.5, $MgCl_2 \cdot 6H_2O$ 3, K-lactobionate 60, taurine 20, $KH_2PO_4$ 10, HEPES 20, sucrose 160 and 1 g/l BSA, essentially fatty acid free, at 25 °C (Gnaiger et al., 2000). The hepatic tissue was blotted dry on filter paper, weighed, and immediately immersed into oroboros respiration chambers filled with 2 mL of MiRO5.

Oxygen was added into the gas phase above media prior to closing chambers to supersaturate oxygen concentration. The Complex I (CI) substrates (5 mM malate, 10 mM glutamate and 5 mM pyruvate) were added to measure state II respiration through CI in the absence of ADP (denoted 'Leak I'). Excess ADP (2.5 mM) stimulated oxidative phosphorylation (OXPHOS -I, state III respiration). Cytochrome c (10 µM) was added to test outer membrane integrity. Phosphorylating respiration with CI and complex II (CI+II) substrates (OXPHOS -I, II) was measured by the addition of succinate (10 mM). The respiratory electron transfer-pathway capacity (denoted 'ETS') was assessed by repeated titrations of carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone (FCCP, 0.5 µM). The activity of CI, II and III complexes were then inhibited by the addition of rotenone (0.5 µM), malonate (15 mM), and antimycin A (1 µM), respectively. Complex IV activity was recorded by addition of 5 mM ascorbic acid and TMPD, passing all electrons to complex IV. RCR was calculated as ETS/leak state ratio. See the Figure 2 for the representative protocol.

Oxygen uptake and ROS production were simultaneously measured in the same cell preparation using the Oroboros Oxygraph and DatLab 2 software (Oroboros Instruments GmbH, Innsbruck, Austria). Superoxide dismutase (SOD; at 22.5 U $mL^{-1}$) was added to catalyze the reaction of the superoxide produced by the mitochondria and horseradish peroxidase (3 U $mL^{-1}$) was added to catalyze the reaction of hydrogen peroxide with Ampliflu Red (15 µM) and produce the fluorescent product resorufin (detected using an excitation wavelength of 525 nm and ampliometric filter set (AmR); Oroboros Instruments). The resorufin signal was calibrated with additions of exogenous hydrogen peroxide.

### *2.5 Plasma lactate levels*

Plasma samples (≅ 50 µl) were sonicated in 1.5 mL of 6 % PCA and then neutralized with the addition of 2 volumes of 2M $KHCO_3$. The solution was shaken vigorously and centrifuged at 10000 g for 5 min 4°C. The supernatant was mixed with 0.6M glycine buffer (Sigma G5418) and $NAD^+$ 2.5 mM, and the addition of LDH L2500 (dilute 1:3) started the reaction, which was measured for 30 min spectrophotometrically (340 nm). To obtain the lactate levels, the absorbance was compared with a lactate standard curve (0 to 2 mM lactate). The method was based on reducing $NAD^+$ to NADH (340 nm) in a glycine-hydrazine buffer (MacCormack et al 2006).

### *2.6 Statistical analyses*

Data are presented as mean ± S.E.M. and n = 6 individuals for all treatments. To define and compare the metabolic profile of the species, a One-Way ANOVA was performed considering the species as factors. To assess the effect of intermittent and constant hypoxia on mitochondria respiration, ROS content, lactate, and HSI, another One-Way ANOVA was applied for each species, this time considering oxygen concentration as a factor. Data were first tested for normality in both cases, and a Tukey post hoc test was used to identify the differences. A non-parametric Kruskal Wallis test was applied when the ANOVA premises were violated. The significance level adopted was p=0.05).

## 3. Results

### *3.1 Metabolic profile*

The three species showed different metabolic patterns under normoxic conditions. The *P. pardalis* showed the lower RMR (60±5.1 $mgO_2\ kg^{-1}\ h^{-1}$), indicating a low oxygen consumption of this species compared to *A. ocellatus* and *C. macropomum* (Table 1). Despite values being close for all three species, $PO_{2crit}$ was significantly lower in *A. ocellatus*, which suggests this species has a greater tolerance to hypoxia (Figure 1). The $P_{LOE}$ was similar in *A. ocellatus* and *P. pardalis* but was significantly higher for *C. macropomum* (Table 1).

### *3.2 Mitochondrial respiration, ROS content, lactate, and HSI*

All three species showed a significant increase in lactate in both hypoxic regimens, indicating that anaerobic metabolism was necessary when submitted to metabolically hypoxic conditions (Table 2). The species studied here seem to show a response pattern to chronic hypoxia, which was little dependent on the hypoxic regimen tested (constant or intermittent hypoxia) for most species. In *A. ocellatus*, for example, both hypoxic regimes induced an increased in the mitochondria respiration in CI+ CII and ETS (Figure 3A), whereas only the intermittent hypoxia exposure increased HSI (Table 2). For *C. macropomum*, the physiological effects of both constant and intermittent hypoxia were similar. The mitochondria were uncoupled, as observed by the increases in leak respiration, total leak (Figure 3B), and RCR (Table 2), even though ROS content was decreased only during hypoxia exposures (Figure 4). On the other hand, *P. pardalis* showed RCR values really low at less than 2, indicating a low coupling efficiency rate. In addition, this specie decreases mitochondria respiration, which was observed by the decrease in CI and ETS in both constant and intermittent hypoxia (Figure 3C). However, the respiration in CIV was decreased in *P. pardalis* only in intermittent exposures, whereas only hypoxia treatment was able to reduce the ROS content (Figure 4).

## 4. Discussion

Amazonian aquatic environments show significant variations in DO concentration, where recurrent hypoxic conditions drove an essential evolutionary force

in developing hypoxia tolerance mechanisms in these species. Here, the three species analyzed are considered tolerant to hypoxia. Our findings showed that the metabolic profiles between the three fish species are different, affecting their capacity to extract oxygen from the environment and directly influencing mitochondrial activity in highly metabolic organs such as the liver.

### *4.1 Hypoxic-tolerance is dependent upon metabolic profile*

In aquatic organisms, variations in $\dot{M}O_2$ and PO2 crit are appropriate indicators of hypoxia tolerance and provide information on the capacity to extract environmental $O_2$ (Borowiec et al., 2020). The three Amazon fish species studied showed distinct metabolic profiles (table 1). *A. ocellatus* is a species known to increase hypoxia tolerance throughout ontogeny by activating anaerobic metabolism and is well known to use a massive depression of $\dot{M}O_2$ under hypoxia. The relatively high RMR recorded in normoxia here could suggest that this species would be more hypoxia sensitive, perhaps due to its small size (≅ 10g). However, *A. ocellatus* was the most tolerant species examined, presenting the lowest $PO_{2\ crit}$ and $P_{LOE}$. Although some species appear to be oxyconformers, with $\dot{M}O_2$ declining progressively as $PO_2$ falls from normoxic levels (Urbina et al., 2012; Wood, 2018), $PO_{2\ crit}$ still is a legitimate measure of hypoxia tolerance (Regan et al., 2019; Rogers et al., 2016). Thus, the proximity of the $PO_{2\ crit}$ values of *C. macropomum* and *P. pardalis* could indicate a similarity in tolerance to hypoxia between these species. However, *P. pardalis* presented much lower RMR values than *C. macropomum*, indicating a lower oxygen dependence by *P. pardalis* and, therefore, suggesting a higher tolerance in conditions where oxygen is scarce.

*C. macropomum* showed a higher $P_{LOE}$ than *A. ocellatus* and *P. pardalis*, and exhibited both high RMR and rates of mitochondria oxygen consumption ($mt\dot{M}O_2$), confirming that this fish is more dependent on aerobic metabolism in normoxic conditions (Figure 3B). Some studies support the idea that mitochondria from hypoxia-adapted organisms have high $O_2$ affinity, thereby providing maintenance of aerobic respiration even at lower $O_2$ tensions (Chung et al. 2017; Lau et al. 2017; Sokolova et al., 2019). Intracellular $O_2$ gradients depend on the overall rate of aerobic metabolism ($\dot{M}O_2$) so that cellular $O_2$ tensions tend to be lower in metabolically active tissues and cells (Gnaiger

2001). During normoxic conditions, *A. ocellatus* showed a high RMR but low mt$\dot{M}O_2$ rates, suggesting an important adaptation of this fish specie to keep your mitochondrial respiration levels down. However, hypoxia exposure drops the $MO_2$ by under 90%, at the same time that a low $PO_2$ gradient from plasma to mitochondria is observed (Driedzic et al., 2021), letting us believe that the increase in mt$\dot{M}O_2$ observed (CI+CII and ETS) at low blood $O_2$ levels previously described for this specie during hypoxia, would indicate high $O_2$ affinity. Thus, the mitochondrial affinity of different species might reflect differences in the intracellular $O_2$ tensions determined by the balance of $O_2$ delivery and tissue $O_2$ consumption rather than adaptations to the external $PO_2$. However, are needed future investigations in a broad comparative framework controlling for the species' phylogeny and overall metabolic rates and the actual intracellular $PO_2$ in different tissues and organisms to test this hypothesis.

On the other hand, *P. pardalis* showed both a low RMR and mt$\dot{M}O_2$, indicating that the energy demand of this species is low, a characteristic that can be advantageous for tolerating episodes of hypoxia. Differences in metabolic responses of *A. ocellatus* and *P. pardalis* were recently described by Driedzic et al., (2021), highlighting an intense hypometabolism is sustained by *A. ocellatus* in hypoxia, reducing the heart performance and further reducing energy demand achieved by decreases in ATP demanding processes, while *P. pardalis* generally maintain cardiac performance under hypoxia. Thus, the strong metabolic depression of *A. ocellatus* in hypoxia (~90%) can explain the difference in $PO_{2crit}$ between that species and *P. pardalis*, considering that the lower oxygen consumption became this Cichlidae less oxygen-dependent and, therefore, reaching the breakpoint to oxyconformist belatedly. On the other hand, the higher $PO_{2\ crit}$ of *P. pardalis* can be related to the lower hability to decrease the $\dot{M}O_2$ in such a large variation, despite the similar $P_{LOE}$ and mt$\dot{M}O_2$ observed between these species. Therefore, the normoxic low RMR may be advantageous in hypoxia tolerance for this species. *C. macropomum* was the least tolerant of the three species and demonstrated the most dependence on aerobic metabolism. Thus, from the metabolic profile of these species, we can infer that under conditions of continuous and adequate oxygen supply, *A. ocellatus* was the most tolerant species, presenting lower $PO_{2\ crit}$, $P_{LOE}$, and very, possibly high mitochondrial oxygen affinity.

***4.2 Hypoxic tolerance determines mitochondrial adjustments during continuous and***

***intermittent hypoxia***

Fluctuations in $O_2$ strongly affect mitochondrial respiration dynamics and the mitochondrial and cellular redox balance, and these physiological adjustments are essential to enhancing the animal's tolerance to hypoxia (Sokolova et al., 2019). In general, physiological responses to hypoxia are often associated a reduction in tissue O2 demand to match the reduced $O_2$ supply (Kalogeris et al. 2012). However, in the present work, *A. ocellatus* increased respiration in CI+CII and ETS under both hypoxic conditions. The metabolic profile responses have shown that *A. ocellatus* is highly tolerant, with the lowest $PO_{2\ crit}$ and more efficient use of oxygen, thereby ensuring aerobic respiration at lower $O_2$ tensions.

The increase in ETS shows a higher capacity of the electron transfer system an open-circuit operation of the transmembrane protonmotive force, indicate an elevated electron flux in constant and intermittent hypoxia. In addition, the increase in liver mitochondrial respiration in CI+CII by *A. ocellatus* can indicate a higher respiratory capacity to deal with ROS. This is because succinate accumulation causes ROS production to increase by the backflow of electrons through mitochondrial Complex I (Chouchani et al. 2014). Thus, the greater respiration of CI+CII, the fewer electrons will accumulate, and ROS levels will be kept within the ideal range for the maintenance of necessary cellular functions. Although the mitochondria responses were similar for the two hypoxic regimes, the more significant proportion of the liver in the intermittent treatment due to the increase in the HSI suggests that glycogen reserves are being used. In intermittent hypoxia, glycogen reserves are likely being replenished during the normoxic periods. Thus, despite the higher metabolic requirement in the intermittent hypoxia treatment, the ability to use the glycogen as an energy source indicates that the fish can recover between bouts of hypoxia and may survive this treatment indefinitely. On the other hand, under chronic hypoxia, they can’t replenish their glycogen reserves, so they eventually run out of fuel and die.

Furthermore, difference approximately 5-fold in HSI between *A. ocellatus* and *P.*

*pardalis* is due of their bony structures, once the body mass of *P. pardalis* almost 40% armor (MacCormack et al., 2017; Harter et al., 2014), indicating that these two species have approximate weights and makes them comparable. However, previous studies show that *P. pardalis* has a 14% higher yield of ATP derived from glycolysis than other species (Driedzic et al 2021), demonstrating a high anaerobic potential, which was supported in this work by the higher lactate levels in this species, even under normoxic conditions. Thus, in contrast to what was observed for *A. ocellatus* and *C. macropomum*, the reduction in mitochondrial CI respiration and decrease of stimulation of maximum electron flux of *P. pardalis* in both hypoxic regimes tested may be associated with passive factors, including $O_2$ limitation and/or damage to CI proteins, or active factors like the down-regulation of electron flow in CI.

Intermittent hypoxia seems to be a challenge to *P. pardalis*, which showed a decrease in OXPHOS by reducing CIV activity. Moreover, cyclic hypoxia events may have posed an additional energetic cost for this species since in hypoxia *P. pardalis* goes to the surface of the water column, swallows air, and extracts $O_2$ from it via the vascularized stomach. Therefore, the hypoxia exposure experiments were carried out without access to air, to keep the animal in real hypoxia. Thus, the intensity of reoxygenation may have been different from natural environmental conditions, since the oxygen concentration in atmospheric air is much higher than the DO in water.

The lower intensity of restoration of oxygen may conditionate the liver mitochondria of this animal in an endurance process after seven days of hypoxic cycles. Furthermore, this mechanism would work with successive daily episodes of hypoxic cycles where the lack of oxygen followed by the gradual reoxygenation could generate resistance to future low $PO_2$ conditions (Borowiec et al., 2015). Thus, the successive episodes of the reduction in the $O_2$ tension could decrease the release of $H^+$, such as observed by the ETS decreases. As a result, the suppression of COX activity is observed when the intermittent hypoxia, the gradual restoration of oxygen leads to the substrate ($O_2$) limitation of COX, slowing down the electron transfer rate in the ETS, decreasing the rate of the proton pumping (Semenza 2007).

The increment in leak respiration to avoid ROS formation is another component reducing the coupling between $O_2$ flux and ATP turnover (Gnaiger, 2020). Leak respiration increased in both hypoxic regimes for *P. pardalis* and, in addition, RCR rates were the lowest compared with *A. ocellatus* and *C. macropomum*, indicating an increase in uncoupling efficiency. Although ROS production rates did not change in intermittent hypoxia, they were lower in continuous hypoxia, indicating the presence of a regulatory mechanism in the mitochondria of this species, since damaged mitochondria during hypoxia or reoxygenation may result in elevated ROS emission, ATP deficiency, a lower threshold for apoptosis, or release of damage-associated molecular pattern molecules (such as HSP60 or mtDNA) that induce inflammation and ROS production (Stotland & Gottlieb 2015).

The mitochondrial mechanism of *C. macropomum* to deal with constant and intermittent hypoxia was focused on uncoupling activation, both in the absence of ADP (leak respiration) and after inhibition of the phosphorylation system by oligomycin (total leak). In addition, RCR decreased in both hypoxic regimes. Coupling and uncoupling are key components of mitochondrial respiratory control (Gnaiger 2020). The higher mt$\dot{M}O_2$ indicates a greater oxygen demand for this fish species and, consequently, a higher electron slip, which can increase ROS production. Then, the uncoupling activation in *C. macropomum* is related to ROS reduction, such as observed in hypoxia exposures, as the compensatory mechanism to decrease the surrounding electrons in the system.

### *4.3 The systematics and evolution of Amazon fish can determine the mitochondrial responses to hypoxia*

Since the Cambrian period, hypoxic and anoxic conditions were prevalent in the Amazon floodplain due to low atmospheric oxygen concentrations (Almeida-Val et al., 2006; Randall et al., 1981). During the Cenozoic (upper tertiary), oscillations in atmospheric oxygen levels occurred more frequently than in previous periods and were reflected in aquatic environments (Randall et al., 1981). This was when most fish species

that live in the Amazon today originated (Albert & Reis, 2011; Randall et al., 1981). The Amazon aquatic environments were kept hypoxic from then to now, but for different reasons (Braz-Mota & Almeida-Val 2021; Almeida- Val & Farias, 1996; Val & Almeida-Val, 2006), meaning that hypoxia was a significant selective force for the evolution for Amazon fish species (Randal et al., 1981).

If we consider the systematics and evolution of Amazon fish, we can see that *C. macropomum* (Characiformes) is the least derived species, followed by *P. pardalis* (Siluriformes) and then *A. ocellatus* (Cichliformes), the most derived species from the three studied here (Fink & Fink 1978). Therefore, the adaptive pattern of mitochondrial response observed may be related to the emergence of Amazonian fish on the evolutionary scale. Although all three species are known to be tolerant to hypoxia, their mitochondrial and metabolic tolerance mechanisms show differences that may be associated with the adaptive capacity of these species over evolutionary time. The least derived species, *C. macropomum*, was the most dependent on aerobic metabolism, showing higher mt$\dot{M}O_2$ and mechanisms related to mitochondrial uncoupling, maintaining mitochondrial respiration, but possibly showing reduced energy levels. On the other hand, *P. pardalis* has adaptive mechanisms to deal with hypoxia by capturing oxygen from the atmosphere, thus ensuring a continuous supply of oxygen to carry out mitochondrial functions, since this species also has a low metabolic rate. At the same time, *A. ocellatus* is part of the recent group of cichliforms known for their high tolerance to hypoxia (Almeida-Val et al., 1999; Almeida-Val et al., 2000), presenting specialized mechanisms in liver mitochondria to maintain the flow of electrons and manage ROS content. Thus, the mitochondrial tolerance responses can be related to the phylogenetic scale of these three Amazon tolerant fish.

## 5. Conclusion

Comparing mitochondrial adjustments to constant and intermittent hypoxia exposure in three Amazonian fish species with a different tolerance mechanism reveals some adaptive traits. For example, increases in ETS capacity (observed in *A. ocellatus* in both hypoxic regimes), the suppression of ROS producing electron flux (observed in *P. pardalis* and *C. macropomum* in constant hypoxia exposure), reversible suppression of OXPHOS activity (verified in *P. pardalis* by the decrease in CIV), and uncoupling

activation to guarantee the mitochondrial quality (observed in *C. macropomum* during hypoxia exposures). Therefore, our findings showed that the mitochondrial adaptations to intermittent and constant hypoxia are very similar after seven days. Such results indicate that the experimental conditions tested here mimicked those seen by these fish in the wild once they were already prepared to deal with the common oxygen variation observed in their natural environment. In addition, the metabolic profile (as observed by the $PO_{2\ crit}$) of the species supported the mitochondrial adaptations responses to deal with both hypoxic regimes and that the evolutionary history of these three tolerant fish species can help explain mitochondrial strategies when exposed to the same physiological level of hypoxia.

## Competing interests

The authors declare no competing or financial interests.


## Acknowledgments

The authors are grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Amazonas (FAPEAM), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) which support INCT/ADAPTA (CAPES – Finance Code 001; CNPQ process #465540/2014-7; FAPEAM process #062.01187/2017). SBM is the recipient of a doctoral fellowship from CAPES. TJM is supported by a New Brunswick Environmental Trust Fund grant. ALV and VMFAV are supported by INCT ADAPTA (FAPEAM/CNPq/CAPES) and receive a research fellowship from CNPq.


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## *LIST OF RESULTS*

**Table 1.** Body masses of fish used to define the hypoxia tolerance through the metabolic profile (routine metabolic rate ($\dot{M}O_2$), critical oxygen tension ($PO_{2crit}$), and oxygen concentration at the loss of equilibrium (LOE)) of *A. ocellatus*, *C. macropomum*, and *P. pardalis* under normoxic conditions (6.24 mg $O_2$ $l^{-1}$). Different letters represent the statistical difference between species ($p<0.05$). See text for further statistical details.

| | ***Body mass (g)*** | ***Temperature (°C)*** | ***RMR ($mgO_2\ kg^{-1}\ h^{-1}$)*** | ***$PO_{2crit}$ ($mgO_2\ kg^{-1}\ h^{-1}$)*** | ***LOE (mg $O_2$ $l^{-1}$)*** |
|---|---|---|---|---|---|
| *A. ocellatus* | 10.02±3.12 | 25.81±0.30 | 173±12[a] | 1.01±0.07[a] | 0.21±0.02[a] |
| *C. macropomum* | 22.62±2.61 | 26.06±0.26 | 162±16[a] | 1.24±0.24[b] | 0.28±0.007[b] |
| *P. pardalis* | 43.40±2.07 | 26.05±0.60 | 60±5.1[b] | 1.37±0.28[b] | 0.22±0.01[a] |

**Table 2.** Body masses, experimental water conditions (temperature, oxygen and ammonia concentrations), lactate levels and hepatic somatic index of the *A. ocellatus*, *C. macropomum*, and *P. pardalis* exposed to normoxia, intermittent hypoxia or constant hypoxia for 7 days. Different letters represent the statistical difference between different oxygen regimes ($p<0.05$).

| | | ***Body mass (g)*** | ***Temperature (°C)*** | ***Oxygen (mg $l^{-1}$)*** | ***Ammonia (µM)*** | ***RCR*** | ***Lactate (mM)*** | ***Hepatic somatic index (HSI)*** |
|---|---|---|---|---|---|---|---|---|
| *A. ocellatus* | Normoxia | 9.64±2.24 | 25.91±0.20 | 6.16±0.07 | 50.82±12.32 | 10.45±6.57 | 2.61±0.45[a] | 0.031±0.004[a] |
| | Intermittent hypoxia | 8.82±2.45 | 24.96±0.29 | 0.83±0.26 | 47.14±22.32 | 7.00±3.22 | 7.11±2.65[b] | 0.040±0.005[b] |
| | Constant hypoxia | 12.07±2.45 | 25.22±0.29 | 0.9±0.31 | 58.76±18.49 | 6.15±6.16 | 5.28±1.25[b] | 0.028±0.004[ac] |
| *C. macropomum* | Normoxia | 25.02±2.27 | 25.56±0.22 | 6.36±0.1 | 45.52±15.23 | 8.85±2.49[a] | 1.09±0.32[a] | 0.010±0.001 |
| | Intermittent hypoxia | 23.62±1.94 | 25.30±0.25 | 1.0±0.17 | 59.51±15.19 | 4.54±1.33[b] | 5.16±1.29[b] | 0.009±0.001 |
| | Constant hypoxia | 20.41±1.94 | 25.90±0.22 | 0.9±0.13 | 61.21±16.69 | 4.32±2.05[b] | 4.38±0.47[b] | 0.010±0.001 |
| *P. pardalis* | Normoxia | 43.40±2.07 | 25.50±0.31 | 6.10±0.09 | 69.95±15.87 | 2.76±0.61 | 3.72±0.05[a] | 0.007±0.001 |
| | Intermittent hypoxia | 47.85±1.94 | 25.33±0.25 | 1.1±0.20 | 83.54±27.38 | 1.34±0.31 | 4.39±0.28[b] | 0.006±0.001 |
| | Constant hypoxia | 47.24±2.07 | 25.00±0.25 | 1.0±0.09 | 76.94±9.52 | 1.38±0.22 | 4.43±0.23[b] | 0.006±0.001 |

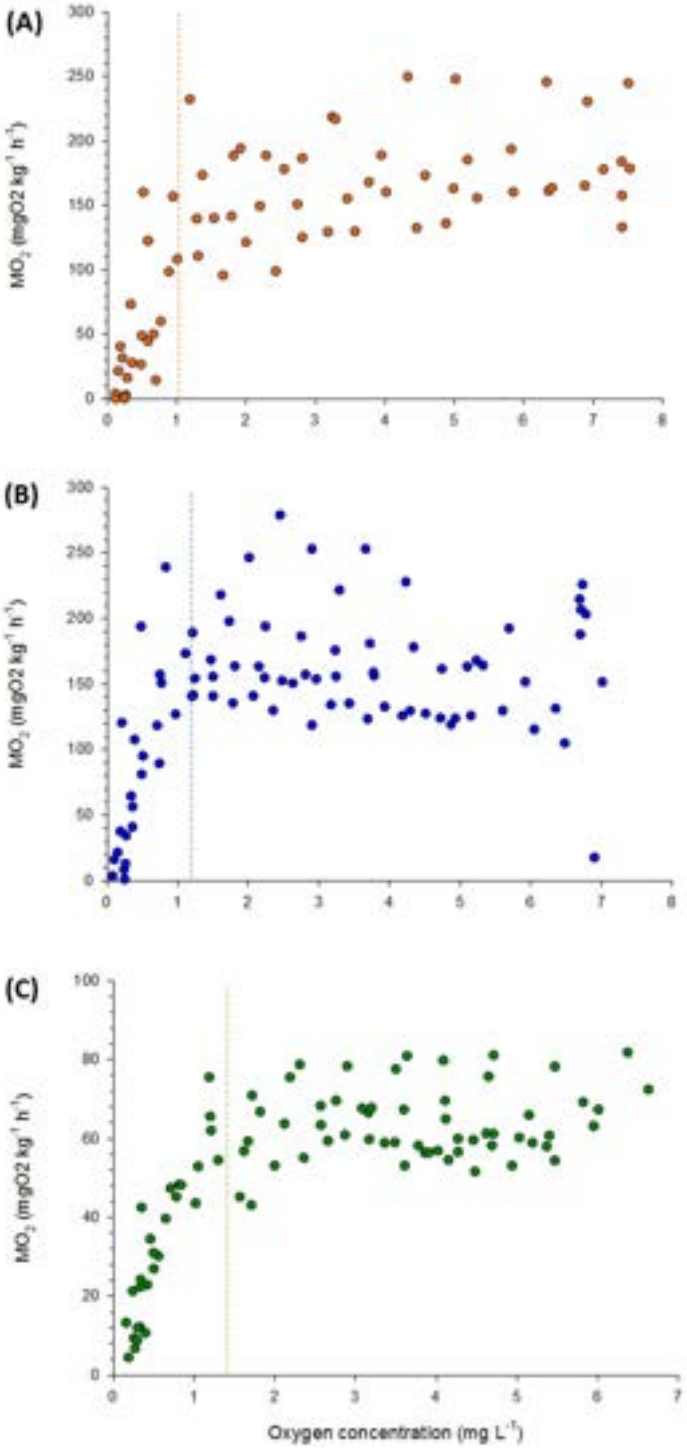


**Figure 1. Hypoxia-tolerant Amazon species have approximate $PO_{2crit}$ values.** Critical oxygen tension ($PO_{2crit}$) of the *A. ocellatus* (A), *C. macropomum* (B), and *P. pardalis* (C) under normoxic conditions (6.24 mg $O_2$ $l^{-1}$) were, respectively: (in mg $O_2$ $l^{-1}$) 1.01±0.07; 1.24±0.24 and 1.37±0.28. For the experimental conditions, 80% of the $PO_{2crit}$ for each specie was used for the constant and intermittent hypoxia exposures.

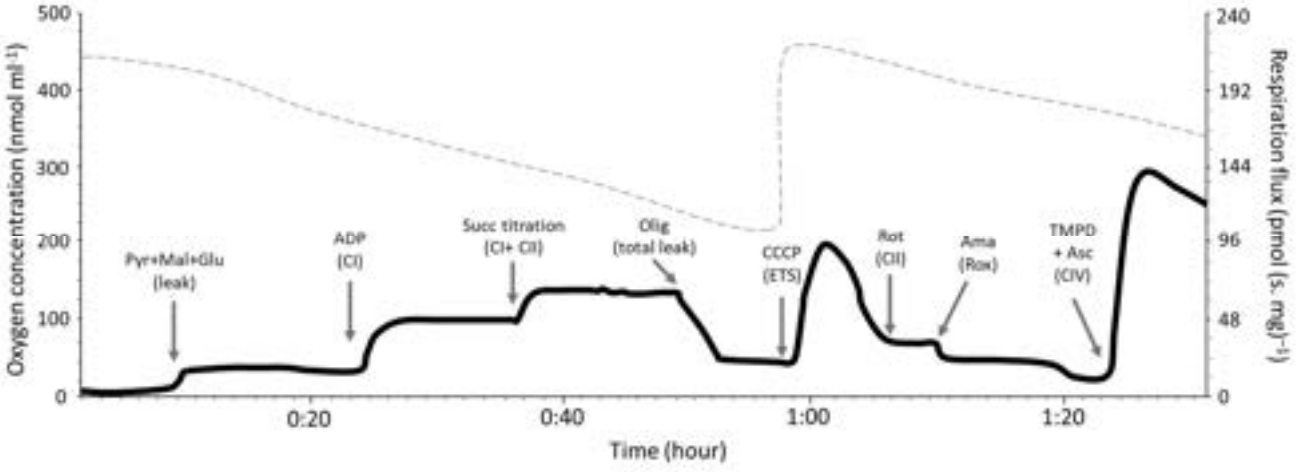


**Figure 2**. **A representative trace of a multiple substrate uncoupler inhibitor protocol (SUIT) performed in this study.** The mitochondria respiration and reactive oxygen species (ROS) emission in liver permeabilized fibers of *A. ocellatus*, *C. macropomum*, and *P. pardalis* exposed to normoxia, intermittent hypoxia, or constant hypoxia for seven days. The addition of the pyruvate (pyt), malate (mal), and glutamate (glu), made the leak respiration. The presence of the ADP stimulated oxidative phosphorylation of the complex I (CI). The oligomycin (Olig) was added to induce mitochondria in the total leak state (total leak). The respiratory electron transfer-pathway capacity (ETS) was assessed by titration of CCCP. The CII was verified by inhibiting the CI addicting the rotenone (rot), and the inhibition of the CIII was obtained by adding antimycin A (Rox). Complex IV activity was recorded, adding ascorbic acid and TMPD, passing all electrons to complex IV. ROS emission rate was concurrently measured by the fluorescent detection of resorufin, produced from mitochondrial superoxide when in the presence of superoxide dismutase, horseradish peroxidase, and Ampliflu Red. See Materials and Methods for additional details.

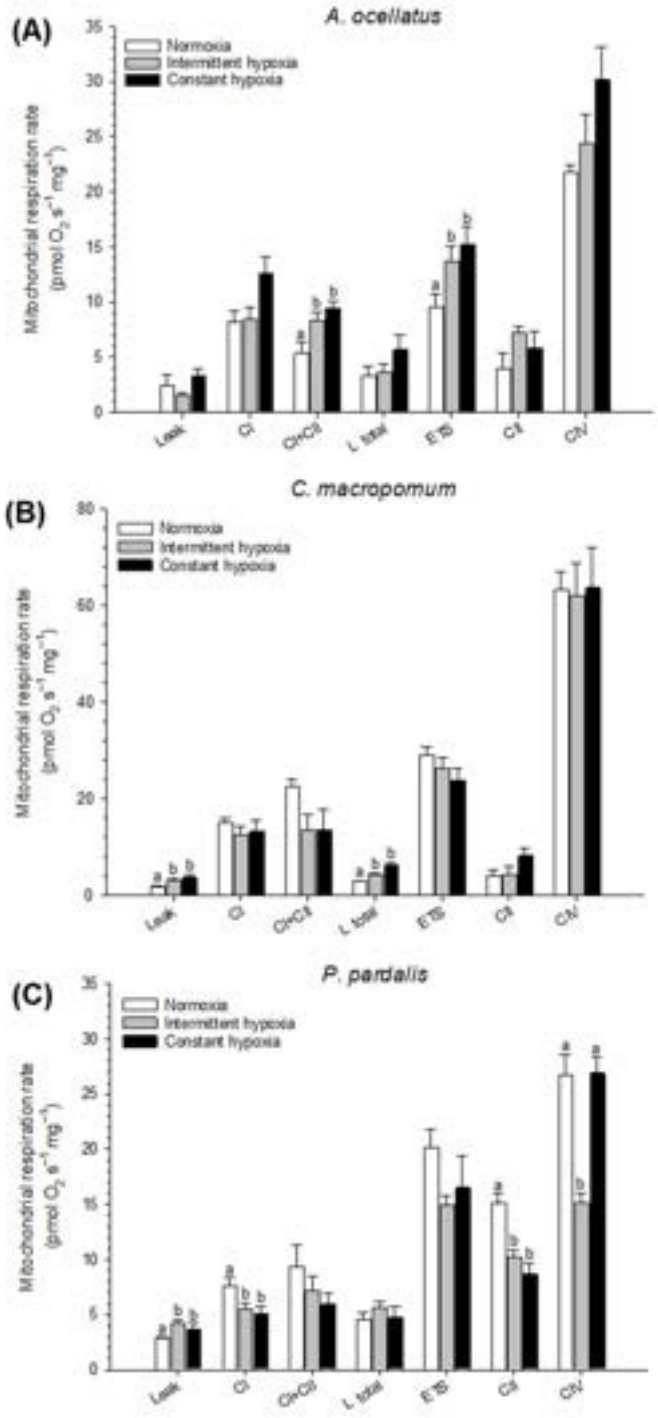


**Figure 3. Different mitochondrial mechanisms act in the determination of hypoxia tolerance in tolerant fish.** Effect of exposed to normoxia, intermittent hypoxia or constant hypoxia for seven days on leak respiration (leak), complex I (CI), complex CI+CII (CI+CII), total leak (L total), Electron transfer capacity (ETS), Complex II (CII and Complex IV (IV) of the amazon fishes *A. ocellatus* (A), *C. macropomum* (B) and *P. pardalis* (C). Different letters represent the statistical difference between different oxygen regimes ($p<0.05$).

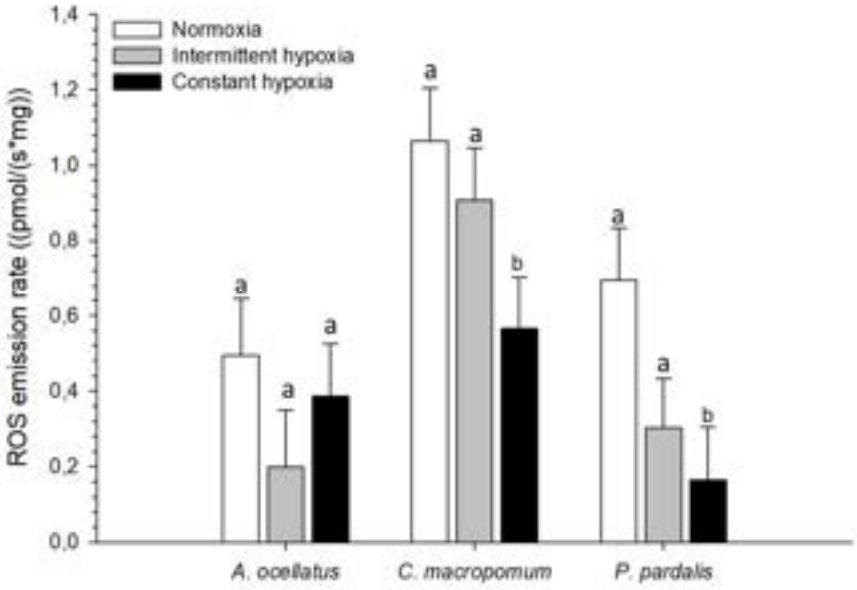


**Figure 4. Reactive oxygen species (ROS) emission from liver mitochondria was reduced by hypoxia acclimation in *C. macropomum* and *P. pardalis***. Effect of exposed to normoxia, intermittent hypoxia or constant hypoxia for seven days on mitochondrial complexes of the amazon fish *A. ocellatus* (A), *C. macropomum* (B) and *P. pardalis* (C). Different letters represent the statistical difference between different oxygen regimes ($p<0.05$).

## 6. CAPÍTULO 4

*Artigo formatado de acordo com as normas da revista *Journal of Experimental Biology.*

# Developmental phenotypic plasticity: Metabolic adjustments and mitochondrial dynamics define the hypoxia tolerance of *A. ocellatus* through ontogeny

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| **List of abbreviations** | |
|---|---|
| ASR | aquatic surface respiration |
| mt$\dot{M}O_2$ | mitochondria oxygen consumption |

| | |
|---|---|
| $\dot{M}O_2$ | oxygen consumption rates |
| CCCP | carbonyl cyanide m-chlorophyll hydrazine |
| CI | mitochondrial complex I |
| CII | mitochondrial complex II (or succinate dehydrogenase, SHD) |
| ETS | Maximum capacity of the electron transfer system |
| Oxphos | oxidative phosphorylation |
| RCR | respiratory control ratio |
| ROS | reactive oxygen species |

**Abstract**

Adults and juveniles of *Astronotus ocellatus* tend to occupy different strata of the water column when exposed to the same level of environmental hypoxia. This behavior has been associated with an increase in tolerance throughout the ontogeny of the species. Despite this, information about metabolic functioning at the level of cellular energy production in highly metabolic organs, such as the liver, is still poorly understood. The objective of the present work was to compare the hypoxia tolerance of adult and juvenile individuals of *A. ocellatus* exposed to hypoxia and reoxygenation, evaluating cellular energetic parameters, mechanisms involved in cell tolerance, and mitochondrial dynamics. Here we assessed the metabolic rate of the animals, the level of metabolites in the plasma, the mitochondrial respiration in the liver, and the cellular morphology of the hepatocytes and gills of *A. ocellatus* adults and juveniles. We observed a difference in oxygen consumption levels between adults and juveniles proportionally associated with mitochondrial respiratory rate. Furthermore, morphological changes in the liver and gills in response to hypoxia were more frequently observed in juveniles when compared to adults. Our results show that the hypoxia tolerance of *A. ocellatus* throughout ontogeny is also associated with important cellular changes that provide a consistent idea about the developmental phenotypic plasticity in this species.

## 1. Introduction

*Astronotus ocellatus* is an Amazon fish that lives mainly in lakes and at the margins of rivers, with a strong preference for lentic environments (Santos et al., 1984). Oxygen gradients are known to develop in Amazon lakes and reach concentrations very

close to zero, particularly at night. This occurs between hypoxic flooded areas and the normoxic main river, directly affecting the ability of the fish to survive (Junk et al., 1983). *Astronotus ocellatus* is a highly hypoxia-tolerant Amazon fish, and their behavior in the natural environment gives us some clues about the physiological mechanisms they use to deal with hypoxia. One of the mechanisms used by small juveniles to deal with hypoxia is aquatic surface respiration (ASR), where they swim close to the water surface to benefit from the thin layer of well-oxygenated water associated with oxygen diffusion from the air (Kramer and Mehegan, 1981; Kramer and McClure, 1982; Sloman et al., 2006). On the other hand, adults remain in hypoxic waters and avoid ASR to decrease the risk of aerial predation and predation by aquatic predators less tolerant of hypoxia (McIntyre and McCollum, 2000; Robb and Abrahams, 2003), but this necessitates either physiological or biochemical responses to enable survival.

The tolerance of *A. ocellatus* has been proposed to increase through ontogeny, where the adults show more physiological mechanisms to deal with hypoxic conditions (Almeida-Val et al., 2000; Almeida-Val et al., 1999). Some responses of *A. ocellatus* to hypoxia include a decrease in metabolic rate ($\dot{M}O_2$) (Almeida-Val et al., 1999, 2000, 2011, Chippari-Gomes et al., 2005), regulation of key genes, such as HIF1a (Vasconcelos-Lima et al., 2021; Heinrichs-Caldas et al., 2019; Baptista et al., 2016), increases in anaerobic potential and energy savings in the reduction of ion flux (De Boeck et al., 2013 Wood et al., 2007, 2009; Matey et al., 2011). However, some gaps in our understanding of the regulation of cellular metabolic functions remain, including energy production and mitochondrial redox mechanisms, especially during acute hypoxia and reoxygenation events. Despite the substantial contribution of ATP supplied by anaerobic metabolism to total metabolism during hypoxia for *A. ocellatus*, the dynamic regulation of metabolic pathways, especially in the liver, has a pivotal role in energy metabolism. Thus, mitochondrial quality control systems are essential for maintaining functional mitochondria. At the organelle level, they include mitochondrial biogenesis, fusion, and fission, to compensate for mitochondrial function, and mitophagy, for degrading damaged mitochondria (Yoo & Jung 2018).

Developmental plasticity refers to the property by which the same genotype produces distinct phenotypes depending on the environmental conditions under which development occurs (Lafuente and Beldade, 2019), such as different oxygen concentrations in the same lake. Thus, the organism can produce phenotypes adjusted to what adults experience, providing physiological adjustments to cope with environmental heterogeneity. The objective of the present work was to evaluate if the hypoxia tolerance of *A. ocellatus* throughout ontogeny is associated with metabolic adjustments and mitochondrial dynamics and to characterize the effects of this tolerance on the recovery process. We hypothesized that the adults would have more cellular mechanisms for regulating mitochondrial OXPHOS, electron transport system (ETS), redox status, and mitochondrial quality control. Thus, such cellular responses will be crucial to determine developmental plasticity in *A. ocellatus*, suggesting that they will produce phenotypes adjusted to the hypoxic conditions that adults will experience to cope with environmental heterogeneity.

## 2. Material and Methods

### *2.1 Ethical statement*

All the experimental procedures were approved by the Animal Use Ethics Committee of the Brazilian National Institute for Research of the Amazon (CEUA/INPA) (protocol number: 018/2017).

### *2.2 Animals and housing*

Adults and juveniles of *Astronotus ocellatus* were obtained from a local fish farm (Santo Antonio Fish Farm, AM-010 Km-113, Rio Preto da Eva, Amazonas, Brazil - 2.6982° S, 59.6994° W) and transferred to the Laboratory of Ecophysiology and Molecular Evolution at the National Institute of Amazonian Research (INPA) (Manaus, Amazonas, Brazil - 3.1190° S, 60.0217° W), where the experiments were carried out. In the lab, the animals were maintained indoors for at least 1 month in fiberglass tanks of 450 L (for the juveniles) or 2000 L (for the adults), supplied with continuous aeration and

flow through well water (in μmol $L^{-1}$ ; $Na^+$, 43; $Cl^-$, 31; $K^+$, 10; $Ca^{2+}$, 9; $Mg^{2+}$, 4; pH 6.0, 6.40 mg $O_2$ $L^{-1}$ and 28°C ±1). Well water was vigorously aerated prior to use to reduce dissolved $CO_2$. Throughout the acclimation period fish were fed ground dry commercial trout pellets once a day and held on a 12 h light/12 h dark photoperiod. Feeding was suspended 24 h before experiments. There was no mortality observed during the acclimation period.

### *2.3 Experimental acute exposures to hypoxia and reoxygenation (recovery)*

Groups of six adults or juveniles of *A. ocellatus* were placed individually in six glass experimental tanks that were proportional to their size (either 4 L or 60 L). They remained overnight for recovery from handling (27°C ±0.9). The next day, pairs of tanks were exposed to one of three treatments: normoxia (5.5 ± 0.5 mg $O_2$ $L^{-1}$ for 3h), hypoxia (0.7 ± 0.3 mg $O_2$ $L^{-1}$ for 3h), or recovery (3h of hypoxia at 0.7 mg/L plus 1 hour of reoxygenation ± 0.5 mg $O_2$ $L^{-1}$). The experimental design described above was performed 3 times to generate a sample size of 6 individuals per treatment (n=6) for each size. Nitrogen gas was used to decrease oxygen in the water (rate of decline was 4.36 mg $O_2$ $L^{-1}$ $hour^{-1}$), and the water surface of each aquarium was covered with a bubble plastic to reduce oxygen exchange and maintain the hypoxic condition. Following their respective exposures, animals were removed from the tanks, weighted, and blood sampled from the caudal vein with heparinized syringes and kept on ice. The blood was centrifuged at 10,000 rpm for 5 min, and the plasma was removed and frozen in liquid nitrogen and stored at -80°C for subsequent glucose and lactate analysis. The animals were then euthanized by head concussion followed by rupturing the spinal cord, and the liver and gills were collected. A piece of the liver was immediately immersed in 2 mL of ice-cold relaxing BIOPS buffer (pH 7.1), containing (in mM): CaK2EGTA 2.77, K2EGTA 7.23, Na2ATP 5.77, MgCl2·6H2O 6.56, taurine 20, imidazole 20, dithiothreitol 0.5, K-MES 50, sodium phosphocreatine 15, and sucrose 50. Another piece of the liver and the second gill arch from the right side were collected quickly, rinsed in water, and immediately fixed in cold Glutaraldehyde 2.5% in 0.1M Sodium Cacodylate Buffer, pH 7.4.

### *2.4 Respirometry*

Seven adult (230g) and seven juvenile (10g) *A. ocellatus* were randomly selected, weighed, and placed inside respirometry chambers that were proportional to their size (0.7 L or 15L). Chambers were immersed in a 100 L temperature-controlled (28 ± 0.5°C) reservoir tank in normoxia. The animals remained in this overnight to acclimate and recover from manipulation stress. After that, rates of oxygen consumption ($\dot{M}O_2$) were measured for 4 h under the following conditions: normoxia (during 4 h), hypoxia (1h of normoxia + 3h of hypoxia), and reoxygenation (3 h of hypoxia + 1 h of reoxygenation). The oxygen concentration was controlled directly in the reservoir tank where the respirometry chambers were immersed. Nitrogen gas was used to decrease and maintain the oxygen concentration in the tank (rate of decline was 4.62 mg $O_2$ $L^{-1}$ hour $^{-1}$). The surface of the water was covered with bubble plastic to optimize oxygen decay and maintain the hypoxic condition. Oxygen consumption rate measurements were measured by intermittent flow respirometry, using products and equipment from Loligo® Systems (Viborg, Denmark). The respirometry chambers were coupled with optode sensors that continuously measured the oxygen concentration inside the chamber through a Witrox 4 dissolve oxygen meter. A DAQ-M data acquisition system and AutoResp software were used for real-time analysis. The intermittent-flow respirometry cycle consisted of: a measurement period, in which the chamber was sealed and oxygen consumption by the animal was monitored; a flush period, in which the chamber was flushed and equilibrated with surrounding reservoir; and a wait period, in which the water inside the chamber was allowed to mix entirely before the measurement phase. The measurement time was determined by the time that the oxygen saturation decreased from 100% to 80% saturation for each size, and the flush time was enough to return the oxygen level to 100% saturation. Aerobic metabolic rate ($\dot{M}O_2$) was calculated as: $\dot{M}O_2 = \Delta O_2 * V_{resp} * B^{-1}$, where: $\Delta O_2$ is the rate of change in oxygen concentration (mg $O_2$ $h^{-1}$), $V_{resp}$ is the volume of the respirometry chamber, and B is the mass of the individual (kg). Here, the RMR was defined as the spontaneous activity obtained by the average oxygen uptake rate measurements in animals deprived of food for 24 hours (Chabot et al., 2016; Killen et al., 2021). The measurements were recorded after two hours from the animals were inserted into the chambers (previously defined time for handling recovery), and we considered only correlation coefficient ($r^2$) above 0.9.

### *2.5 Metabolites content measurements*

Lactate and glucose were quantified according to Callaghan et al. (2016). For both assays, plasma was diluted 1:4 (v:v) using 6% perchloric acid (PCA) and centrifuged at 10,000 g for 10 min. For lactate, the supernatant was added to a reaction media containing (in mmol $L^{-1}$: glycine 178, pH 9.2, hydrazine 148, $NAD^+$ 2.17 and 40 U $mL^{-1}$ lactate dehydrogenase). Lactate was measured by the reduction of $NAD^+$ to NADH at 340 nm and changes in absorbance were compared to a standard curve ranging from 0.0 to 2 mmol $L^{-1}$ lactate. For glucose, the supernatant was added to a reaction media containing (in mmol: Imidazole 250, $MgSO_4$ 5, ATP 10, $NADP^+$ 0.8, pH 7.8 and 40 U $mL^{-1}$ both glucose-6-phosphate dehydrogenase and hexokinase). Glucose levels were measured by the reduction of $NADP^+$ to NADPH at 340 nm and changes in absorbance were compared with a standard curve ranging from 0.005 to 2 mmol $L^{-1}$ glucose.

### *2.6 Determination of mitochondria respiration and ROS content*

Fresh liver tissue (≅ 5 mg) was removed from the BIOPS buffer and gently homogenized in 1 ml ice-cold BIOPS along with 50 µg $ml^{-1}$ saponin. After 30 min, the liver was washed thrice for 10 min in 2 mL of modified mitochondrial respiratory medium MiRO5 buffer (pH 7.2), containing (in mM): EGTA 0.5, $MgCl_2 \cdot 6H_2O$ 3, K-lactobionate 60, taurine 20, $KH_2PO_4$ 10, HEPES 20, sucrose 160 and 1 g/l BSA, essentially fatty acid free, at 25 °C (Gnaiger et al., 2000). The hepatic tissue was blotted dry on filter paper, weighed, and immediately immersed into oroboros respiration chambers filled with 2 mL of MiRO5. Mitochondrial physiology was assessed using high-resolution respirometry and fluorometry (Oxygraph-2k with O2k-Fluorescence module, Oroboros Instruments, Innsbruck, Austria). Oxygen was added to the gas phase above the media before closing chambers to maintain supersaturated levels inside the chamber. Complex I (CI) substrates (in mM) malate 5, glutamate10, and pyruvate 5) were added to measure state II respiration through CI in the absence of ADP (denoted 'Leak I'). Excess ADP (2.5 mM) stimulated oxidative phosphorylation (OXP-I, state III respiration). Phosphorylating respiration with CI and complex II (CI+II) substrates (OXP-I, II) was measured by the addition of succinate (10 mM). Oligomycin (Oli) was added to induce mitochondria into total leak state (Ltotal). Then, the mitochondria were chemically uncoupled with the titration of CCCP carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone (FCCP, 0.5 µM) to determine maximum electron transport system capacity (denoted 'ETSmax'). Complex

IV (CIV) activity was recorded by the addition of 5 mM ascorbic acid and TMPD, passing all electrons to CIV. RCR was calculated as ETS/leak state. Oxygen uptake and ROS production were simultaneously measured in the same cell preparation using the Oroboros Oxygraph and DatLab 2 software (Oroboros Instruments GmbH, Innsbruck, Austria). Superoxide dismutase (SOD; at 22.5 U $mL^{-1}$) was added to catalyze the reaction of the superoxide produced by the mitochondria and horseradish peroxidase (3 U $mL^{-1}$) was added to catalyze the reaction of hydrogen peroxide with Ampliflu Red (15 µM) and produce the fluorescent product resorufin (detected using an excitation wavelength of 525 nm and amperometric filter set (AmR); Oroboros Instruments). The resorufin signal was calibrated with additions of exogenous hydrogen peroxide.

### *2.7 Transmission electron micrographs in liver and gills*

After being kept in the fixative buffer for 24 h, the small pieces of liver and gill were washed in 0.1 M sodium cacodylate buffer (3 times for 10 min each at pH 7.2). Tissue fragments were post-fixed with 1% osmium tetroxide in the same buffer for 2 h, washed, and subjected to block contrast using 5% aqueous uranyl acetate for 2 h at 4 °C. The samples were then dehydrated in ascending concentrations of ethanol from 30 to 100 %. The tissue fragments were gradually embedded in resin using increasing proportions of propylene resin every 24 h (2:1; 1:1; 1:2, before being embedded in Durcupan-ACM Fluka© resin). The resin blocks were cut to a thickness of 70 nm using an ultramicrotome. To intensify the contrast of the ultrathin slices, the grids were treated with 5% uranyl acetate for 50 min and counterstained with lead citrate for 5 min. The ultrastructures were visualized at HV=80kV using a transmission electron microscope (JEM 1400 Flash, JEOL BRASIL Instrumentos Cientificos Ltda) and high magnification images acquired using an integrated high-sensitivity camera (Câmera AMT 43 Megapixel Bottom Mount).

### *2.8 Data and statistical Analyses*

All data are reported as mean ± S.E.M. (n=6). A two-way ANOVA was used to evaluate the effect of exposure to acute hypoxia and reoxygenation (recovery) on the two sizes of *A. ocellatus*, using the following factors: oxygen concentration and size. Significant differences were determined using a Tukey post hoc test. When ANOVA

assumptions were violated, a non-parametric Fridman test was applied. A one-way ANOVA was applied to assess the Metabolic Rate Depression ($\dot{M}O_2$ in normoxia/ $\dot{M}O_2$in hypoxia), considering the two different sizes as factors, and a Tukey post hoc test was used. All statistical analyses and graphics employed Sigma Stat and Sigma Plot software (Jandel Scientific, San Jose, U.S.A.).

## 3. Results

### *3.1 Metabolic rate of adult and juvenile exposed to hypoxia and reoxygenation*

The routine metabolic rate (RMR) in normoxia showed that the aerobic energy requirement of adults was 2.34 times lower than the juveniles of *A. ocellatus*, and the same trend was observed between sizes in the hypoxia and recovery treatments ($F=130.65$; $p=<0.001$) (Figure 1A). In addition, hypoxia decreased the $\dot{M}O_2$ for both sizes ($F=15.461$; $p=<0.001$), although reoxygenation was effective only for juvenile fish; adults completely recover their RMR after 1 h of recovery (Figure 1 A). Furthermore, the metabolic rates depression did not show a statistical difference between of the intensity of $\dot{M}O_2$ reduction during hypoxia, despite a certain trend that this was more intense for smaller fish ($F=3.943$; $p=0.073$) (Figure 1B).

### *3.2 Glucose and lactate levels in plasma*

There were no differences in plasma glucose levels between the two sizes ($F=0.444$; $p=0.509$). However, for both adults and juveniles *A. ocellatus*, there was an increase in plasma glucose levels during hypoxia that, even after 1h of reoxygenation, were kept significantly elevated compared to animals in normoxia ($F=10.978$; $p=<0.001$) (Figure 2A). The increase in lactate levels shows that the anaerobic metabolism was activated during hypoxia for both sizes (Figure 2B), although juvenile fish had comparatively much higher levels than adults during hypoxic exposures ($F=14.595$; $p=<0.001$). After 1 h of reoxygenation, lactate levels in juveniles significantly decreased compared to those measured under hypoxia, but they did recover to those measured under normoxia (Figure 2B). In adult *A. ocellatus*, lactate levels still high even after 1 h of reoxygenation, that anaerobic metabolism was maintained (Figure 2B).

### *3.3 Mitochondrial respiration rate and ROS content*

The hypoxia exposure increased leak respiration for both sizes of *A. ocellatus* (F=3.265; p=0.049), indicating that low oxygen concentrations can induce uncoupling mechanisms (Figure 3A). Furthermore, the mitochondrial respiration rate showed that after 1 h of reoxygenation, animals from both sizes could recover leak state to normoxic levels (Figure 3A). At the same time, the respiratory control ratio (RCR) is lower in adult *A. ocellatus* in normoxia, expressing that their mitochondria are uncoupled relative to juveniles under these conditions (F=5.020; p=0.003) (Figure 3G). Hypoxia decreased the RCR for juveniles, and even after 1h of reoxygenation, it did not return to normoxic levels (F=7.123; p=0.003). In contrast, adults showed no change in RCR under any treatment condition (Figure 3G). Mitochondrial ROS content was higher in juveniles compared with adults under normoxic conditions (Figure 3H). Both fish sizes decreased ROS production during hypoxia and could not restore to normoxic levels after 1 h of reoxygenation (F=6.139; p=0.006) (Figure 3H).Adults of *A. ocellatus* showed significantly lower respiration in CI+CII during normoxia, hypoxia, and recovery treatments in comparison with juveniles in the same conditions (F=48.575; p=<0.001) (Figure 3C). In addition, the respiration of CI+CII in the juveniles decreased in hypoxic exposures, although it was restored after 1h of reoxygenation (F=3.342; p=0.049). For adult fish, the treatments did not affect CI+CII respiration (Figure 3C). Adults also showed lower levels of stimulation of maximum electron flux (ETS respiration) during normoxia and reoxygenation compared with juveniles (F=49.806; p=<0.001). For juveniles, hypoxia exposure decreased the ETS, suggesting a lower dissipate the energy of $H^+$, and this effect persisted even after reoxygenation (F=10.424; p=<0.001). No alterations were observed in the ETS for adults of *A. ocellatus* (Figure 3E). In addition, adult fish showed rates of electron flow through CIV (cytochrome c oxidase) around three fold lower than juveniles (F=52.968; p=<0.001) (Figure 3F). Hypoxia exposure had no effects on CIV respiration for either size class of fish (F=0.0930; p=0.911) (Figure 3F).

### *3.4 Liver and gill morphology*

Mitochondrial morphology was investigated qualitatively for both liver and gill tissues (n=3). Hepatocyte mitochondria were more uniformly spheres or ovoids in normoxic conditions for both size classes of fish (Figure 4A and D), while long filaments

were noted in hypoxia (Figure 4B and E). After 1 h of reoxygenation, the mitochondria appeared to return to ovoid shape in juveniles, while for adults, the long filament morphology persisted (Figure 4C and F). Qualitatively, mitochondrial density under normoxia seems to be higher in juveniles than in adults (Figure 4A and D). Density appears to decrease after 3 h of hypoxia in both fish sizes (Figure 4B and E) and returns to normal levels after reoxygenation (Figure 4A and D). For adults and juveniles, a large amount of rough endoplasmic reticulum (RER) was observed around the mitochondria during normoxia and reoxygenation (Figure 4A and C). During reoxygenation, many mitochondria were often observed near the cell nucleus in adults and juveniles of *A. ocellatus* (Figure 4C). For the gills, the mitochondria density was also lower in adults of *A. ocellatus* when compared to juveniles in normoxic exposures (Figure 5A and D). Gill mitochondrial density also decreased after 3h of hypoxia, although it was reversed during normoxic recovery for both sizes (Figure 5). While for juveniles, the pavement cells (PVCs) covered mitochondria-rich cells (MRCs) during the hypoxia exposure, the adult individuals analyzed here seemed to have comparatively less exposed MRC crests even during normoxia. This response is potentiated during hypoxia (Figure 5D and E).

## 4. Discussion

### *4.1 Metabolic depression as an indicator of hypoxia tolerance throughout ontogeny*

Size-specific metabolic adjustments were observed for adults and juveniles of *A. ocellatus* under the same hypoxic challenge. Although hypoxia caused a metabolic depression in the juveniles, they completely recovered their RMR after 1h of reoxygenation, demonstrating that, once oxygen concentrations were reestablished, there was no need to resort to anaerobic metabolism. In contrast, adults of *A. ocellatus* showed a significantly lower RMR than the juveniles exposed to both normoxia and hypoxia, revealing the ability of the large fish to reduce ATP requirements. Indeed, this idea can be supported by this fish specie behavior in natural environments. The juveniles of *A. ocellatus* usually increase their activity by going closer to the water surface to search for oxygen-rich areas to avoid hypoxia. While the adults are often found in hypoxic or even anoxic environments, exhibiting a lower oxygen dependence (Sloman et al., 2006). Both

adults and juveniles had similar rates of metabolic depression (Figure 1B), despite the trend towards a smaller decline for adults (p=0.073). This shows that, although oxygen consumption decline rates are similar, the aerobic dependence scale is very different, indicating that the adults don't need to produce as much ATP per gram of body mass.

The plasma glucose and lactate increased during hypoxia and could not return to normal levels after 1h of reoxygenation, suggesting that both fish sizes increased the anaerobic metabolism demand. Increased plasma glucose probably means an effective mobilization of glycogen stored in the liver due to activation of hepatic glycogenolysis, releasing glucose which was probably channeled to anaerobic glycolysis, a strategy commonly used by this species when exposed to hypoxia (Baptista et al., 2016; Chippari-Gomes et al., 2005; Heinrichs-Caldas et al., 2019; Li et al., 2018; Richards et al., 2007). Furthermore, the increase in lactate levels in the same conditions reinforces the idea of a higher anaerobic metabolism since lactate is a product of anaerobic glycolysis metabolism. Our results showed that the juveniles accumulate almost exactly twice as much lactate when compared with the adults and, interestingly, the same pattern was observed for $\dot{M}O_2$, which are roughly twice as high in juveniles than adults. Thus, the juveniles accumulate more lactate because their ATP demand is double that of the adults and this is the reason why they are more sensitive to hypoxia.

Plasma metabolites matched the $\dot{M}O_2$ results of both sizes. In juveniles, the lower metabolic rate in hypoxia corresponded to a compensatory increase of anaerobic metabolisms, such as observed by the high glucose and lactate values. On the other hand, the small fish recover the $\dot{M}O_2$ to the RMR rates after the reoxygenation. In addition, the juveniles were able to reduce blood lactate levels from about 11 mM to about five mM in just 1hour of reoxygenation. This shows a gradual degradation of lactate, either by tissues like the heart or lactate uptake and gluconeogenesis/glycogen resynthesize in the liver. At the same time, although juveniles return to RMR levels during reoxygenation, the decreased rates of RCRs could indicate less production of ATP, and then, some anaerobic metabolism can be required. The RCR values implication will be better discussed later.

However, within the hypoxia exposures, the low lactate values for adults in comparison with the juveniles bring the idea that during the ontogeny, the adults have a combined strategy of a low rate of oxygen consumption, a low requirement for aerobic metabolism, and a high potential for anaerobic metabolism. As for juveniles, since they could accumulate lactate to a level proportional to their metabolic rate, they may have an equally high potential for anaerobic metabolism. Thus, it is just the low RMR and associated low ATP demand that allows the adults to survive longer due to the ability to take longer to accumulate anaerobic end products (e.g., lactate, $H^+$) and consume their glucose/glycogen. Thus, the adults of *A. ocellatus* can activate a hypometabolism during hypoxia, as previously postulated by Driedzic et al (2021), and sustain a more pronounced effect of size on hypoxia, even anoxic, survival time. Indeed, Almeida-Val et al. (2000) found that survival time in severe hypoxia increased with size in this species, from 5 h in 5 g fish to 50 h in 350 g individuals, a scaling exponent of 0.59. Thus, unlike many other species, the *A. ocellatus* shows a positive relationship between physiological tolerance to hypoxia and size. Large *A. ocellatus* was able to withstanding the effects of falling $PO_2$ better than juveniles, supported by a more significant difference anaerobic potential to allow prolonged survival in extreme hypoxia.

### *4.2 Mitochondria oxygen consumption during hypoxia is fish size-dependent*

In the present work the hypoxia exposure lowed the oxygen consumption of adults and juveniles of *A. ocellatus* to cope with limited aerobic ATP production while maintaining vital physiological functions. This strategy requires a fine tuned regulation of metabolic pathways, especially in the liver, which has a pivotal role in energy metabolism. Leak respiration allows the oxygen flow necessary to drive $H^+$ through the mtIM redox pumps to maintain a steady-state proton motive force in the absence of ADP phosphorylation to ATP. Proton leakage rates increased to almost double after 3h of exposure to hypoxia in both sizes, indicating increased inner membrane permeability, which was recovered after 1h of reoxygenation. Although for juveniles, this resulted in a measurable depression of the RCR, which persisted during reoxygenation, suggesting that increased oxygen flow rates are necessary to maintain mitochondrial membrane potentials. The RCR has been used to express coupling in living cells and isolated mitochondria (Gnaiger 2020). These depressed RCR indicates increased decoupling of mitochondria, which should decrease phosphorylation efficiency and capacity. C

Considering that juvenile fish are more aerobic than adults, deficiencies in Oxphos are consistent with the hypoxia response observed for *A. ocellatus* juveniles. After 1h of reoxygenation, despite the juveniles having the same level of $O_2$ consumption that those in normoxia, the decreased RCR values indicate that their mitochondria are uncoupled and, then, producing fewer ATP per unit $O_2$. Such results can be associated with the lactate levels dropping by half under reoxygenation, indicating that, although the juveniles are primarily aerobic, some anaerobic activity was still being maintained once the mitochondrial uncoupling probably decreased the aerobic efficiency. On the other hand, for adults of *A. ocellatus*, hypoxia did not cause changes in the RCR. However, the levels of mitochondrial coupling rates are significantly lower in adult animals, consistent with the drop in the aerobic ATP synthesis capacity observed in adults even in normoxia and greater control of the Oxphos ability of this animal.

An increase in mitochondrial uncoupling constrains oxidative phosphorylation, often reducing energy generation by up to 40%, but it also limits oxidative cell injury by decreasing reactive oxygen species (ROS) production (Brokes 2005; Baffy 2017). Hypoxia can result in the elevated production of ROS due to electron slip from the partially reduced upstream electron transport system (ETS) complexes (Solaini et al. 2010; Cadenas 2018) and/or reverse electron flow through mitochondrial Complex I (Chouchani et al. 2014). ROS can oxidize and damage mitochondrial membranes, as well as ETS and tricarboxylic acid cycle (TCA) enzymes, leading to OXPHOS suppression and ROS overproduction (Aragones et al. 2009; Hernansanz-Agustın et al. 2014). Our results revealed the adults produce fewer ROS then the juveniles, and this lower production may be due to mitochondria of the adults of *A. ocellatus* are naturally uncoupled (low RCR values) relative to the juveniles, suggesting a fewer electrons 'backing up' in the system. In contrast, the juveniles show coupled mitochondria and high ROS levels during normoxia, which let us think that their high metabolic rate made them unable to afford to reduce the efficiency of their OXPHOS and compromise ATP production, such as observed for the adults.

The reduction of succinate dehydrogenase activity observed in juveniles after the hypoxic exposure would diminish the contribution of succinate-CoA ligase to ATP

production via substrate-level phosphorylation and limit electron flow through Complex II (Gnaiger 2020). On the other hand, the gradual increase in CII respiration seen during reoxygenation may be protective against reoxygenation injuries in this size of *A. ocellatus*. Mitochondrial ROS production occurs early in reperfusion (Eltzschig, & Eckle 2011, Chouchani et al., 2013; Zweier et al 1987; Chouchani et al., 2014) so increasing electron flow through CII should help to oxidize metabolites fuelling ROS production. This may also explain why CI capacity of juveniles was not altered during the hypoxia. The lack of alterations in CI+CII in adults may demonstrate that they have better control mechanisms to deal with the low oxygen concentration tested in the present work.

In addition to the lack of change in CI+CII, hypoxia exposure did not affect either the maximum flow of electrons (ETS) or the capacity of cytochrome c oxidase (CIV) in mitochondria of adult *A. ocellatus*, indicating that, at this hypoxia levels, the mitochondria still have the same capacity to produce ATP aerobically after 3h of hypoxia. Thus, the depression of ATP turnover is probably substantial in adults during hypoxia, as observed by the low $\dot{M}O_2$ but is likely not as dramatic as the pronounced decline in aerobic metabolism, suggesting some mitochondrial mechanism for maintaining a mitochondrial affinity for oxygen at a controlled rate, at least during acute 3-h exposure to hypoxia. On the other hand, the ETS decreased juveniles in hypoxia, and the normoxic levels could not be recovered after 1h of reoxygenation, suggesting a higher oxygen dependence for the juveniles. These results were reinforced by the higher mt$\dot{M}O_2$ in juveniles, as observed by the electron flow through CIV (cytochrome c oxidase). Our results show that for the adults of *A. ocellatus*, ATP supply from anaerobic metabolism may make a substantial contribution to total metabolism during hypoxia. At the same time, the juveniles are more aerobic dependent, despite showing refined mitochondrial mechanisms to deal with hypoxia.

### *4.3 Mitochondrial morphology is controlled by metabolism*

Mitochondrial dynamics regulate mitochondrial network connectivity, which depends on the specific metabolic needs of the cell (Miettinen and Björklund, 2017; Rambold and Pearce, 2017). Changes in mitochondrial shape and size, for example, can be associated with the fission and fusion of the mitochondria according to the cell's needs (Trotta and Chipuk, 2017). In the present work, we observed changes in hepatocyte

mitochondria from ovoid forms in normoxia to long filaments after 3h of hypoxia in both size classes of *A. ocellatus*. These morphological alterations can be related to a high fusion between mitochondria, mitigating the effects of hypoxia damage through exchanging proteins and lipids with other mitochondria, thus maximizing the oxidative capacity. Therefore, the fusion allows functional mitochondria to complement dysfunctional mitochondria by diffusion and sharing components between organelles Youle & Van der Bliek 2012). As the aerobic metabolism ($\dot{M}O_2$) of juveniles was rapidly restored after reoxygenation, mitochondrial fission events likely occurred, restoring the oval shape of liver mitochondria. At the same time, adults maintained a more significant number of hepatic mitochondria of elongated shape after the reoxygenation, indicating a sustainable fusion mechanism even after the reestablished the oxygen concentrations.

Adults of *A. ocellatus* also showed lower mitochondria density than the juveniles, which can be associated with the lower mt$\dot{M}O_2$ observed for the larger fish. At the same time, both size classes of animals decreased mitochondrial density after 3h of hypoxia, suggesting that mitochondria quality control (MQC) mechanisms were activated, such as the fusion events mentioned above, or in cases where dysfunctional mitochondria goes beyond a certain threshold, complete degradation by mitophagy. The mitophagy events found in hepatocytes of the juveniles after 3h of hypoxia may reflect a compensatory mechanism for protecting organisms against oxidative damage, as suggested by Lu et al. (2016). Furthermore, the decrease in contact between the ER and mitochondria observed for both adults and juveniles during hypoxia provides some ideas about regulating cell physiology. According to Rowland & Voeltz (2012), such interaction also can indicate an increase in biosynthesis and lipid transfer between these two membranes to maintain the ratios of phospholipids found in each of these organelles. Thus, the decrease in mitochondria membrane lipid environment could compensate, at least in part, the lower energy production during the hypoxia exposure. In addition, the interaction between the ER and mitochondria also controls mitochondrial biogenesis (Csordás et al. 2006). Therefore, the increase in such interactions after reoxygenation can be related to the new mitochondria observed mainly in the juveniles.

Noteworthy, during normoxia and, mainly after one h of reoxygenation, the mitochondria of both sizes of *A. ocellatus* were observed predominantly localized in the central part of the cell, close to the nucleus. At the same time, the mitochondria from the juveniles exposed to hypoxia were frequently found close to the cell membrane. It can be explained by the low availability of dissolved oxygen in those areas of hepatocytes distant from the cytoplasmic membrane. This same pattern of mitochondrial configuration is observed in icefish, where the loss of Mb and Hb increases the mitochondria density close to the lipid membrane due to the enhanced fraction of oxygen supply that occurs through higher diffusive oxygen flux (O'Brien1 & Mueller 2010). Some regions of higher electron density were observed in hepatocyte nuclei in both size classes of *A. ocellatus* after 1h of hypoxia recovery, suggesting that chromatin became condensed. This process is associated with enhanced transcription rates, increasing protein synthesis as previously observed for this species (Cassidy et al., 2018; Lewis et al., 2007). This may compensate for reductions in protein synthesis that occur during hypoxia as part of the animal's strategy to decrease energy demand and reduce $\dot{M}O_2$.

Gill tissue was also marked by reduced mitochondrial density after hypoxia exposure for both size classes of *A. ocellatus*. Also, there was a reduction in mitochondria-rich cells (MRCs) for both sizes, which was also previously observed by Matey et al. (2011), suggesting that mitochondrial fusion also occurred in the gill cells. In addition, fewer mitochondria were observed for adults of *A. ocellatus* than juveniles under normoxic conditions, reinforcing the idea that gill metabolic demand is also lower in large fish. We also observed an increase in pavement cells (PVC) over MRCs during hypoxia exposure for both sizes, decreasing the superficial area of the MRCs and preventing the loss of ions. In addition, after the reoxygenation, the juveniles showed an increase in the regions of close contact between the ER and mitochondrial membranes. This could be related to $Ca^{2+}$, as this element is released from the ER to mitochondria at such contact sites. This seems to be essential for mitochondrial function, division, and regulation of apoptosis (Rizzuto et al., 1993). Thus, the re-established of the oxygen can stimulate the mitochondrial division by changes in $Ca^{2+}$ concentrations in gills epithelium besides that some of the factors that are found at ER-mitochondria contact sites and are required for proper mitochondrial morphology are regulated by $Ca^{2+}$ binding (Rowland & Voeltz, 2012).

## 5. Conclusion

In the present work, adults and juveniles of *A. ocellatus* produce distinct physiological adjustments when exposed to the same hypoxic challenge. Our data show that juveniles accumulate almost twice as much lactate as adults. At the same time, a similar pattern is observed in the $\dot{M}O_2$, which are roughly twice as high in juveniles than adults, showing that the juveniles accumulate more lactate because their ATP demand is double that of the adults. This explains at least part, why they are more sensitive to hypoxia. The $\dot{M}O_2$of the adults have a much slower metabolism under all conditions, so $O_2$ demand is lower, indicating that the adults of *A. ocellatus* need less ATP overall, so they do not need to resort to anaerobic metabolism and accumulate lactate as a result. In addition, due to the high $\dot{M}O_2$of the juveniles, they could afford to reduce the efficiency of their OXPHOS and compromise ATP production, making this another reason why the juveniles are more sensitive to hypoxia. Although the juveniles were able to accumulate lactate to a level proportional to their metabolic rate and probably have an equally high potential for anaerobic metabolism, the low RMR and associated low ATP demand in adults allows them to survive longer in hypoxia due they take longer to accumulate anaerobic end-products and consume their glucose/glycogen. In addition, the high tolerance of adults also is due to the low RCR levels, indicating that uncoupled mitochondria relative to the juveniles also show low ROS levels by the fewer electrons backing up in the system. Thus, the mitochondria physiology of the *A. ocellatus* revealed that the adults with remarkable hypoxia tolerance rely instead on low $O_2$ demands. At the same time, the mitochondrial redistribution within the mitochondria affords the increase of the density of this organelle to moving to close to the membrane to enhance cellular $O_2$ uptake. This process was combined with mitochondria fusion and fission to sustain the low-energy cellular demands arising from hypoxia for both sizes, particularly juveniles. Our findings bring the concept that *A. ocellatus* can modify their physiological responses through ontogeny to adjust to the conditions that adults will experience, defining developmental plasticity that can provide the means to cope with the heterogeneity of the Amazon environments.

## Competing interests

The authors declare no competing or financial interests.

**Acknowledgments**

The authors are grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Amazonas (FAPEAM), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) by supporting INCT/ADAPTA (CAPES – Finance Code 001; CNPQ process #465540/2014-7; FAPEAM process #062.01187/2017). SBM is the recipient of a doctoral fellowship from CAPES. TJM is supported by a New Brunswick Environmental Trust Fund grant. ALV and VMFAV were supported by INCT ADAPTA (FAPEAM/CNPq/CAPES), and both received a research fellowship from CNPq.

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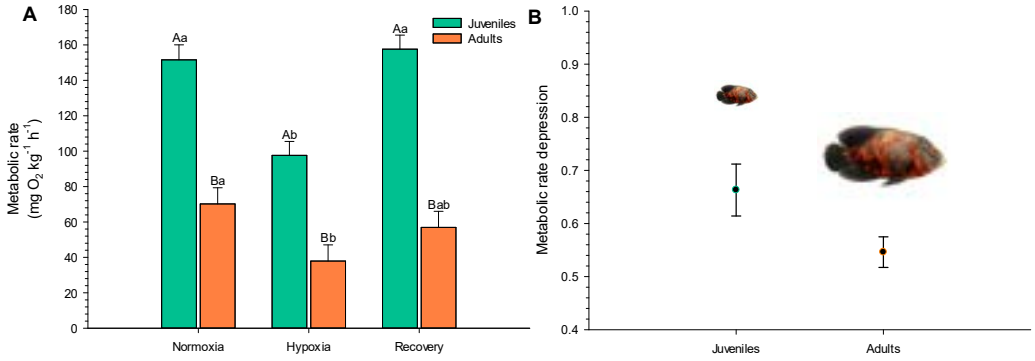


**Figure 1. Adults (orange bars) of *A. ocellatus* have a lower metabolic rate routine than juveniles (green bars), although the metabolic rate of depression has not changed**. (A) The metabolic rate ($\dot{M}O_2$) was measured in both sizes (230g and 10g) of *A. ocellatus* after being exposed for 3 h to one of the three treatments: normoxia, hypoxia (0.7 mg $L^{-1}$), and recovery (3h of hypoxia followed by 1 h of reoxygenation). Uppercase letters indicate a statistical difference between adult and juvenile fish within the same treatment, while lowercase letters indicate a significant difference between treatments within the same size (p=0.05). Both sizes reduced RMR in hypoxia, but juveniles could restore RMR after 1 h of reoxygenation, while for adults, 1h was not enough time to regain their RMR levels. (B) Metabolic rate depression ($\dot{M}O_2$ in normoxia/ $\dot{M}O_2$in hypoxia) in hypoxia exposure for both sizes of *A. ocellatus*. despite the trend there was no difference in the Metabolic rate depression between the two sizes (F=3.943; p=0.073).

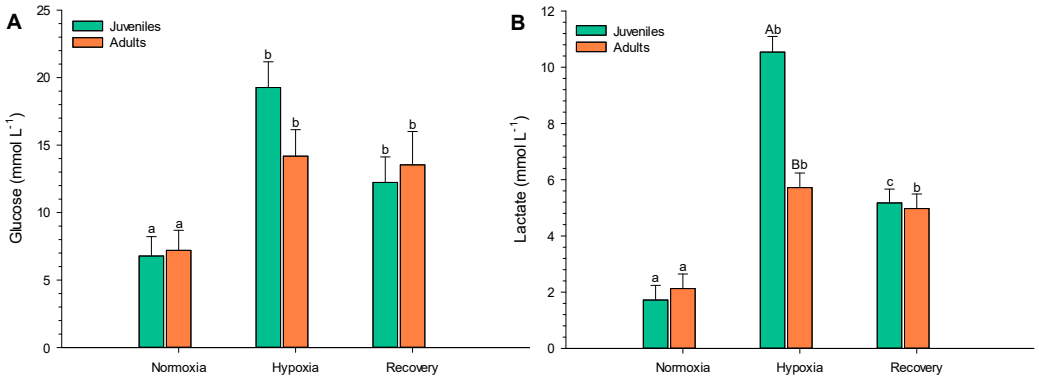


**Figure 2. Hypoxia increased glucose and lactate levels in both sizes of *A. ocellatus*, and 1h of reoxygenation was not time enough to recover.** (A) Plasma glucose levels and (B) Plasma lactate levels were measured in both sizes (230g and 10g) of *A. ocellatus* after being exposed for 3 h to one of the three treatments: normoxia, hypoxia (0.7 mg $L^{-1}$), and recovery (3h of hypoxia followed by 1 h of reoxygenation). Uppercase letters indicate a statistical difference between adult and juvenile fish within the same treatment, while lowercase letters indicate a significant difference between treatments within the same size (p=0.05).

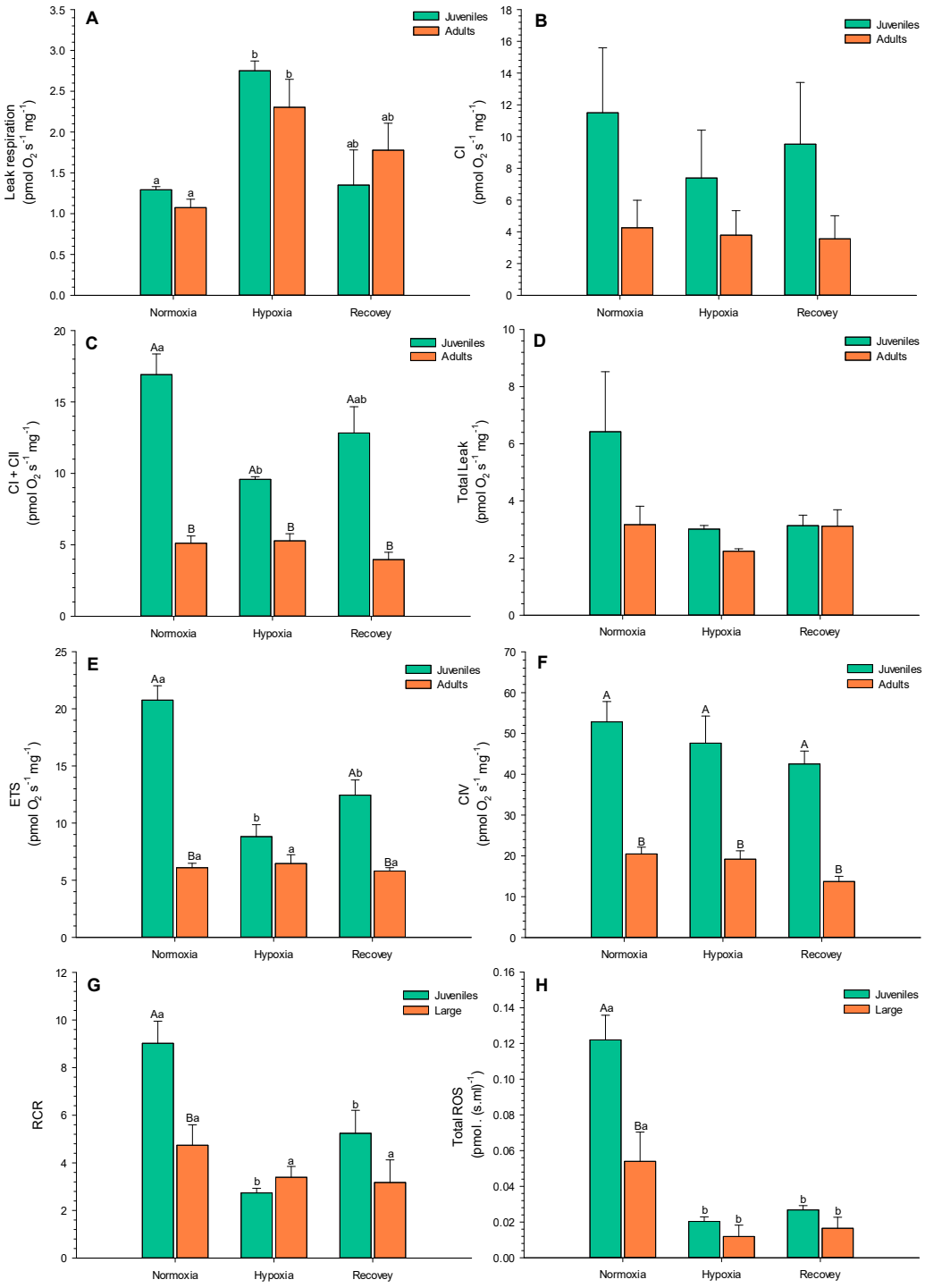


**Figure 3. The mitochondria of the *A. ocellatus* adults were slightly affected by hypoxia and showed a lower oxygen consumption, naturally uncoupled mechanism, and lower ROS content than the juveniles.** Mitochondrial respiration rate of (A) Leak respiration, (B) Complex I (CI), (C) Complexes I and II (CI+ CII), (D) Total leak, (E) Electron transfer-system capacity (ETS), (F) the electron flow through cytochrome c oxidase (CIV) and (G) Respiratory control rates (RCR) and (H) Ros content in

permeabilized liver of *Astrontous ocelattus* adults and juveniles (230g and 10g) exposed for 3 h to one of the three treatments: normoxia, hypoxia (0.7 mg $L^{-1}$), and recovery (3h of hypoxia followed by 1 h of reoxygenation). Uppercase letters indicate a statistical difference between adult and juvenile fish within the same treatment, while lowercase letters indicate a significant difference between treatments within the same size (p=0.05).

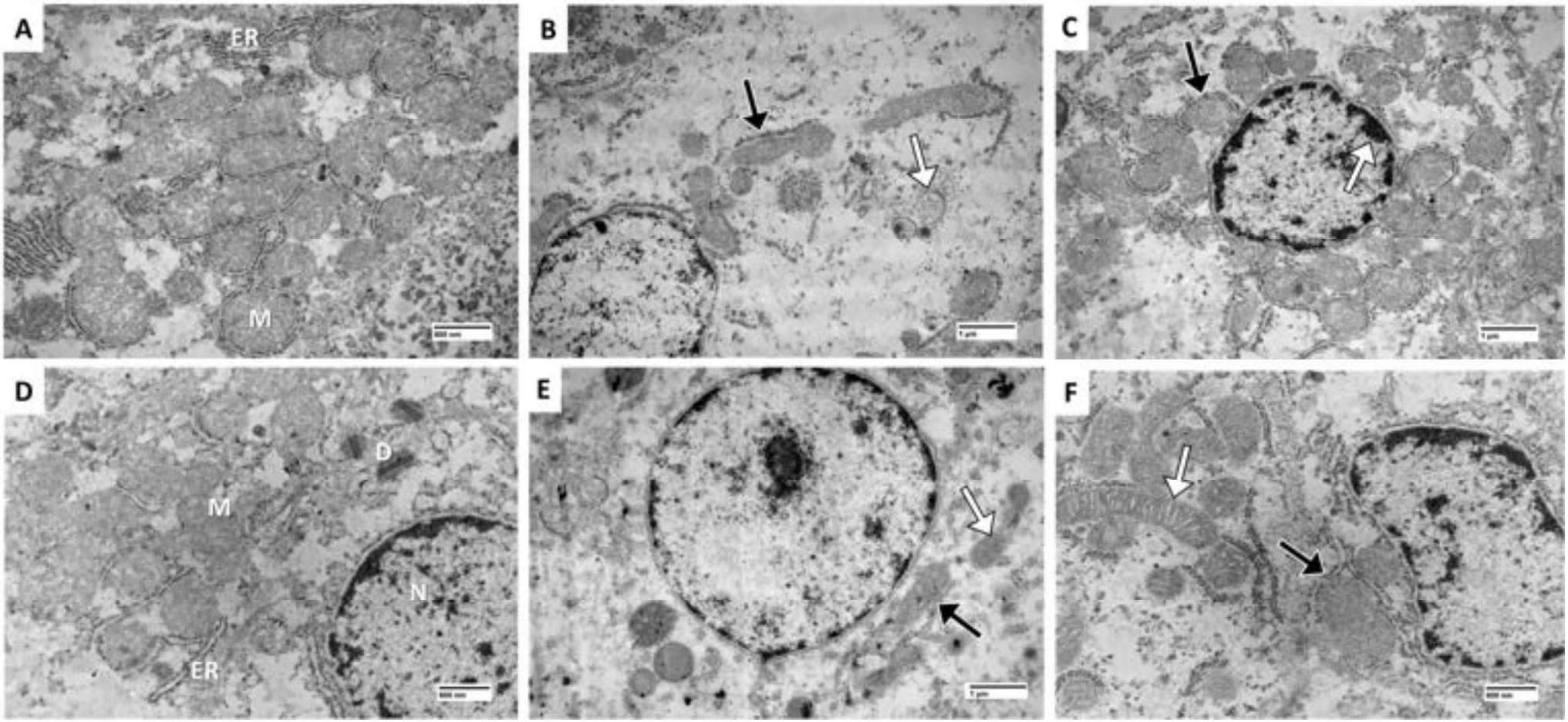
A
ER
M
B
C
D
D
M
N
ER
E
F

**Figure 4. The hepatocyte mitochondria changed from ovoid forms for long filaments suggesting an increase of fusion after hypoxia exposure for both fish sizes. Although the juveniles return to oval shape after reoxygenation, the long filament's morphology appears to persist in adults.** Ultrastructure of hepatocytes of juveniles (A, B and C) and adults (D, E, and F) of *A. ocellatus* after being exposed for 3 h to one of the three treatments: normoxia, hypoxia (3h of hypoxia at 0.7 mg $L^{-1}$), and recovery (3h of hypoxia at 0.7 mg $L^{-1}$ followed by 1 h of reoxygenation). (A) the regular arrangement of cell morphology in juveniles *A. ocellatus* hepatocytes; (B) cellular morphology of juveniles exposed to hypoxia, showing a reduced number of circulating mitochondria, the change in mitochondrial structure for long filaments (black arrow), and possible mitophagy induced by hypoxia (white arrow); (C) after 1h of reoxygenation, cellular arrangement of juveniles shows an increase in mitochondria density as well as the contact between the ER and mitochondrial membranes (black arrow) and some regions more electron-density in hepatocyte nucleus suggesting that the chromatin becomes condensed (white arrow); (D) the regular arrangement of cell morphology in adults *A. ocellatus* hepatocytes; (E) cellular morphology of adults exposed to hypoxia, showing a reduced number of circulating mitochondria, mitochondrial structure with long filaments (white arrow and black arrow); (F) after 1h of reoxygenation, the cellular arrangement of adults recovered the mitochondria density, and the long filament's morphology appears to persist in adults (white arrow). In addition, contact between the ER and mitochondrial membranes increased (black arrow). Note the cell morphology of the mitochondria (M), endoplasmic reticulum (ER), desmosomes (D) and nucleus (N).

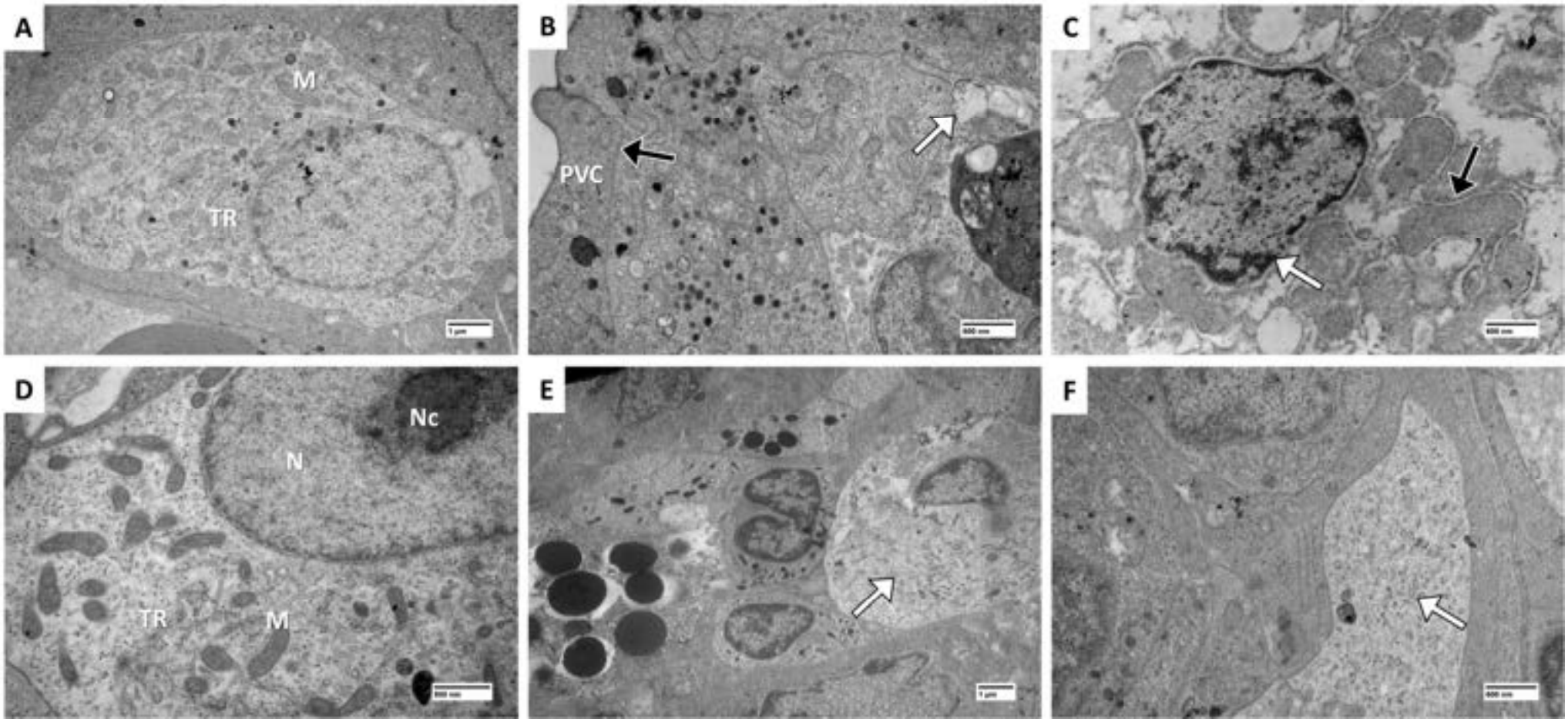
A
M
TR
B
PVC
C
D
Nc
N
TR
M
E
F

**Figure 5. The hypoxia promotes an increase in pavement cells (PVC) over MRCs for both sizes, decreasing the superficial area of the MRC and after the reoxygenation, the juveniles showed an increase in the regions of close contact between the ER and mitochondrial membranes.** Ultrastructure of the gill epithelium of juveniles (A, B and C) and adults (D, E, and F) of *A. ocellatus* after being exposed for 3 h to one of the three treatments: normoxia, hypoxia (3h of hypoxia at 0.7 mg $L^{-1}$), and recovery (3h of hypoxia at 0.7 mg $L^{-1}$ followed by 1 h of reoxygenation). (A) the regular arrangement of gill cell morphology in juveniles *A. ocellatus*; (B) cellular morphology of juveniles exposed to hypoxia, showing an increase in pavement cells (PVC) over MRCs (black arrow) and a region of mitophagy (white arrow); (C) after 1h of reoxygenation, cellular arrangement of juveniles shows an increase in mitochondria density as well as the contact between the ER and mitochondrial membranes (black arrow) and a change from the normal circular shape of the nucleus (white arrow); (D) the regular arrangement of gill cell morphology in adults *A. ocellatus*; (E) hypoxia promotes a reduction in the number of circulating mitochondria (white arrow) and (F) after 1h of reoxygenation, the cellular arrangement of adults could not recovered the mitochondria density (white arrow). Note the gill cell morphology of the mitochondria (M), tubular reticulum (TR), pavement cells (PVC), nucleus (N) and nucleolus (Nc).

**Supplementary material (S1).** ANOVA results (F) and significance values (p) for each analysis performed.

| | **Statistic** | | | **p value** | | |
|---|---|---|---|---|---|---|
| | Size | $[O_2]$ | Size x $[O_2]$ | Size | $[O_2]$ | Size x $[O_2]$ |
| Metabolic rate | F=130.650 | F=15.461 | F=2.888 | <0.001 | <0.001 | 0.069 |
| Metabolic rate depression* | F=3.943 | - | - | 0.073 | - | - |
| Glucosis | F=0.444 | F=10.978 | F=1.366 | 0.509 | <0.001 | 0.266 |
| Lactate | F=13.114 | F=69.100 | F=14.595 | <0.001 | <0.001 | <0.001 |
| Leak respiration | F=5.054 | F=3.265 | F=1.239 | 0.032 | 0.049 | 0.304 |
| CI | F=44.345 | F=2.489 | F=1.610 | <0.001 | 0.100 | 0.217 |
| CI+CII | F=48.575 | F=3.211 | F=3.342 | <0.001 | 0.054 | 0.049 |
| L total | F=1.268 | F=1.201 | F=0.667 | 0.269 | 0.315 | 0.521 |
| ETS | F=49.806 | F=9.635 | F=10.424 | <0.001 | <0.001 | <0.001 |
| CII | F=4.070 | F=1.699 | F=0.0447 | 0.053 | 0.201 | 0.956 |
| CIV | F=52.968 | F=1.469 | F=0.0930 | <0.001 | 0.246 | 0.911 |
| RCR | F=5.020 | F=7.132 | F=2.837 | 0.003 | 0.003 | 0.074 |
| ROS | F=1.604 | F=6.139 | F=1.247 | 0.215 | 0.006 | 0.302 |

*One-Way ANOVA

## 7. CONCLUSÃO GERAL

O presente trabalho de tese mostrou que, além dos diversos mecanismos comportamentais, fisiológicos, bioquímicos e morfológicos já descritos até o momento, os peixes da Amazônia também apresentam diversas adaptações mitocondriais para lidar com eventos de hipóxia e reoxigenação. Nossos resultados mostram um aumento da respiração mitocondrial cardíaca das espécies em seu ambiente natural durante o período das cheias, quando os níveis de oxigênio dissolvido (DO) são mais baixos em comparação com a estação seca, onde os DO são mais altos. Esse padrão contraria nossa hipótese inicial de que os peixes reduziriam a respiração mitocondrial na época das cheias e que as respostas dependeriam de sua posição predominante na coluna d'água. Entretanto, essas respostas apresentam uma importante estratégia de manutenção da função cardíaca para compensar a redução metabólica gerada pela hipóxia, melhorando o rendimento de ATP por mol de oxigênio consumido e, assim, otimizando a eficiência da produção de energia no miocárdio. Uma vez que entendemos que, para a maioria das espécies, a eficiência cardíaca é otimizada em condições ambientais naturais, buscamos entender se a comparação dos ajustes mitocondriais de peixes da Amazônia tolerantes à hipóxia revela características adaptativas comuns quando em situação de hipóxia e reoxigenação.

Para esse aspecto, mostramos que, após sete dias, as adaptações mitocondriais à hipóxia intermitente e constante são muito semelhantes entre as três espécies com diferentes mecanismos de tolerâncias morfológica e comportamental à hipóxia, indicando que as condições testadas aqui mimetizaram as observadas por esses peixes na natureza, uma vez que já estavam preparados para lidar com a variação comum de oxigênio observada em seu ambiente natural. Além disso, o perfil metabólico das espécies (conforme observado pelo $PO_{2crit}$) sustentou as respostas de adaptações mitocondriais para lidar com ambos os regimes hipóxicos e, assim, a história evolutiva de *A. ocellatus*, *C. macropomum* e *P. pardalis* podem ajudar a explicar as estratégias mitocondriais quando expostas ao mesmo nível fisiológico de hipóxia.

Além disso, pra entender as respostas mitocondriais são dependentes da tolerância à hipóxia, avaliamos adultos e juvenis de *A. ocellatus* (que apresentam aumento da tolerância à hipóxia através da ontogenia). Observamos o fato de os adultos apresentarem metabolismo muito mais lento em todas as condições é o ponto chave para a sua tolerância, pois a demanda de $O_2$ é menor, indicando que os adultos de *A. ocellatus* precisam de menos ATP em geral, de modo que não precisam recorrer ao metabolismo anaeróbico e acumular lactato como resultado. Além disso, devido ao alto $\dot{M}O2$ dos

juvenis, eles poderiam reduzir a eficiência de seus OXPHOS e comprometer a produção de ATP, tornando esta outra razão pela qual os juvenis são mais sensíveis à hipóxia. Além disso, a tolerância dos adultos também se deve aos baixos níveis de RCR, indicando que as mitocôndrias desacopladas em relação aos juvenis também apresentam baixos níveis de ROS devido ao menor número de elétrons no sistema. Assim, os peixes amazônicos apresentam refinados mecanismos metabólicos e mitocondriais para lidar com a hipóxia, bem como são capazes de rapidamente ativar mecanismos de reparo após eventos de reoxigenação. Esse trabalho apresenta, pela primeira vez, mecanismos de respiração mitocondrial nunca antes descritos para as espécies aqui estudadas, relacionando essas respostas aos padrões de taxa de consumo de oxigênio e lançando luz sobre a tolerância celular dos peixes amazônicos em seus ambientes naturais.

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