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Plasticidade fenotípica em espécies de peixes ornamentais: relacionando ecologia, fisiologia e epigenética

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Manaus - Amazonas

Outubro, 2021

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Plasticidade fenotípica em espécies de peixes ornamentais: relacionando ecologia, fisiologia e epigenética

Orientador: VERA MARIA FONSECA DE ALMEIDA E VAL

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Manaus - Amazonas

Outubro, 2021

ATA DE DEFESA

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PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA, CONSERVAÇÃO E BIOLOGIA EVOLUTIVA

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Sinopse

Este estudo teve como objetivo avaliar o quanto a exposição à hipóxia, natural e experimental, pode influenciar a tolerância de espécies de peixes ornamentais e os mecanismos fisiológicos, genéticos e epigenéticos (inter e transgeracional) envolvidos nessa tolerância.

Palavras-chave: hipóxia, tolerância, aerobiose, anaerobiose, respiração mitocondrial, Amazônia, microRNA, epigenética.

Aos meus pais, Eva e Waldir

Com todo o amor da minha vida.

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“It is not enough to discover and prove a useful truth previously unknown, but that it is necessary also to be able to propagate it and get it recognized.”

Jean-Baptiste Lamarck

RESUMO

As respostas dos peixes à hipóxia parecem estar relacionadas à diversidade dos ambientes aquáticos, que apresentam variações diárias e sazonais na concentração de oxigênio dissolvido. Dentre a adaptação desses animais à hipóxia, as respostas comportamentais, metabólicas, fisiológicas e bioquímicas são bem conhecidas para algumas espécies. Muitas dessas respostas são controladas por mecanismos genéticos e, possivelmente, epigenéticos, como microRNAs (MiRNAs). MiRNAs são mecanismos epigenéticos que agem na interface entre o ambiente e o transcriptoma e são conhecidos por conduzirem respostas rápidas frente a estressores ambientais, conferindo plasticidade fenotípica aos animais. As funções dos dos miRNAs permanecem pouco exploradas quanto à sua ação na tolerância à hipóxia, sua ação em diferentes sexos após a exposição à hipóxia e seus efeitos como reguladores da expressão gênica em peixes após exposição à hipóxia, de forma inter e transgeracional. Neste trabalho, visamos identificar como dois ambientes aquáticos diferentes, igarapés e lagos, "ditam" as respostas à hipóxia para duas espécies de ciclídeos, *Mesonauta festivus* e *Aequidens pallidus*. No primeiro capítulo, os resultados mostram que o *A. pallidus* é menos tolerante à hipóxia, o que parece estar relacionado ao ambiente normóxico natural desse animal. Embora esta espécie module a respiração mitocondrial para um melhor aproveitamento do oxigênio disponível, também apresenta menor queda da taxa metabólica quando exposta à hipóxia e não ativa o metabolismo anaeróbio. Diferentemente, a espécie *M. festivus* mostra uma maior queda na taxa metabólica, reduzindo a demanda por oxigênio, e apresenta uma ativação do metabolismo anaeróbico. Os dados revelam que o oxigênio dissolvido natural influencia a tolerância à hipóxia e a tolerância da espécie está relacionada à capacidade da espécie em deprimir o seu metabolismo. Além disso, os resultados mostram pouca influência da respiração

mitocondrial nesses processos. Considerando os resultados obtidos quanto a tolerância à hipóxia encontrada para ambas as espécies, o segundo capítulo teve como objetivo avaliar a expressão de miRNAs considerados importantes na regulação gênica em resposta à hipóxia. São eles: miR-210, miR-181c, miR-18a, miR-21 e let-7d. Esses miRs foram avaliados nas duas espécies amazônicas estudadas no primeiro capítulo que apresentaram tolerâncias distintas à hipóxia. Os resultados mostram que, quando exposta à hipóxia, a espécie *M. festivus* altera a expressão de miR-181c e let-7d no fígado, enquanto *A. pallidus* altera a expressão de miR-181c e miR-210 no fígado. Os resultados também mostram que a mudança na expressão desses miRNAs está relacionada à estratégia desses peixes para suportar a hipóxia. O terceiro capítulo deste estudo teve como objetivo identificar diferenças na expressão de mRNA e miRNA na geração F_1 do zebrafish (*Danio rerio*) 1 hpf (hora pós-fertilização) após a exposição de machos e fêmeas da F_0 a 2 semanas de hipóxia contínua (45%). Em geral, embriões F_1 em 1 hpf demonstraram diferenças na expressão de mRNA e miRNAs relacionadas ao sexo da geração F_0, a qual foi exposta à hipóxia. A análise de bioinformática de miRNA previu que as possíveis vias afetadas pela expressão destes miRNAs indicam modificações em vias conhecidas de sinalização de hipóxia e do metabolismo mitocondrial. Esta pesquisa demonstra a importância de examinarmos as contribuições de machos e fêmeas na variação fenotípica nas gerações subsequentes e fornece evidências de que há contribuição materna e paterna de miRNA através de óvulos e espermatozoides.

ABSTRACT

Fishes' responses to hypoxia seem to be related to the diversity of aquatic environments, which present daily and seasonal variations in the dissolved oxygen concentration. Amongst these fishes' adaptation to hypoxia, behavioral, metabolic, physiological, and biochemical responses are well known for some species. Many of these responses are controlled by genetic and possibly epigenetic mechanisms, such as microRNAs (miRNAs). MiRNAs are epigenetic mechanisms that act at the interface of the environment and the transcriptome and are known to drive plasticity in the responses following environmental stressors. An area of miRNA that has remained underexplored is the function of miRNAs in hypoxia tolerance, its sex specific action following hypoxia exposure and its effects as gene expression regulator in fishes. In this work, we aimed to identify how two different aquatic environments, normoxic forest streams and hypoxic lakes, dictate the responses to hypoxia for two cichlid species, *Mesonauta festivus* and *Aequidens pallidus*. In the first chapter, the results show that *A. pallidus* is less tolerant to hypoxia, which seems to be related to this animal's natural normoxic environment. Even though this species modulates the mitochondrial respiration in order to improve the oxygen use, it also shows a lower decrease in metabolic rate when exposed to hypoxia, and no activation of the anaerobic metabolism. Instead, *M. festivus* showed a higher decrease in metabolic rate and an activation of the anaerobic metabolism after hypoxia exposure. These data reveal that the natural dissolved oxygen influences the hypoxia tolerance and that species' tolerance is related to its ability to perform metabolic depression. The interesting results are the absence of mitochondrial respiration influence in these processes. Following the hypoxia tolerance found for both these species, in the second chapter, this work aimed to evaluated the

expression of miR-210, miR-181c, miR-18a, miR-21, and let-7d in these two Amazon species, which have distinct hypoxia tolerance strategies. The results show that, when exposed to hypoxia, *M. festivus* changes the expression of miR-181c and let-7d in the liver, while *A. pallidus* changes the expression of miR-181c and miR-210 in the liver. The results also confirm that the changes in expression of these miRNAs are related to these fishes' strategy to endure hypoxia. The third chapter in this study aimed to identify differences in mRNA and miRNA expression in the F_1 generation of zebrafish (*Danio rerio*) at 1 hpf after either F_0 parental male and female exposed to 2 weeks of continuous (45%) hypoxia. In general, F_1 embryos at 1 hpf demonstrated differences in mRNA and miRNAs expression related to the stressor and to the specific sex of the F_0 that was exposed to hypoxia. Bioinformatic pathway analysis of predicted miRNA:mRNA relationships indicated responses in known hypoxia signaling and mitochondrial bioenergetic pathways. This research demonstrates the importance of examining the specific male and female contributions to phenotypic variation in subsequent generations and provides evidence that there is both maternal and paternal contribution of miRNA through eggs and sperm from one generation to the next when the first is exposed to hypoxia.

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1. INTRODUÇÃO GERAL

1.1. Mais de 50 anos de pesquisa

Em 1848, Alfred Russel Wallace chegou ao rio Amazonas e iniciou sua viagem pela Amazônia, “Uma terra longínqua onde reina o verão sem fim”. Durante sua viagem, Wallace descreveu com detalhes a população local, a floresta, as espécies que encontrou e caracterizou os rios. Em seu livro (Wallace, 1889), Wallace caracterizou as águas como pretas, marrons e azuis (as azuis são atualmente descritas como brancas). Essas águas possuíam características físico-químicas diferentes, o que foi posteriormente bem descrito por Sioli (1984) com base na cor da água, transparência, pH e condutividade Ao lado dessas características, as águas amazônicas também apresentam variação de temperatura e nos níveis de oxigênio nos distintos ambientes como lagos, florestas alagadas, igapós e várzeas, e pequenos riachos localizados em terra firme.

Em 1929, Carter e Beadle realizaram uma expedição nos rios amazônicos e iniciaram os estudos com a respiração da Piramboia (*Lepidosiren paradoxa*) e sua capacidade de, como descrito por eles, permanecer enterrada durante os períodos de seca (Carter e Beadle, 1930). Já em 1946, Paulo Sawaya continuou os estudos a respeito da biologia de peixes de respiração aérea, onde neste trabalho, além dos estudos com a Piramboia, também foram feitas observações a respeito do Pirarucu (*Arapaima gigas*) (Sawaya, 1946).

A grande revolução com os estudos da fisiologia dos peixes amazônicos aconteceu pouco tempo depois com as expedições do R/V Alpha Helix II, de 1976 a 1977, liderada por pesquisadores que acreditavam que muitos problemas fundamentais da biologia poderiam ser melhor investigados onde eles ocorriam, o que foi feito nessa excursão por

um time de cientistas com apoio de um laboratório flutuante, o barco de pesquisa Alpha Helix, que curiosamente recebeu este nome em homenagem à estrutura do DNA. Em relação aos organismos aquáticos, o principal foco da expedição foi à cerca das características da hemoglobina dos peixes Amazônicos, além da capacidade de algumas espécies de respirar gás atmosférico, tópico extensivamente estudado por J.B. Graham em seus trabalhos com peixes Amazônicos, o que em 1997 culminou no livro "Air-Breathing Fishes – Evolution, Diversity and Adaptation" (Graham, 1997). Nos anos seguintes, diversos trabalhos foram publicados e os peixes, denominados como uma "mina de ouro" amazônica, começaram a ser explorados, com diversos estudos reunidos em livros como "Physiology and Biochemistry of the Fishes of the Amazon" (Val et al., 1996) e "Biology of Tropical Fishes" (Val e Almeida-Val, 1999). À medida que os estudos avançaram, contando também com novas tecnologias, novas perguntas surgiram quanto aos peixes da Amazônia e suas respostas fisiológicas, bioquímicas, respiratórias, comportamentais e genéticas diante das alterações de seu ambiente natural. Tais respostas continuam a ser estudadas atualmente por meio do projeto ADAPTA (Adaptação da Biota Aquática), o qual já se encontra em sua segunda edição. A seguir, passamos a detalhar melhor algumas características necessárias para o embasamento desta tese.

1.2. O oxigênio dissolvido no ambiente aquático Amazônico

A bacia hidrográfica amazônica consiste no maior ecossistema de água doce do mundo, com tamanho aproximado de 700,000 km^2 (Santos e Ferreira, 1999), sendo constituída por rios, lagos, igarapés, praias, várzeas e igapós (Sioli, 1994). Dentre esses ambientes, alguns, como rios, lagos e áreas inundadas, apresentam drásticas variações em suas características físicas e químicas, com variações diárias (dioturnas) e sazonais nos níveis de oxigênio e temperatura, estas últimas ocorrendo, provavelmente, em

decorrência dos ciclos nos níveis das águas ou pulso de inundação (Junk et al., 1983; Val e Almeida-Val, 1996).

Em alguns desses ambientes, os níveis de oxigênio podem ir de normóxia a hipóxia em menos de 24 horas, ou mesmo passar toda a estação de inundação com concentrações próximas à anóxia (Gessner, 1961; Junk, 1983). As variações diárias de oxigênio dissolvido podem ser mais críticas, variando de uma concentração supersaturada ao meio dia, a zero durante a noite, o que é resultado de diversos fatores, como a presença de macrófitas, a variação de temperatura, incidência solar, pluviosidade e profundidade das águas (Junk et al., 1983; Val e Almeida-Val, 1996).

As variações nos níveis de oxigênio e o ciclo das águas estão intimamente relacionados. Durante o período de cheia, observa-se uma redução nos níveis de oxigênio, com exceção de ambientes que apresentam acúmulo de macrófitas, fator crítico na determinação dos níveis de oxigênio dissolvido em florestas de várzea e igapós (Junk, 1984; Val e Almeida-Val, 1996). As alterações nos níveis de oxigênio dos rios e lagos da bacia amazônica são cíclicas e muito mais acentuadas que em rios de zonas temperadas, que, apesar de passarem por situações de hipóxia, não apresentam temperaturas elevadas como os rios de regiões tropicais, aumentando os desafios para os animais (Junk et al., 1983; Val e Almeida-Val, 1996). Por outro lado, a bacia amazônica também compreende ambientes aquáticos com características mais estáveis, como pequenos cursos d'água, conhecidos regionalmente como igarapés, os quais apresentam pouca ou nenhuma variação nas concentrações de oxigênio e temperatura (Sioli, 1984; Walker, 1995), exceto em períodos nos quais chuvas intensas levam a inundações momentâneas, ocorrendo a formação de novos micro habitats tais como poças laterais (Pazin et al., 2006), com características físico-químicas distintas das encontradas no

canal principal dos igarapés (Lowe-McConnell, 1991; Mendonça et al., 2005; Espírito-Santo et al., 2009).

Os igarapés formam um mosaico de diferentes habitats e micro habitats determinados por fatores físicos e espaciais como a entrada de água, velocidade de fluxo, profundidade, tipo de sedimento e detritos, fatores que influenciam diretamente a estrutura biótica destes ambientes, levando a uma grande diversidade de espécies (Araújo-Lima et al, 1995; Sabino e Zuanon, 1998; Lowe-McConnell, 1999; Castro, 1999; Mendonça et al., 2005).

1.3. A hipóxia e a fisiologia dos peixes amazônicos

A ictiofauna da região amazônica é composta por animais que variam em tamanho, forma e estratégia de vida, uma resposta adaptativa à enorme complexidade ambiental do local onde esses peixes vivem. Nesta região, estima-se que exista em torno de 3.000 espécies descritas (Reis et al., 2003). Os igarapés que cortam as florestas de terra firme também abrigam uma grande variedade de espécies de peixes, com importantes representantes de peixes ornamentais que apresentam grande dependência da floresta, tanto para a obtenção de alimentos quanto para a formação de abrigos (Zuanon et al., 2015).

A ictiofauna apresenta diversas adaptações para compensar a baixa disponibilidade de oxigênio no ambiente, como por exemplo modificações fisiológicas, bioquímicas e genéticas, as quais são fundamentais para a sobrevivência dessas espécies (Bickler e Leslie, 2007). Duas estratégias se mostram fundamentais: (1) os animais podem diminuir o consumo de oxigênio, diminuindo assim a demanda por ATP; ou, (2) ativar o metabolismo anaeróbico, reduzindo, consequentemente, a demanda por

oxigênio (Hochachka, 1992; Val e Almeida-Val, 1996; Bickler e Leslie, 2007;), apesar de que alguns estudos mostram que certas espécies de peixes apresentam as duas estratégias simultaneamente (Almeida-Val et al., 1993; Heinrichs-Caldas et al., 2016). Além de compensações fisiológicas, algumas espécies apresentam adaptações comportamentais, refletidas em diferentes estratégias respiratórias, como a respiração aérea obrigatória ou facultativa e a respiração na superfície aquática (ASR, do inglês, Aquatic Surface Respiration). A ASR ocorre quando o animal exposto à hipóxia se desloca até a superfície da coluna d'água para captar a porção mais rica em oxigênio (Saint-Paul, 1984). Além disso, quando em hipóxia, alguns animais aumentam a frequência dos batimentos operculares, uma adaptação comportamental bem descrita para peixes amazônicos (Rantin et al., 1992; de Salvo Souza et al., 2001; Oliveira et al., 2004; Chippari-Gomes et al., 2005; Heinrichs-Caldas et al., 2016).

O oxigênio desempenha um papel crucial na respiração celular aeróbica, sendo fundamental para a glicólise, rota metabólica produtora do substrato piruvato que é, posteriormente, direcionado ao ciclo dos ácidos cítricos que geram poder redutor para que se dê a fosforilação oxidativa com geração de grande quantidade de ATP. Na ausência ou escassez de oxigênio, diversas adaptações são observadas nos diferentes tecidos dos peixes, com intuito de viabilizar a sobrevivência desses animais. Por exemplo, durante hipóxia ou atividade física, os músculos e outros órgãos ativam o metabolismo anaeróbico, utilizando suas próprias reservas de glicogênio ou de outras fontes (Jensen et al., 1993; Val e Almeida-Val, 1995) para dar continuidade à glicólise com formação de piruvato e consequente redução a lactato, produto final da anaerobiose. A transformação de piruvato a lactato é possibilitada pela enzima LDH (lactato desidrogenase) a qual utiliza como cofator o NADH (forma reduzida da nicotinamida adenina dinucleotideo). Assim, a cada molécula de piruvato reduzido, uma

molécula de NADH é oxidada a NAD que será reutilizada na glicólise, permitindo que a rota continue ininterruptamente até que o oxigênio se torne novamente disponível para a célula. Mais especificamente o fígado, órgão anfibólico que tem a função de gerar e armazenar glicogênio, utiliza suas reservas para suprir a glicólise anaeróbica quando o animal é exposto à hipóxia, mantendo assim a produção de ATP e o poder redutor sem consumo de oxigênio em outros tecidos (Hochachka, 1988).

Almeida-Val e colaboradores (1993) mostraram que o tambaqui exposto à hipóxia por duas horas sem acesso à superfície eleva os níveis de ácido lático no sangue, mostrando uma dependência da glicólise anaeróbica durante o episódio de hipóxia; quando o ambiente é reoxigenado, em duas horas o tambaqui tem seus níveis de ácido lático plasmático de volta aos níveis normóxicos. Em outro trabalho, realizado com o ciclídeo *Cichlasoma amazonarum*, Almeida-Val e colaboradores (1995) observaram mudanças na distribuição das isoformas da enzima LDH, as quais têm papel crucial na regulação do metabolismo anaeróbico (Hochachka e Somero, 1984), em diferentes tecidos. Na espécie estudada por Almeida-Val e colaboradores (1995), a isoforma A_4 foi mais ativa no cérebro e no coração e a isoforma B_4 mais ativa no fígado, permitindo que esse animal, quando em hipóxia, utilize duas adaptações metabólicas: aumento do metabolismo anaeróbico e supressão da demanda energética. Heinrichs-Caldas e colaboradores (2016) observaram que o ciclídeo *Astronotus crassipinnis*, para sobreviver à hipóxia, aumenta o seu metabolismo anaeróbico e regula sua expressão gênica, aumentando a expressão do gene *hif-1α* no fígado e mantendo alta sua expressão nos músculos. Este gene está altamente relacionado à sobrevivência à hipóxia pois controla a expressão de muitos genes pertencentes a várias vias metabólicas responsáveis pela homeostase da célula quando os níveis de oxigênio baixam a condições hipóxicas ou anóxicas (Semenza, 2000).

1.4. Hipóxia, genética e epigenética

As adaptações descritas na sessão anterior englobam respostas de uma poderosa maquinaria gênica, com relações entre RNA mensageiro (mRNA) e micro RNA (miRNA), como foi descrito recentemente por Zhang e colaboradores (2016) em um estudo com *Pelteobagrus vachelii*, uma espécie tolerante à hipóxia. Nesse trabalho, os autores demonstraram o papel regulador dos miRNAs na pós-transcrição de mRNAs de diversas vias metabólicas tais como a via de sinalização do HIF-1, a da glicólise e da gliconeogênese, além de apresentar correlações negativas entre miRNA e seus mRNAs alvos, onde o aumento da expressão de um miRNA leva à diminuição da concentração de seu mRNA alvo, fornecendo novas respostas pós transcricionais à regulação gênica.

MicroRNAs são uma classe de RNA não codificantes, compostos por 17 a 22 nucleotídeos, que regulam a expressão gênica, agindo como moduladores epigenéticos, através de processos pós-transcricionais de silenciamento ou degradação de mRNA específicos, como pode ser observado na **figura 1** (Joo et al., 2014).

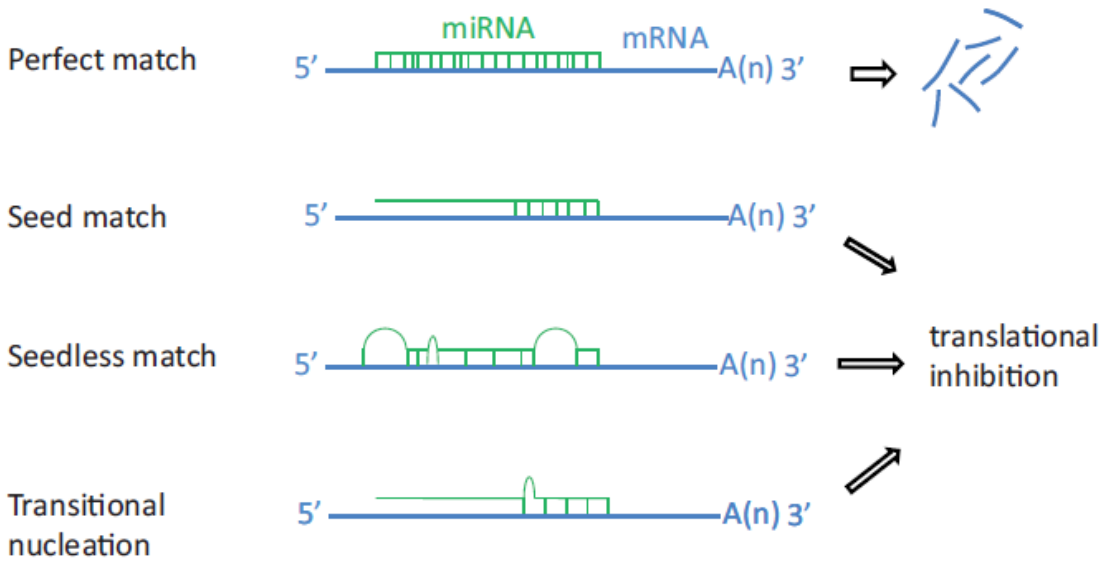

Figura 1. Quatro modelos de interações entre micro RNA e RNA mensageiro e seu resultado final. Um pareamento perfeito entre miRNA e mRNA resulta na degradação do mRNA alvo (perfect match). Por outro lado, pareamentos imperfeitos (seed match, seedless match e transitional nucleation) resultam na inibição da tradução dessas sequências (Bizuayehu e Babiak, 2014).

Os microRNAs possuem um papel fundamental em muitos processos celulares como metabolismo, proliferação e diferenciação celular e apoptose, com estudos recentes demonstrando a ligação entre a expressão de miRNAs específicos e as respostas à hipóxia, com profundas implicações epigenéticas (Kulshreshtha et al., 2008; Du et al., 2015), o que mostra a importância desses marcadores em estudos de expressão gênica. MicroRNAs também têm funções no desenvolvimento animal e possuem efeito transgeracional. Mesmo que o mecanismo permaneça desconhecido, a exposição paterna a diferentes estressores ambientais estimulam marcas epigenéticas nas células germinativas, incluindo miRNAs, que impactam diretamente os processos biológicos da próxima geração, o que caracteriza este mecanismo como epigenético (Rodgers et al., 2015). Mudanças epigenéticas podem permanecer por pelo menos uma geração e podem persistir mesmo depois que o estressor não esteja mais presente, o que depende do estressor ambiental em estudo (Casier et al., 2019). Durante o desenvolvimento, estudos têm demonstrado que os miRNAs são essenciais para o desenvolvimento normal; a falta de miRNAs maduros em células-tronco embrionárias de camundongo impede a célula de se diferenciar, *in vivo* e *in vitro* (Kanellopoulou et al., 2005).

1.5. A relação entre o ambiente e a tolerância à hipóxia

Todas as adaptações observadas estão fortemente relacionadas aos ambientes em que esses peixes vivem, selecionados através de processos evolutivos a se adaptarem, mesmo em estágios juvenis e larvais, às pressões ambientais locais. Diversos estudos

mostram que as respostas fisiológicas dos animais estão relacionadas ao tipo de ambiente aos quais estão expostos.

No trabalho de McBryan e colaboradores (2016), indivíduos de *Fundulus heteroclitus* coletados em ambientes com variações diárias e sazonais distintas de temperatura e oxigênio apresentam diferentes respostas. Os animais que estão expostos a maiores variações se mostraram mais tolerantes à hipóxia, com menores níveis de pressão crítica de oxigênio (PO_{2crit}) e maior tempo de exposição sem perder o equilíbrio (LOE_{hip}), além de apresentarem maior superfície lamelar. Animais do gênero *Paracheirodon* que habitam ambientes com altas temperaturas (até 35°C) se mostraram mais tolerantes à hipóxia que seus congêneres que habitam ambientes com temperatura máxima de 30°C, apresentando um maior polígono de tolerância térmica, uma maior taxa metabólica e atividade enzimática indicando uma maior capacidade anaeróbica, demonstrando que esses animais são mais tolerantes a variações de oxigênio e temperatura (Campos et al., 2016).

Ao estudar uma população de *Micropogonias undulatus*, Thomas e colaboradores (2007) observaram que os peixes que habitam ambientes de hipóxia recorrente apresentam maior regulação da expressão do gene *hif-1α,* quando comparados com animais de regiões em constante normóxia. Resultados similares foram observados com uma população de *Callionymus valenciennei* distribuída entre zonas hipóxicas e normóxicas, onde animais de regiões com baixa disponibilidade de oxigênio dissolvido demonstram maior capacidade de regulação deste gene (Kodama et al., 2012).

A exposição a um ambiente pode alterar fatores regulatórios epigenéticos, tais como a expressão de microRNAs. Como a sensibilidade da regulação epigenética pode ser maior durante as fases de desenvolvimento (Marsit, 2015), a exposição de animais a

estressores em fases larvais pode alterar a maquinaria gênica, trazendo impactos aos processos biológicos. Em trabalho realizado por Donelson (2015), foi observado que indivíduos da espécie *Premnas biaculeatus*, em estágio de desenvolvimento larval, quando expostos a +1,5 e +3°C que a temperatura de aclimatação, exibiram um aumento na capacidade térmica máxima (CT_{max}) sugerindo que a aclimatação a um estressor pode aumentar a capacidade de tolerância do animal. Um trabalho feito com zebrafish (*Danio rerio*) mostrou que a exposição crônica de larvas à hipóxia aumentou a tolerância desses animais a essa condição, aumentando, consequentemente, a sobrevivência com uma maior regulação do gene *hif-1α*, indicando uma ativação maior das vias de resposta, sendo que a aclimatação também conferiu aos animais uma maior capacidade cardíaca (Kopp et al., 2014). Ainda com o *Danio rerio,* embriões deste animal foram expostos à hipóxia durante o desenvolvimento e, já na fase adulta, foram novamente expostos à hipóxia e foi observado que esses animais possuíam uma maior capacidade de regular o metabolismo anaeróbico e aeróbico, além de possuírem menor PO_{2crit} e taxa metabólica comparados aos animais que não haviam sido previamente expostos à hipóxia na fase embrionária (Barrionuevo et al., 2010). De acordo com Ho e Burggren (2012), além da exposição prévia de embriões e larvas levar a um aumento na capacidade de tolerância à hipóxia, este aumento também pode ser transmitido à próxima geração a partir da exposição parental a um estressor, o que pode possivelmente demonstrar a relação entre esses trabalhos e a adaptação por marcas epigenéticas.

Esses trabalhos demonstram que as respostas à adaptação local podem surgir durante o desenvolvimento, o que pode explicar maiores tolerâncias de animais que se desenvolvem em locais com variações extremas de oxigênio, conferindo à prole uma maior tolerância na vida adulta. Apesar da importância de se entender tais processos

adaptativos relacionados à expressão diferencial, tanto de mRNA quanto de miRNA, de animais expostos a essas condições durante o desenvolvimento, até o presente momento os resultados obtidos nesta tese são os primeiros a expor a relação direta entre a variação natural de oxigênio nos ambientes aquáticos e a tolerância à hipóxia em duas espécies de peixes amazônicos, além de expor alguns outros mecanismos que possuem influência nesta tolerância, como as defesas antioxidantes, a respiração mitocondrial e ação intergeracional dos microRNAs. Além disso, este trabalho mostra o papel transgeracional da expressão dos microRNAs após a exposição da F_0 à hipóxia, demonstrando ainda que animais de diferente sexo são influenciados diferencialmente por esse estressor, e como esse fator se reflete na expressão dos microRNAs da geração seguinte.

2. Objetivos

2.2. Objetivo geral

O presente trabalho objetivou avaliar o quanto a exposição à hipóxia, natural e experimental, pode influenciar a tolerância de espécies de peixes ornamentais e os mecanismos fisiológicos, genéticos e epigenéticos (inter e transgeracional) envolvidos nessa tolerância.

3. Objetivos específicos

3.1. Capítulo I

Avaliar a influência de ambientes com diferentes regimes de concentração de oxigênio sobre a tolerância à hipóxia de duas espécies amazônicas de Cichlidae: *Aequidens pallidus* e *Mesonauta festivus*.

3.2. Capítulo II

Investigar a função da expressão de microRNAs sobre a tolerância à hipóxia de duas espécies de Cichlidae, *Aequidens pallidus* e *Mesonauta festivus*, com diferentes níveis de tolerância a esse estressor.

3.3. Capítulo III

Investigar a expressão de microRNAs na F_1 de zebrafish, *Danio rerio*, uma hora após a fertilização após a exposição de machos e fêmeas da F_0 a duas semanas de hipóxia.

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Capítulo I

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

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

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

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

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Introduction

Several aquatic environments undergo different dissolved oxygen concentration variations worldwide, presenting from moderate to severe hypoxia, dur- ing shorter and longer periods (Mandica and Regan 2018). The Amazon River basin is diverse in aquatic environments, and most of these environments endure daily and seasonal fluctuation in water oxygen concentration fluctuation. These environments have different daily and seasonal dynamics such as *várzeas*, lakes, flooded forests, and rivers main (Junk et al. 1983). The variations of oxygen levels in these environments can go from moderate to severe and last for short and long periods, bringing considerable differences in O_2 availability between these environments (Val and Almeida-Val 1996). Even though most of the Amazonian aquatic environments go through oxygen

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

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

Most of the Amazonian fishes' responses to hypoxia seem to be related to their environment characteristics, responding at several biological levels, from behavioral to respiratory, physiological, biochemical, and genetic adjustments (Val et al. 1998). Although some species chose to avoid hypoxic environments, others present physiological and biochemical adaptations to endure such stress. During development, as a defense against predator, some species can use hypoxic environments as refugee (Anjos et al. 2008). When exposed to hypoxia, the characidae *Colossoma macropomum* can extend its lower lip in order to extract the water from the upper water column, which is more oxygenated (Saint-Paul 1984; Val and Almeida-Val 1996). When exposed to hypoxia, Amazonian fishes can show different strategies: (1) decrease the ATP demand by decreasing the aerobic respiration, or (2) increase the anaerobic metabolism (Almeida-Val et al. 2000; Hochachka and Somero 2002), even though some species show both strategies, which seem to be related to their tol- erance levels (Almeida-Val et al. 1993; Almeida-Val and Hochachka 1995; Chippari-Gomes et al. 2005). For instance, while decreasing the overall metabolic rate in order to decrease energy demand, the cichlid *Astronotus ocellatus*, which is one of the vertebrate species that can cope with hypoxia and anoxia for long periods of time and survives under oxygen deprivation in nature, increases its anaerobic metabolism, increasing blood levels of lactate, as lactate dehydrogenase is recruited to increase its levels (Almeida-Val et al. 2000; Sloman et al. 2006). These results have been confirmed by several other works with the Oscar species, *Astronotus crassipinnis*, which results in this species high tolerance to hypoxia (Heinrichs-Caldas et al. 2019).

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

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

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

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

Material and methods

Experimental fish

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

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

Respirometry and loss of equilibrium

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

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

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

To achieve the time of loss of equilibrium in hypoxia, $LOE_{(hyp)}$, eight animals of each species were kept in the chambers described above. Then, the oxygen concentration was set at 0.79 mg O_2.L^{-1} ± 0.8 for *M. festivus* and 1.25 mg O_2.L^{-1} ± 1.3 for *A. pallidus*, until the animal was not able to maintain the equilibrium. If the animal did not respond after 3 gentle touches to the chamber, the time to loss of equilibrium was determined (McBryan et al. 2016).

Hypoxia exposure

For the experiment, both species were divided into two groups, normoxia and hypoxia (n = 8). Each ani- mal was randomly placed in a 1.5 L individual plastic container 1 day before the experiment to acclimate from handling. During the acclimation, the animals were kept at 28 °C ± 0.3 with constant aeration (O_2 concentration: 6.82 mg O_2.L^{-1} ±0.12). For the hypoxia group, the oxygen concentration was slowly decreased by N_2 pump directly in the water for around1:30 h, and after the oxygen concentration reached 0.73 mg O_2.L^{-1} ± 0.9 for *M. festivus* and 1.23 mg O_2.L^{-1} ± 0.5 for *A. pallidus*, the animals in the hypoxia group were exposed to 3 h of hypoxia. Meanwhile, the normoxic group was kept under constant aeration 6.89 mg O_2.L^{-1} ± 0.27. Dissolved oxygen concentration was monitored with an Oxymeter 5512-Ft (YSI, USA, Ohio, Yellow Springs). The 3-h exposition to hypoxia was chosen based on a preview exposition, for both species, in the respirometry, to assure that this was enough for metabolic depression and oper- cular movements increase, which ensure that the ani- mals were in hypoxia. For each group and each spe- cies, eight animals were sampled and euthanized by head concussion followed by a cut in the spine cord. Then, the liver was excised and stored at − 80 °C until the following assays. For the mitochondrial respira- tion, a small piece of the liver was immediately put in BIOPS buffer (2.77 CaK_2EGTA, 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56 $MgCl_2.6H_2O$, 20 taurine, 20 imidazole, 0.5 dithiothreitol, 50 K-MES, 15 sodium phosphocreatine, and 50 fructose, all measured in mM). This experiment was performed in the morning and is in accordance with CONCEA Brazilian Guide for Animals Use and Care under INPA's authorization (CEUA: protocol #054/2017).

Liver mitochondria respiration and ROS production

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

30 min. After this, the livers were dried, weighed, and immersed in 0.5 mL of MiR05 buffer (pH 7.24) at 20 °C: 0.5 EGTA, 3.0 $MgCl_2 \cdot 6H_2O$, 60 potassium lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 160 sucrose, and 1.0 BSA 1.0 (all in mM), essentially fatty acid free, and homogenized by hand. This homogenate was then diluted in 2.5 mL MiR05 in order to measure the oxygen consumption in an Oxygraph 2K Oroboro (Innsbruck, Austria) (Gnaiger et al. 2000).

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

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

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

Enzyme activities and lipoperoxidative damage

Lactate dehydrogenase (LDH; E.C. 1.1.1.27) and citrate synthase (CS; E.C. 4.1.3.7) were measured in the liver following a well-established protocol for Amazonian fish tissues (Driedzic and Almeida-Val 1996) modified for plate spectrophotometer reader. For the assay, 0.01 g of tissue was homogenized in a 4× imidazole buffer (50 mM imidazole, 1 mM EDTA, and 1% Triton x-100 at pH 7.4) and centrifuged at 10.000×g in a Refrigerate Centrifuge 5430 R (Eppendorf, Hamburg, GE) for 15 min at 4 °C. LDH assays were performed in 0.2 mL 96-well plate containing 0.15 mM NADH, 1 mM KCN, and 50 mM imidazole, pH 7.4 at 25 °C. The LDH reactions started with 1 mM pyruvate. CS assays were performed in a 0.2 mL96-well plate with 0.25 mM DTNB and 75 mM Tris base, pH 8.0 at 25 °C, and the reactions were initiatedwith 0.4 mM acetyl Co-A and 0.5 mM oxaloacetate.

To measure SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6) activities, and lipid peroxidation (LPO) levels, 0.01 g of tissue was homogenized in a buffer containing Tris base 20 mM, EDTA 1.0 mM, dithiothreitol 1.0 nM, sucrose 50 nM, and KCl 150 nM. Then, the homogenates were centrifuged at 15,000 g for 20 min at 4°C and were used in the following proportions: 1:10 w/v for SOD, 1:4 w/v for CATand LPO. SOD activity was determined according to Turrens (1997) based on the cytochrome *c* reduction rate inhibition by superoxide radical at 550 nm and 25 °C. SOD enzyme activity is expressed as U SOD mg $protein^{-1}$, where 1 U of SOD corresponds to the quantity of enzyme that promoted the inhibition of 50% of cytochrome *c*. To measure CAT activity, the H_2O_2 decomposition inhibition rate was measured at 240 nm, and the results were expressed as µmol H_2O_2

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

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

Data analysis

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

Results

Environment description

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

Respirometry and loss of equilibrium

There was difference in the PO_{2crit} in these two species. *M. festivus* showed a lower (P=0.006) PO_{2crit}, 1.01 ± 0.07 mg $O_2.L^{-1}$, while *A. pallidus* PO_{2crit} was 1.80 ± 0.2 mg $O_2.L^{-1}$.

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

Regarding the $LOE_{(hyp)}$ values, *M. festivus* presented a higher (P=0.001) $LOE_{(hyp)}$ value, needing 121.14 ± 12 min to lose equilibrium when oxygen concentration was below 1 mg $O_2.L^{-1}$, while for *A. pallidus* species, the loss of equilibrium was with 62.12 ± 4.4 min.

Mitochondrial respiration and ROS production

The mitochondrial respiration (Fig. 3) showed no difference between species and treatments for complex I (P=0.213, F = 1.657), complex IV (P=0.407, F=0.718), and H+ Leak (P=0.317, F=1.055). Complex I+II was higher for *M. festivus* in nor- moxia (P=0.015, F=3.772) when comparing both species. *A. pallidus* decreased ETS in hypoxia (P=0.006, F=4.323). CIII was higher for *M. festi- vus* in normoxia and hypoxia compared to *A. pal- lidus* (P=0.008, F=4.15); the same happened to RCR, which was higher for *M. festivus* in both treat- ments (P=0.012, F=3.93).

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

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

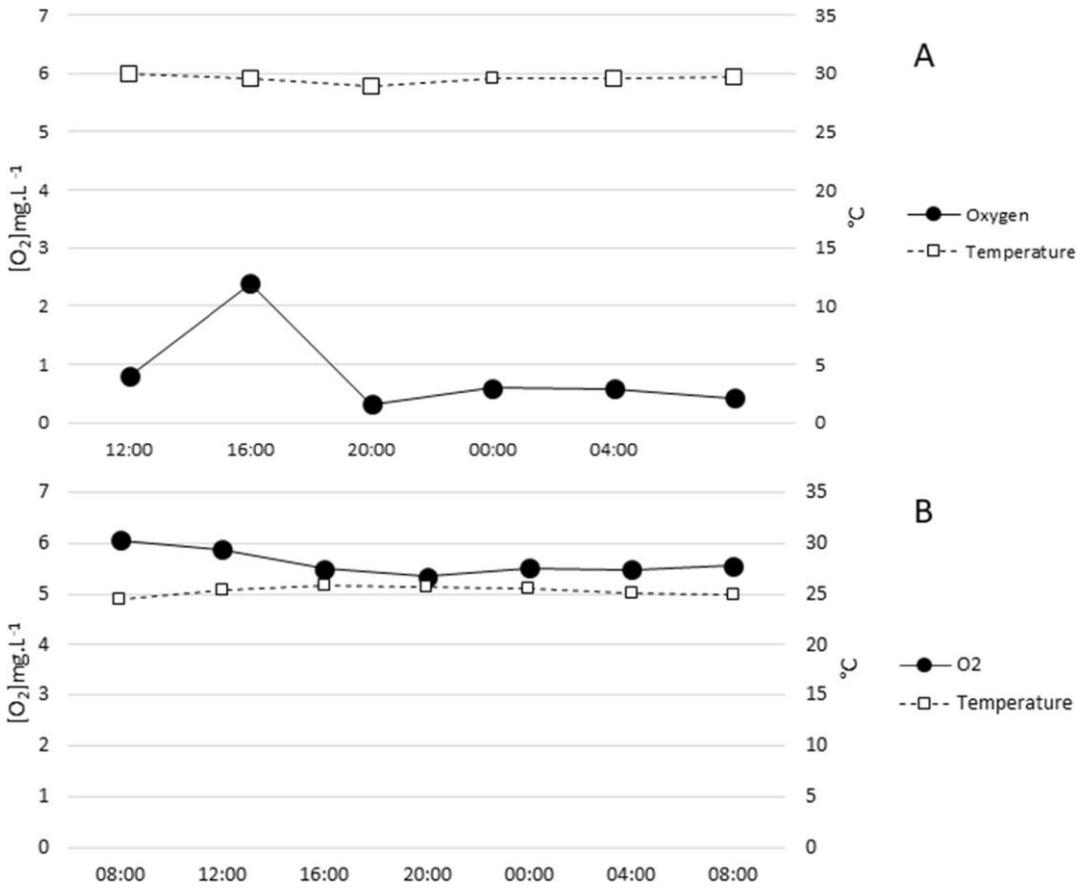

Fig. 1 Oxygen concentration and temperature measurements in both fish sampling sites (Catalão Lake, 3°10′S, 59°54′W, and Reserva Florestal Adolpho Ducke forest streams, 02°53′S, 59°58′W) during 24 h. These data were collected during the fishes sampling.

Enzyme activities and lipoperoxidative damage

Enzyme activities showed different patterns forboth species. LDH activity was higher (P=0.021, F = 15.02) for *M. festivus* during hypoxia exposure (P=0.048, F=2.32), while *A. pallidus* showed no difference between treatments (P=0.774, F=0.41). CS showed no difference (P = 0.913, F=0.174) in hypoxia or normoxia in both species. LDH/CS was higher (P=0.006, F = 6.89) for *M. festivus* under hypoxia, and *A. pallidus* showed no difference between treatments (P=0.156, F=2.04). SOD activ- ity showed no difference between species (P=0.290, F=1.52), even though *A. pallidus* increased SOD activity during hypoxia (P=0.021, F=3.48). CAT activity was lower in *M. festivus* in both treatments when comparing to *A. pallidus*. (P<0.001, F=7.46). *A. pallidus* increased CAT activity under hypoxia (P<0.001, F=9.53), while *M. festivus* showed no differences between treatments (P=0.605, F=0.741). LPO increased in hypoxia for *M. festivus* (P<0.001, F=12.031) and *A. pallidus* (P<0.001, F=8.046), and, in hypoxia, it was higher (P=0.001, F=5.21) for *M. festivus*. All data are shown in Table 1.

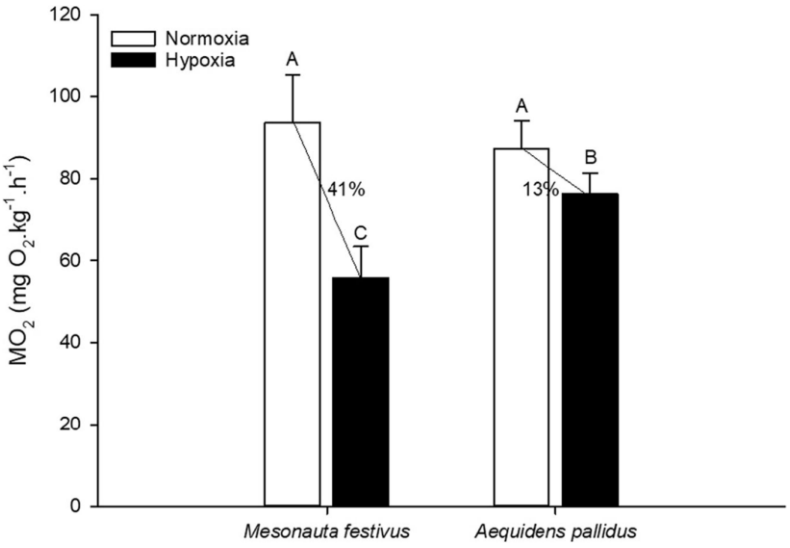

Fig. 2 *Mesonauta festivus* (*n*=8) and *Aequidens pallidus* (*n*=8) MO_2 during 3 h of normoxia and hypoxia. The decrease in MO_2 is indicated by the % symbol. The statistical significance was analyzed using a two-way ANOVA. Letters indicate the differences between species and treatments ($P<0.05$).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

Discussion

Hypoxia tolerance and metabolic strategies

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

The species studied in this article showed differ- ent strategies to cope with hypoxia, which are related to their life history. By exhibiting a lower PO_{2Crit}, a higher depression of the MO_2 under hypoxia and a longer $LOE_{(hyp)}$ time, *M. festivus* showed to be more tolerant to this stressor than its related species, *A. pallidus*. A lower PO_{2crit} and a higher MO_2 depression indicate a higher capacity to extract O_2 from the environment and to maintain cellular function, which is reflected by the $LOE_{(hyp)}$, a direct measure that shows how long an animal can keep its ecological function under hypoxic pressure. Even though the use of PO_{2crit} to determine hypoxia tolerance is under debate (Wood 2018; Regan et al. 2019), in the present work, it showed to be a reliable measurement, since it corresponded to the higher metabolic depression and higher $LOE_{(hyp)}$ time for *M. festivus*. A behavioral response observed in this work is the increase in *A. pallidus* movements in the aquaria when the animal is in hypoxia, other than that, during recovery phase, after hypoxia exposure, half of the *A. pallidus* died, while *M. festivus* showed no death after PO_{2crit} and $LOE_{(hyp)}$ trials.

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

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

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

Oxidative stress and defenses

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

In the present work, the oxidative damages and the activation of antioxidant defenses are determined by the oxygen regime of species natural environment. While *M. festivus* showed no difference in CAT and SOD activities, *A. pallidus* increased the activity of both enzymes when exposed to hypoxia, even though both species increased LPO levels. Otherwise, expos-ing *A. pallidus* to hypoxia increased the catalase activity, which catalyzes H_2O_2 decomposition to H_2O. Thus, we hypothesize that the decrease in total mitochondrial ROS accumulation in this species aims to avoid the appearance of oxidative stress in the liver. The increase in SOD activity for *A. pallidus* also indi-cates an oxidative stress as well, since this enzyme is responsible for general defenses against superox-ide. The activation of both enzymes, CAT and SOD, in hypoxia was also observed in killifish (*Fundulus heteroclitus*), when exposed to different hypoxia pat-terns, in order to mitigate the ROS imbalance (Du et al. 2016). Changes in these and other antioxidant enzymes were also observed in *Perccottus glenii* when exposed to hypoxia (Lushchak and Bagnyuk- ova 2007). Instead, after hypoxia exposure, *Cyprinus carpio*'s liver did not change CAT or SOD activities (Lushchak et al. 2005). Herein, the increase in LPO levels for both species indicates the occurrence of oxi-dative lipid damage, resulting in an oxidative stress for both species under hypoxia, which suggests that, although in different intensities, none of the studied species went through hypoxia free of oxidative dam-age. LPO is an example of ROS-induced damage with the polyunsaturated fatty acids reacting with free rad-icals. An increase in LPO levels was already observed for *Mytilus galloprovincialis* and *Mugil cephalus* when exposed to hypoxia (Ekambaram et al. 2016; Woo et al. 2013). The increase in these enzyme activ-ities and LPO levels in *A. pallidus* liver depicted the loss of cellular homeostasis in hypoxia. We can also hypothesize that the increase in LPO for both species is related to the lipid utilization by the animal during hypoxia. As observed by De Boeck et al. (2013), in Oscar (*A. ocellatus*) exposed to hypoxia, the main energy source comes from lipids, which might reflect in the LPO levels.

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

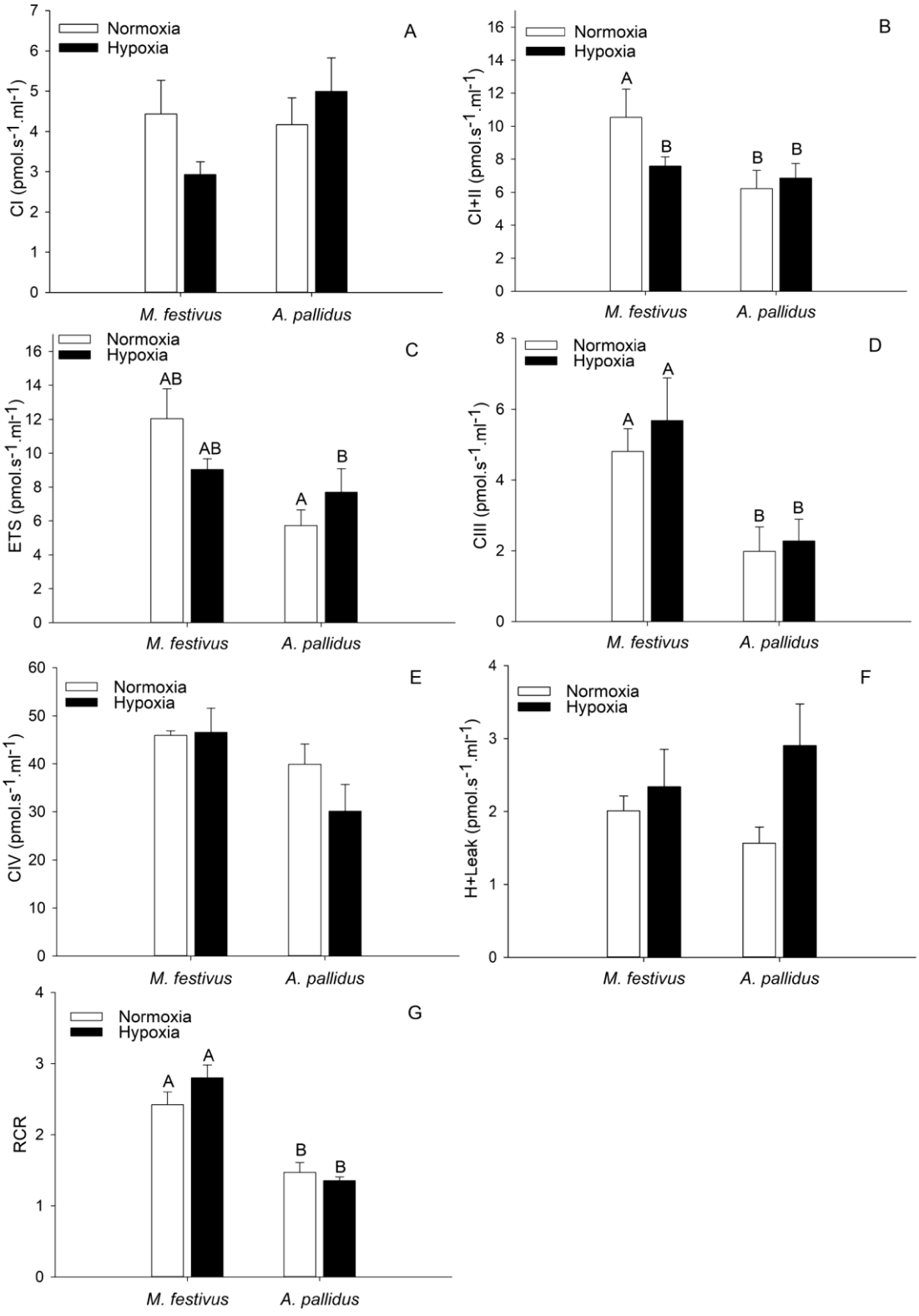

Fig. 3 Complexes I (**A**), I+II (**B**), ETS (**C**), III (**D**), and IV (**E**), H+Leak (**F**), and RCR (**G**) in both species (*Mesonauta festivus* and *Aequidens pallidus*) liver mitochondria when the animals were exposed to 3 h of normoxia and hypoxia. The statistical significance was analyzed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

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

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

Mitochondrial respiration

The mitochondrial liver respiration responses observed in our results showed to be species-specific and related to the hypoxia tolerance (Figure 3). While *M. festivus* showed no acute regulation in most of its mitochondrial respiration complexes, except in CI+II, *A. pallidus*, which is sensitive to hypoxia, increased the ETS in order to endure hypoxia. Mitochondrial oxidative phosphorylation (OXPHOS) plays a cen- tral role regulating cell energy production. In the OXPHOS, mitochondrial complexes I, III, and IVgenerate proton-motive force, while complex II trans-fers electrons to CIII, which by proton-motive force will generate ATP along with the electron transport system (ETS), making the OXPHOS a key compo- nent in oxygen usage in hypoxia (Paradis et al. 2016). However, protons can migrate to the matrix inde-pendent of this process, a mechanism named "proton leak," which uncouples substrate oxygen from ATP generation. The OXPHOS showed few differences forboth species in this study. While *M. festivus* decreasedthe CI+II, *A. pallidus* showed no change, even though
A. pallidus CIII was lower in hypoxia in comparison to *M. festivus*.

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

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

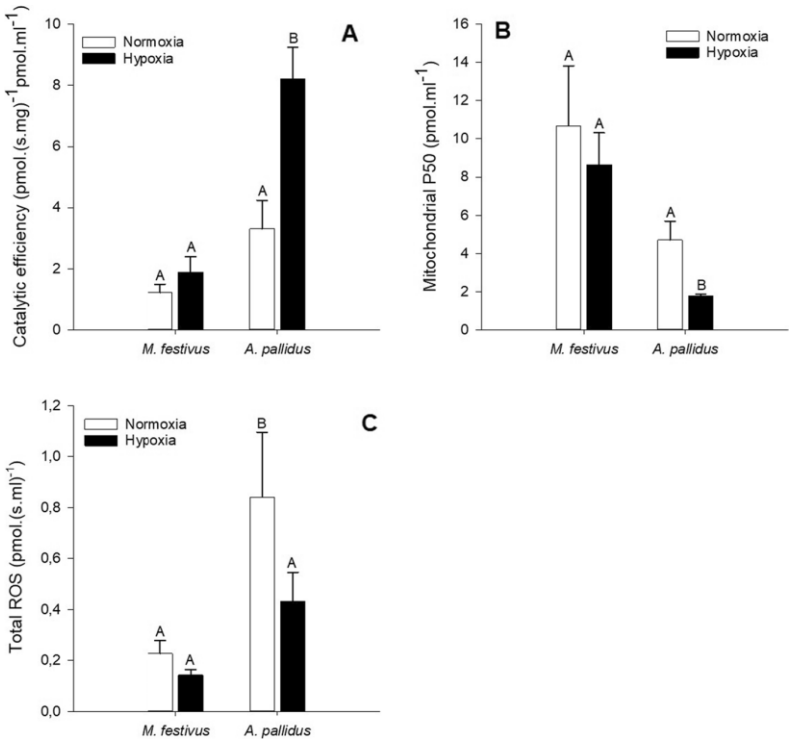

Figure 4 Catalytic efficiency (**A**), mitochondrial P_{50} (**B**) and total mitochondrial ROS production (**C**) in liver mitochondria from both species (*Mesonauta festivus* and *Aequidens pallidus*) when the animals were exposed to 3 h of normoxia and hypoxia. The statistical significance was analyzed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

Table 1 Enzyme activity of lactate dehydrogenase (LDH), citrate synthase (CS), and the ratio between LDH and CS (LDH/CS), superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (LPO) in liver of *M. festivus* (n=8)and *A. pallidus* (n=8) exposed to three hours of normoxia and hypoxia. The statistical significance was analyzed using a two-way ANOVA. LDH, CS and LDH/CS enzyme activity were expressed as µmol.min-1.mg prot^{-1}. SOD activity was expressed as U min^{-1}.mg prot^{-1}.CAT activity was expressed as mM^{-1}.mg prot^{-1}. LPO was expressed as µM cumene hydroperoxide.mg prot^{-1}. Letters indicate differences between species and treatments (*P<0.05*).

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

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

The respiratory control ratio (RCR) for both species showed no difference between treatments (Fig. 1) but was higher, both in hypoxia and nor- moxia, for *M. festivus* mitochondria. RCR is usually measured to indicate the leak rate or ETS coupling efficiency of mitochondria (Rolf and Brown 1997). These results suggest that *M. festivus* mitochondria have a higher respiratory control ratio than *A. pallidus* mitochondria, and this feature might be related to its higher tolerance, same as observed by Hickey et al. (2012) when comparing a higher tolerant species (Epaulet shark) to a lower tolerant one (Shovelnose ray).

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

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

A brief discussion about Cichlidae phenotypic plasticity

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

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

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

Conclusion

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

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

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

Code availability Not applicable

Declarations

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

Competing interest The authors declare no competing interests.

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It is not about the size: microRNA expression influences the hypoxia tolerance of two amazon cichlid species

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Abstract

Gene expression controls several responses to hypoxia; from behavioral, to respiratory, physiological, and biochemical levels. These controls occur through regulations in pre- and post-transcriptional levels. One of the regulatory mechanisms that remain underexplored in hypoxia is the microRNA regulatory capacity, which is already known to be involved in several stress responses related to the environmental changes, such as hypoxia. In the present work, we evaluated the expression of miR-210, miR-181c, miR-18a, miR-21, and let-7d in two Amazon species with distinct hypoxia tolerance levels. Our results show that, when exposed to hypoxia, *M. festivus* changed the expression of miR-181c and let-7d in the liver, while *A. pallidus* changed the expression of miR-181c and -210 in the liver. Our results also show that the change in expression of these miRNAs is related to these fish's strategy to endure hypoxia.

Introduction

Gene expression is responsible for a variety of animal's responses; from behavioral, to respiratory, physiological, and biochemical levels. These controls occur through regulations in pre- and post-transcriptional levels. The post-transcriptional regulation is controlled by several factors, including microRNAs (miRNA) activity. MiRNAs comprehend a class of highly conserved no-coding RNAs that are synthesized in the nucleus through a canonic or alternative pathway, and participates in the regulation of gene expression by inhibiting or enhancing messenger RNAs' (mRNA) translation, by either blocking, or signaling the degradation of the mRNA (Bizuayehu and Babiak, 2014). In terms of length, miRNAs are composed by 17-22 nucleotides, and, regarding the regulation process, one miRNA can regulate several mRNAs, and one mRNA can be regulated by several miRNAs (Bartel, 2009)

Even though information is still scarce, it is already known that miRNAs are involved in the adaptations to environmental stress. The modulation process by miRNAs are involved in thermal, low oxygen, salinity, pollutant and diseases, in fishes (Bizuayehu and Babiak, 2014). When it comes to hypoxia, some microRNAs are well known in function, and are characterized as hypoxiamirs (Nallamshetty et al., 2013). For example, F_0 hypoxia exposition results in differential miRNA expression in zebrafish F_1, affecting more the F_1 when the female F_0 is exposed to this stressor (Heinrichs-Caldas et al., in prep). Acute air exposure can also change miRNA expression in different tissues, such as head kidney, liver, and plasma, bringing predicted changes to metabolic and cell signaling pathways (Cadonic et al., 2020; Ikert et al., 2021).

Oxygen availability is a determinant stressor in aquatic environments. Worldwide, aquatic environments go through hypoxic, or even anoxic events with different times and intensity (Mandic and Regan, 2018). The Amazon River basin comprehend a great

diversity of aquatic environments that suffer daily and seasonally extreme dissolved oxygen variations throughout its distinct environments, such as várzea, lakes, flooded forests, main rivers, and small forest streams (Junk et al., 1983). To survive these dissolved oxygen dynamic changes, the Amazon aquatic biota developed multiple adaptations, including genetics, biochemical, physiological, respiratory, morphological, and behavioral (Val et al., 1998); so, which of these adaptive characteristics are related to the oxygen variation where these species live (Mandic and Regan, 2018)?

Environmental stress dictates the aquatic organisms' responses and adaptations. Regarding hypoxia adaptations, the responses to oxygen availability are related to the animal tolerance and are species-specific. *Colossoma macropomum*, the Tambaqui, when exposed to hypoxia, extends its lower lip in order to get access to the most oxygenated part of the water column (Saint-Paul, 1984, Val and Almeida-Val, 1996), *Astronotus crassipinnis* and *Symphysodon aequifasciatus* depress the oxygen consumption when exposed to hypoxia (Chippari-Gomes et al., 2005). When it comes to metabolic changes, Amazonian fishes can rely on two strategies: increase the anaerobic glycolysis, and/or decrease the ATP demand by depressing the aerobic metabolism throughout the reduction of oxygen consumption (Heinrichs-Caldas et al., 2019, Hochachka and Somero 2002, Almeida-Val et al., 1993). Heinrichs-Caldas & Almeida-Val (2021) found that the hypoxia tolerance is related to the different environmental dissolved oxygen fluctuation that two cichlid species (*Mesonauta festivus* and *Aequidens pallidus*) are naturally exposed, which dictates different strategies to endure such stressor. All of these adaptations are related to the natural hypoxic stress, and are controlled by gene expression, which can be regulated by miRNA activity.

In the present work, we compare the expression of miR-181a, Let-7, miR-210, miR-21, and miR-18a in the liver of two cichlid species which present different degrees of

tolerance to hypoxia; *Mesonauta festivus* and *Aequidens pallidus*. Each miRNA was measured in both fish species exposed to low oxygen levels aiming to find out if the miRNA expression is related to the hypoxia tolerance and adaptations of each species response.

Material and methods

Experimental Fish

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

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

Hypoxia Exposure

For the experiment, both species were divided in two groups, normoxia and hypoxia (n = 6). Each animal was randomly placed in a 1.5 L individual plastic container one day before the experiment to acclimate from handling. During the acclimation, the animals were kept at 28 °C ± 0.3 with constant aeration (O_2 concentration: 6.82 mg $O_2.L^{-1}$ ± 0.12). For the hypoxia group, the oxygen concentration was slowly decreased with N_2

pump directly into the water during approximately 90 min, and, when the oxygen concentration reached 0.73 mg $O_2.L^{-1}$ ± 0.9 for *M. festivus* and 1.23 mg $O_2.L^{-1}$ ± 0.5 for *A. pallidus*, these animals were kept in hypoxia during 3 hours, while the normoxic group were kept under constant aeration (6.89 mg $O_2.L^{-1}$ ±0.27). Dissolved oxygen concentration was monitored with an Oxymeter 5512-Ft (YSI, USA, Ohio, Yellow Springs). These hypoxia oxygen concentrations were chosen according to species' critical oxygen tension; representing 70% of both species PO_{2crit}, so these conditions ensured the same degree of hypoxia to both species. Three hours under low oxygen was chosen based on a previous works, for both species, in the respirometry, to ensure that this was enough for metabolic depression and opercular movements increase, ensuring that the animals were under hypoxia (Heinrichs-Caldas & Almeida-Val, 2021). For each group and each species, six animals were sampled and euthanized by head concussion followed by a cut in the spine cord. Then, the liver was excised and stored at -80 °C until the following assays. This experiment was performed in the morning and is in accordance with CONCEA Brazilian Guide for Animals Use and Care under INPA's authorization (CEUA: protocol #054/2017).

RNA and miRNA extraction and first-strand (cDNA) synthesis

Total RNA was isolated from liver using Trizol reagent (ThermoFisher, Cat#15596026) and Qiagen miRNeasy mini kit (Qiagen, Cat# 2170004), according to manufacturer's instruction, and all samples were stored at -80 °C for further analysis. Total RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and, $A_{260/280}$ values were above 2.500 ng of RNA. These amounts were used for the subsequent cDNA synthesis. cDNA synthesis was obtained with Qiagen's miScript II Rt kit (Cat# 218161), according to manufacturer's

instruction, in order to generate miRNA-specific cDNA to be used for qPCR amplification. We used HiFlex buffer to transcribe both miRNA and RNA.

Real-time quantitative PCR

The primers used are listed in Table 1, which shows also the primer's efficiency. The amplification of gene *12S* was constant between treatments, qualifying it as appropriate housekeeping gene for this experiment. All the genes were tested for dilution and melt curve. Initial cDNA was diluted 10x to fit the standard curve and 1 µL was used in a 10 µL PCR reaction (5 µL 2x Fast SYBR^{TM} Green Master Mix (ThermoFisher, Cat# 4385612), 1 µL of 10 µM forward primer and 1 µL of 10 µM reverse primer, and 2 µL molecular grade water). The qPCR analysis was conducted with a Viia7 Dx PCR-System (Applied Biosystems, Foster City, CA, USA), which incubated samples at 95 °C for 30 s, denature at 95 °C for 10 s, anneal at 60 °C for 20 s, and then detect fluorescence (denaturation, annealing, and fluorescence detection occurred for 40 cycles). Following each run, a melt curve analysis was performed by increasing the temperature from 65 °C to 95 °C in 0.5 °C increments every 5 s, with fluorescence measurements occurring at each temperature increment. Samples were run in duplicate and only samples with a standard deviation < 0.35 between technical replicates were used. The relative quantification was calculated using $2^{-\Delta\Delta Ct}$ method and it was normalized to the geometric mean of the housekeeping genes (Livak and Schmittgen, 2001).

Results

MiRNA expression results are shown in figure 1. MiR-181c (Fig. 1A) showed differences between species ($P < 0.001$, $F = 24.914$). *M. festivus* increased 1.09-fold miR-181c expression in hypoxia ($P = 0.035$), while *A. pallidus* also decreased 1.1-fold this gene expression ($P = 0.016$). This miRNA expression was different in normoxia ($P < 0.001$) but showed no difference in hypoxia ($P=0.301$), when comparing both species. MiR-21 (Fig. 1B) showed no differences between species and treatments ($P = 0.297$, $F = 1.160$). MiR-18a (Fig. 1C) showed no differences between species ($P = 0.554$, $F = 0.346$) and treatments ($P = 0.051$, $F = 4.407$). Let-7d (Fig. 1D) showed no differences between species ($P = 0.543$, $F = 3.85$), but showed differences between treatments ($P = 0.025$, $F = 6.079$). *M. festivus* increased let-7c expression increased 1.08-fold in hypoxia ($P = 0.029$). MiR-210 (Fig. 1E) showed no differences between species ($P = 0.084$, $F = 3.402$), but showed differences between treatments ($P = 0.012$, $F = 8.030$). *A. pallidus* increased 1.1-fold miR-210 expression in hypoxia ($P = 0.028$).

Discussion

In a previous work, we described different levels of hypoxia tolerance between *M. festivus* and *A. pallidus*, showing that distinct strategies to face low oxygen concentration is related to species' habitat (Heinrichs-Caldas & Almeida-Val, 2021). *M. festivus*, the most tolerant among both species, inhabit lakes and rivers, where the oxygen concentration goes through drastic oxygen variation, daily and seasonally. As a strategy to survive hypoxia, this species depresses its metabolism, slowing down its oxygen consumption and activating the cellular anaerobic metabolism. On the other hand, *A. pallidus*, the less tolerant species, which inhabits forest streams, where the water is more oxygenated and normoxic year-round, presents a preference for the aerobic metabolism, mainly relying on the mitochondrial respiration when exposed to

hypoxia and, as already mentioned, shows low capacity to survive hypoxia compared to *M. festivus*. The subsequent part of this discussion will focus on how these distinct levels of hypoxia tolerance are related to miRNAs expression in the liver of both species.

Natural oxygen variation brings several changes in the animal metabolism and behavior, altering important processes such as gene expression and protein synthesis. When it comes to hypoxic stress, HIF-1 (Hypoxia inducible factor-1) expression acts as master regulator in metabolic changes and, consequently, hypoxia tolerance. MiR-18a shows a direct relation with *hif-1α* activity that produces HIF subunits, and relies on the oxygen concentration for its stabilization. We know from literature that acting as a direct inhibitor of *hif-1α* expression, the increase in miR-18a expression decreases *hif-1α* expression, subsequently reducing metastasis through a HIF-1A-dependent pathway (Krutilina et al., 2014; Wu et al., 2015). Studies with *hif-1α* expression in Amazon fishes showed that some species change *hif-1α* expression in order to survive hypoxia (Baptista et al., 2016; Heinrichs-Caldas et al., 2019; Silva et al., 2019). When comparing both species and treatments, miR-18a expression did not present differences between species or between oxygen conditions (Fig. 1C). We hypothesize that the maintenance of miR-18a levels induces to the lack of regulation in *hif-1α* expression, which is needed to face hypoxia in nature in the Amazon fishes. In prior works we could show, as above mentioned, that animals exposed during 3 hours to hypoxia presented increases on *hif-1α* expression.

Hypoxia exposure did not change miR-21 (Fig. 1B) expression in the present work either. This miRNA shares with others studied miRNAs several functions in controlling hypoxia responses, such as apoptosis, cell cycle and cell survival (Nallamshetty et al., 2013). This miRNA expression is related to inflammatory responses, increases in

angiogenesis, and works directly with *hif-1α* and *vegf* expression (Liu et al., 2011; Xu, 2019). Angiogenesis is a pivotal response to hypoxia since tissue oxygen maintenance is related to vascular development (Risau and Flamme, 1995). We believe that the lack of responses of miR-21 in the present work reflects an adaptation to hypoxia in fishes of the Amazon, since they deal with variation in oxygen levels and the hypoxia event is not a definite state, as in other cases of hypoxic cells induced by diseases. Even though miR-21 expression did not change in the present work, other miRNAs are also related to angiogenesis, like miR-210 and miR-181, which shows that one pathway can be affected by several miRNAs (Ivan and Huang, 2014).

Several hypoxia responses are related to miR-210 expression, such as cell proliferation, mitochondrial respiration, vascularization, angiogenesis, and DNA repair (Chan et al., 2012), features that make this miRNA one of the most studied when it comes to hypoxia events (Ivan and Huang, 2014). In the present work, miR-210 expression increased in *A. pallidus* liver after exposure to hypoxia (Fig. 1E), and this could be related to this species lower tolerance to hypoxia. An increase in the expression of miR-210 may result in a down-regulation of several predicted pathways, which are important to hypoxia tolerance. However, when miR-210 increases and presents over-expression, it brings protective responses to hypoxia exposition. The marine medaka (*Oryzias melastigma*) showed an increase in this gene expression as a protection against cell apoptosis (Tse et al., 2015), while the up-regulation of this microRNA in *Litopenaeus vannamei* showed several inductions of hypoxia responses in different cell types (Wang et al., 2019). The results observed in the present work show that *A. pallidus* increase in miR-210 expression is related to its low tolerance to hypoxia, and acts as a protective manner, since this animal will not show a variety of adaptations related to low oxygen. Even though this miRNA is known as the master hypoxiamir, *M. festivus* did not change

its expression in hypoxia. Herein, we hypothesize that this is species relies on other genetic mechanisms in order to regulate the cell metabolic shift and oxygen consumption observed in our previous work.

The miR-181c is the other miRNA that changed its expression in the present work. This miRNA expression increased in the liver of the hypoxia tolerant species *M. festivus*, and decreased in *A. pallidus*, which is not tolerant to the lack of oxygen (Fig. 1C). MiR-181 family, among other functions, is known as a nuclear encoded miRNA that acts in the mitochondria, affecting mitochondrial function and regulating mitochondrial gene expression (Das et al., 2014). In our previous work, *A. pallidus* changed mitochondrial oxygen affinity, demonstrated by the change in mitochondrial P_{50} and its catalytic efficiency while *M. festivus* did not change mitochondrial respiration (Heinrichs-Caldas and Almeida-Val, 2021). These opposite responses presented by miR-181c expression in the liver of both species under hypoxia, reveal that mitochondrial respiration changes are controlled by this miRNA expression in particular, since it is related to hypoxia tolerance, showing the relation of miRNA expression and hypoxia tolerance.

Taken together the results of miR-210 and -181c expression in *A. pallidus* and *M.festivus* liver one can hypothesize that miR-210 increase, combined with the miR-181c decrease, resulted in some increase of angiogenesis for the species. Angiogenesis is essential to increase the oxygen delivery to the tissues. MiR-210 overexpression increases angiogenesis in adult mouse brain (Zeng et al., 2014), while the suppression of miR-181c expression was demonstrated to increase the vascular formation and the expression of *vegfa* (Solly et al., 2019). Our previous results with this species for mitochondrial respiration show dependence between the cell function maintenance with oxygen availability in water, what would be sustained by angiogenesis in the cell, as we observed in the expression pattern of miR-210 and -181c in the present work.

The present results show an increase in let-7d expression in *M. festivus'* liver in hypoxia. The let-7 miRNA family function is usually related to tumorigenesis and development, and its function in response to hypoxia remains poorly understood, even though some works already showed that the hypoxia exposition results in different patterns, depending on the cell-type being studied (Kolenda et al., 2014; Kulshreshtha et al., 2008, 2007). In relation to metabolism, it's known that the overexpression of this miRNA regulates the glucose metabolism (Zhu et al., 2011), which can be related to the strategy adopted by *M. festivus* when exposed to hypoxia. When exposed to hypoxia, this animal decreases the oxygen consumption and increases anaerobic glycolysis, which results in this animal's higher tolerance to hypoxia compared to *A. pallidus*. Thus, we hypothesize that the increase in let-7d expression is related to the metabolic shift presented by this species when exposed to hypoxia in our prior work (Heinrichs-Caldas and Almeida-Val, 2021).

Conclusion

Hypoxia tolerance seems to be linked to several metabolic changes and strategies, varying in a tissue- and species-specific manner. As stated before, one microRNA can regulate several pathways, and a pathway can be regulated by several microRNAs, which is shown by the miRNAs' expression pattern observed in the present work. When comparing two cichlid species with different hypoxia tolerance levels acclimatized to environments with distinct oxygen concentration, we observed a differential expression of miRNAs. *A. pallidus* changed the expression of miR-181c and -210 in the liver during hypoxia exposition and these changes might result in predicted phenotypic changes related to mitochondrial respiration and angiogenesis, both features that are related to this species non tolerance to hypoxia. Meanwhile, when exposed to hypoxia, *M. festivus* changed the expression of miR-181c and let-7d, showing a relation between

miRNAs expression and this animal's strategy to change the metabolism from aerobic to anaerobic when exposed to hypoxia. Taken together, these results show that miRNA expression influences in the hypoxia tolerance of both species studied in this work, been necessary further *in silico* analysis in order to predict the metabolic pathways that might be regulated by these miRNAs.

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Table

Table 1. MicroRNAs used for both species for qPCR analysis. The % represents the primer efficiency.

Species	Target	Forward primer sequence	Reverse primer sequence	Efficiency%
A. pallidus	Let-7d-5p	TGAGGTAGTAGGTTGTATAGTT	Qiagen Universal Primer	98.7
	miR-181c-5p	AACATTCAACGCTGTCGGTGAG		98.0
	miR-21	TAGCTTATCAGACTGGTGTTGGC		96.5
	miR-18a	TAAGGTGCATCTAGTGCAGATA		96.6
	miR-210-5p	AGCCACTGACTAACGCACATTG		99.8
	12S	TCGTCGGCAGCGTCAGATGTG	GTCTCGTGGGCTCGGAGATGTG	96.01
M. festivus	Let-7d-5p	TGAGGTAGTAGGTTGTATAGTT	Qiagen Universal Primer	97.2
	miR-181c-5p	AACATTCAACGCTGTCGGTGAG		96.1
	miR-21	TAGCTTATCAGACTGGTGTTGGC		99.3
	miR-18a	TAAGGTGCATCTAGTGCAGATA		95.8
	miR-210-5p	AGCCACTGACTAACGCACATTG		99.4
	12S	AAGAGACAGGTCGGTAAAACTC	CAGCATAGTGGGGTATCTAATC	104.8

Figures

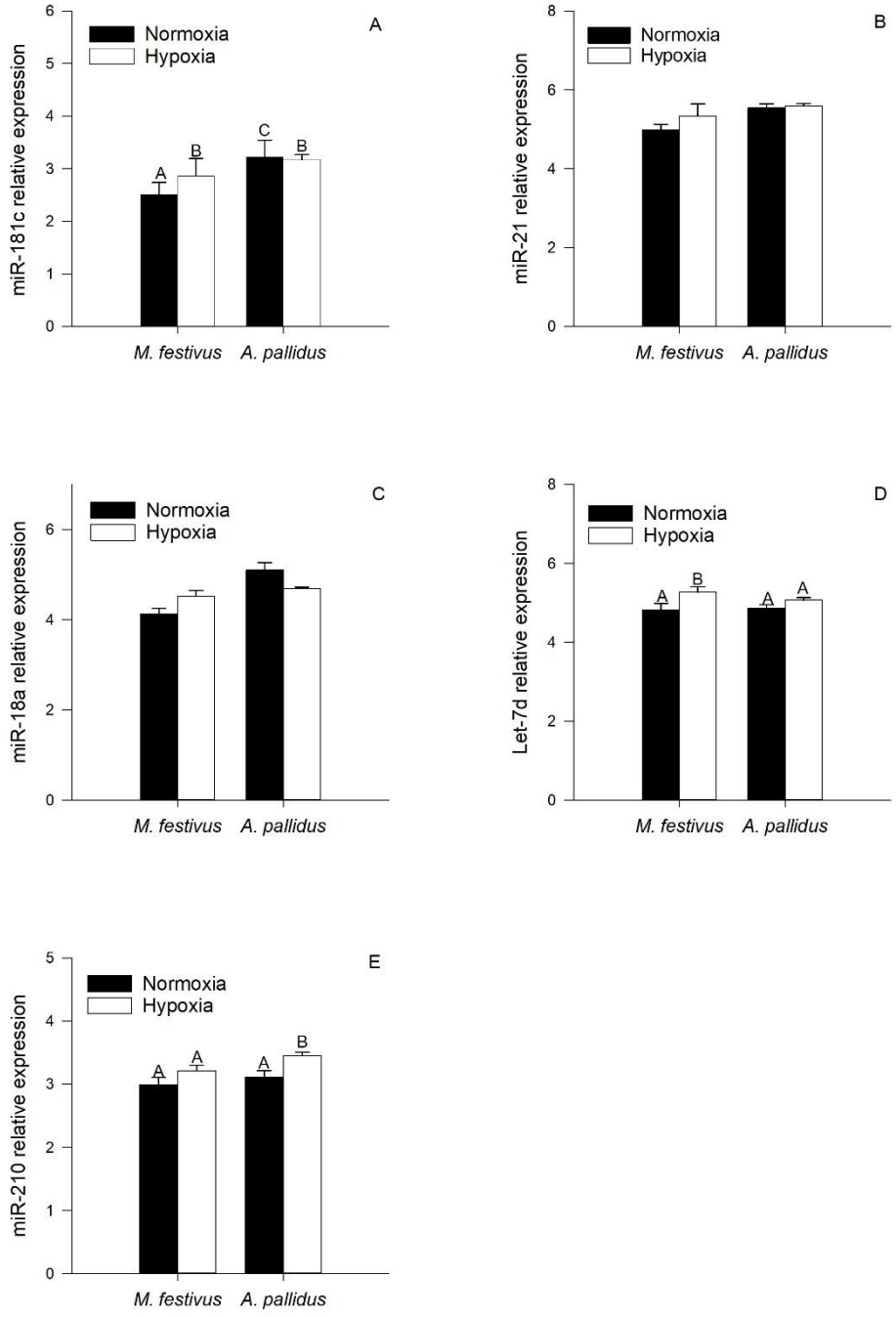

Figure. 1. Relative expression of miR-181c (A), miR-21 (B), miR-18a (C) Let-7d (D), and miR-210 (E) in the liver of *Mesonauta festivus* and *Aequidens pallidus* exposed to normoxia and 3 hours of hypoxia. The statistical significance was analyzed using a Repeated Measure Tow-Way ANOVA. Different letters indicate differences between

species and treatments ($P<0.05$). Deviation bars represent the standard error of the mean.

Sex matters: Gamete-specific contribution of microRNA following parental exposure to hypoxia in zebrafish.

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Abstract

Oxygen availability varies among aquatic environments, and oxygen concentration has been demonstrated to drive behavioral, metabolic, and genetic adaptations in numerous aquatic species. MiRNAs (miRNAs) are epigenetic mechanisms that act at the interface of the environment and the transcriptome and are known to drive plastic responses following environmental stressors. An area of miRNA that has remained underexplored is the sex specific action of miRNAs following hypoxia exposure and its effects as gene expression regulator in fishes. This study aimed to identify differences in mRNA and miRNA expression in the F_1 generation of zebrafish (*Danio rerio*) at 1 hpf after either

F_0 parental male and female were exposed to 2 weeks of continuous (45%) hypoxia. In general, F_1 embryos at 1 hpf demonstrated differences in mRNA and miRNAs expression related to the stressor and to the specific sex of the F_0 that was exposed to hypoxia. Bioinformatic pathway analysis of predicted miRNA:mRNA relationships indicated responses in known hypoxia signaling and mitochondrial bioenergetic pathways. This research demonstrates the importance of examining the specific male and female contributions to phenotypic variation in subsequent generations and provides evidence that there is both maternal and paternal contribution of miRNA through eggs and sperm.

Introduction

Changes in oxygen concentration occurs in most of the aquatic environments of the world with different duration and intensity, from anoxia to hypoxia, and even hyperoxia (Mandic and Regan, 2018). The oxygen availability in these systems is affected by natural and anthropogenic influences, bringing a myriad of responses and adaptation of the aquatic species present (Hochachka & Somero, 2002). The adaptations to anoxic and hypoxic environments range from genetic to metabolic, respiratory, and behavioral responses (Anjos et al., 2008; Heinrichs-Caldas et al., 2019), and vary according to the intensity and duration of the exposure, which can stimulate different adaptative responses. Distinct physiological strategies are used to cope with constant and intermittent hypoxia. Killifish (*Fundulus heteroclitus*), when exposed to hypoxia, decreases their critical O_2 tension, resting O_2 consumption rate, and reduces the O_2 concentration at loss of equilibrium (Borowiec et al., 2015). Meanwhile, the killifish has been shown to regulate mitochondrial physiology to decrease reactive oxygen species (ROS) production, after hypoxia acclimation, in order to increase the hypoxia tolerance (Borowiec et al., 2015). When exposed to 30 days of constant and

cycling hypoxia, *Spinibarbus sinensis* increases their hypoxia tolerance by decreasing the PO_{2crit} (the ambient oxygen tension at which a fish can no longer maintain aerobic respiration), showing that hypoxia acclimation can even be beneficial for this species growth performance (Dan, 2014). Goldfish (*Carassius auratus*) have also been shown to increase hypoxia tolerance when exposed to both hypoxia and sustained exercise, by decreasing the PO_{2crit}, increasing gill lamellar surface area, and increasing red blood cell hemoglobin concentrations (Fu et al., 2011). The differences in hypoxia tolerance can also be related to sex specific response; male zebrafish, after 48 hours in hypoxia (10% O_2 levels) increased their hypoxia tolerance 9-fold, while females in the same conditions, increased only 3-fold, effectively demonstrating that there is a sex-specific hypoxia acclimation and tolerance response (Rees et al., 2001). Ho and Burggren (2012) showed that, after the exposure of zebrafish F_0 to two weeks in hypoxia, the embryos (F_1) developed a higher hypoxia tolerance, with an increase in the time of loss of equilibrium in hypoxia, although the specific contribution of males or females to these phenotypic responses was not explored. Combined, these studies demonstrate that hypoxia acclimation induces different sex-specific biochemical and physiological responses, which varies according to hypoxia intensity and duration.

Most biochemical and physiological adaptations to hypoxia are controlled by transcriptional responses, with hypoxia-inducible factor-1 (HIF-1) being the main contributor in maintaining O_2 homeostasis (Semenza 2001). HIF-1 is a heterodimer composed by two subunits (α and β) that binds to specific sites in the DNA to accelerate the transcription of oxygen regulated genes (Semenza, 2000) and controls cell responses to hypoxia through the activation of other genes like *vegf* (vascular endothelial growth factor) in order to stimulate the angiogenesis (Shweiki et al., 1992). Furthermore, two subunits, *hif-1α* and *hif-2α*, have been shown to be essential in animal development and

cellular control of O_2 homeostasis (Iyer et al., 1997; Compernole et al. 2002). In fish, HIF-1a plays an essential role in the hypoxic ventilatory responses in early larval stages of zebrafish that likely contributes to the reduction in critical O_2 (Mandic et al 2020). At early developmental stages, zebrafish exposed to 4 hours hypoxia increases hsp70a gene expression, which can act as a protective measure against this stressor (Levesque et al., 2019). This suggest that HIF-1, *vegf*, and *hsp70* are critical in early life history development and response to hypoxia, although little information exists on the parental contribution of these transcripts upon the developing embryo.

Another factor that remains underexplored is the actions of the microRNAs (miRNAs) under hypoxia exposure and its capacity as a gene expression modulator. MiRNA is a noncoding RNA class, composed by 17-22 nucleotides in length and act as epigenetic modulators, which affect protein levels of target mRNAs without modifying the gene sequence (Yao et al., 2019). The regulation by miRNAs can act as an inhibitor of messenger RNA (mRNA) translation (Bizuayehu and Babiak, 2014), this regulation occurs by biding the miRNA to a mRNA which can either block the translation or signal the degradation of the mRNA. In the regulation process, one miRNA can target several mRNAs, and several miRNAs can target one mRNA (Bartel, 2009). Under hypoxia, some miRNAs are well known in function, and are characterized as hypoxamirs (Nallamshetty et al., 2013). These miRNAs are induced in response to low oxygen and hypoxia-inducible-factor mechanisms, and act as proapoptotic signals, induce metabolic reprogramming, angiogenesis and several other cellular adaptations to hypoxia (Bizuayehu and Babiak, 2014; Nallamshetty et al., 2013; Pocock, 2011).

MiRNAs also have functions in animal development and show a transgenerational effect (Luu et al., 2021). Even though the mechanism remains unclear, paternal exposure to different environmental stresses stimuli epigenetic marks in germ

cell, including miRNAs, which impacts directly in the biology of the next generation (Rodgers et al., 2015). Epigenetic changes can remain for at least one generation, and can persist even after the stressor is no longer present, which depends on the environmental stressor being studied (Casier et al., 2019). During development, studies have shown that miRNAs are essential for the normal development; the lack of mature miRNAs in mouse embryonic stem cells impairs the cell from differentiating, *in vivo* and *in vitro* (Kanellopoulou et al., 2005). Zebrafish (*Danio rerio*) lacking Dicer1, the miRNA-processing enzyme, suffer a developmental arrest, showing how the miRNAs are essential for this species development (Wienholds et al., 2010). Therefore, miRNAs can be inherited and play an important role in development.

Knowing that exposure to hypoxia alters phenotypic straits, and these traits that might be controlled by genetic and epigenetic regulation, the objective of this study was to investigate miRNA expression in F_1 zebrafish one-hour post fertilization (1hpf), after either male and female F_0 fish were exposed to two weeks of hypoxia. This allows us to understand the sex-specific paternal and maternal influences on miRNA expression in F_1 embryos, and, by predictive miRNA:mRNA bioinformatic analysis, how it can influence the phenotypic response to hypoxia.

Material and methods

Animal care and experiment

Mixed sex adult zebrafish (*Danio rerio*) were purchased from Big Al's Aquarium Supercenters (Kitchener, ON) and housed in an AHAB (Pentair) recirculating water system with controlled temperature (27^{o}C, pH 7.3, 525 μS/cm). Breeding pairs were established prior to exposure, and breeding pairs that produced more than 100 eggs were selected for the exposure. Pairs were identified and separated into two groups; one

group the female was exposed to hypoxia, and in the other group the male was exposed (Figure 1). Prior to placement into the experimental hypoxia and normoxia tanks, these couples were bred and the eggs were sampled 1 hour after breeding to acquire the pre-hypoxia group. Following, the fish were exposed to hypoxia for two weeks. There were 5 males and 5 females caged individually in the hypoxia tanks (O_2: 3.98 $mgO_2.l^{-1}$ ± 0.9, T= 27 °C ± 0.5), and 5 males and 5 females caged individually in normoxia tanks (O_2: 8.7 $mgO_2.l^{-1}$ ± 0.8, T= 27 °C ± 0.4). The fishes were kept separated to make sure that the same couples would be bred after the exposure. After two weeks, the pre-assembled couples were put back together for another breeding and the eggs were sampled again 1 hpf, characterizing the post-hypoxia groups. During the breeding and the hypoxia exposure, the couples were fed twice a day with GEMMA Micro 300 (Skretting, St. Andrews) zebrafish food. The light cycle used to stimulate the breeding was 14h:10h light:dark.

All experimental procedures were approved and carried out in accordance with the University of Waterloo Animal Care and Canadian Council of Animal Care guidelines (Animal Utilization Project Protocol #40989).

RNA extraction and first-strand (cDNA) synthesis

Total RNA was isolated from 1 hpf embryos (± 100 eggs from each couple) using Qiazol reagent (Qiagen, Cat#79306) and Qiagen miRNeasy mini kit (Qiagen, Cat# 2170004), according to manufacturer's instruction, and all samples were stored at -80 °C for further analysis. Total RNA concentration was determined using a SpectraDrop Micro-Volume Microplate (Molecular Devices) and to ensure that $A_{260/280}$ values were above 2. Next, 50 ng of RNA was used for cDNA synthesis using Qiagen's miScript RTII kit (Cat# 218161). The HiFlex buffer protocol was used in order to transcribe both miRNA and RNA.

Real-time quantitative PCR

The primers used are specific to *Danio rerio* and are listed in Table 1, together with the primer's efficiency`. All the miRNAs chosen for this work are based in their function as hypoxiamirs. The amplification of housekeeping genes (*18S* and *eif1α*) was constant between treatments qualifying them as appropriate housekeeping for this experiment. All the genes were tested for dilution and melt curve. Initial cDNA was diluted 10x to fit the standard curve and 1 µL was used in a 10 µL PCR reaction (5 µL 2x SsoAdvanced Universal SYBR Green Supermix (BioRad Laboratories, Canada), 1 µL of 10 µM forward primer and 1 µL of 10 µM reverse primer, and 2 µL molecular grade water). The qPCR analysis was conducted with a BioRad CFX96 Touch Thermal Cycler (Bio-Rad Laboratories, Canada), which incubated samples at 95 °C for 30 s, denature at 95 °C for 10 s, anneal at 60 °C for 20 s, and then detect fluorescence (denaturation, annealing, and fluorescence detection occurred for 40 cycles). Following each run, a melt curve analysis was performed by increasing the temperature from 65 °C to 95 °C in 0.5 °C increments every 5 s, with fluorescence measurements occurring at each temperature increment. Samples were run in duplicate and only samples with a standard deviation < 0.35 between technical replicates were used. The relative quantification was calculated using $2^{-\Delta\Delta Ct}$ method and it was normalized to the geometric mean of the housekeeping genes (Livak and Schmittgen, 2001).

Bioinformatic analysis of targets for microRNAs

To analyze possible physiological effects of altered miRNAs in this work, the miRanda algorithm was used in order to determine each mRNA target for each of the miRNAs (Enright et al., 2003; Ikert et al 2021). First, mature miRNAs sequence was input into the miRanda tool and compared against a curated list of *Danio rerio* 3' untranslated region (UTRs). The mRNA targets identified using miRanda were filtered to only

include matches with a pairing score greater than or equal to 140 and free energy score (ΔG) less than or equal to −20 (Kostyniuk et al., 2019). A higher paring score is associated with increased complementarity between the miRNA and target 3' UTR while a lower ΔG is more energetically favorable for binding. Once mRNA targets were identified, these were converted from NCBI identifiers to UniprotKB identifiers using the Retrieve/ID mapping tool (www.uniprot.org/uploadlists). The analysis was conducted for miRNAs that were significantly altered in females or significantly altered in males. KEGG and DAVID were used to analyze pathways impacted or trends in their expression. This methodology was based on previous work by Cadonic et al. (2020) and Ikert et al (2021).

Statistics

Data are expressed as mean ± S.E.M. The normality and homogeneity of the variances were tested before parametric testing. Relative expression of the genes was examined by a Repeated Measures Two-Way ANOVA, followed by a Tukey post-hoc test. Statistical analyses were performed in SigmaStat (v. 3.5) and the graphs were built in SigmaPlot software (v. 11.0).

Results

mRNA expression

Heat shock protein 70 (*hsp70*) showed no difference between sexes (P=0.833, F=0.0734). Both male F_0 exposed to hypoxia embryos (P=0.018) and female F_0 exposed to hypoxia embryos (P=0.018) increased (1.4-fold male, 1.5-fold female) *hsp70* gene expression (Figure 2A). *Hif-1α* expression showed no difference between groups (P=0.59, F=0.300), but showed difference between treatments, with male F_0 exposed to hypoxia increasing the expression 1.8-fold in the F_1 embryos (P=0.030; Figure 2B).

Vegf showed difference between groups (P<0.001, F=31.232). Both male F_0 exposed to hypoxia embryos (P=0.016) and female F_0 exposed to hypoxia embryos (P=0.001) increased (5-fold male, 7.5-fold female) gene expression (Figure 2C).

MicroRNA expression

MiR-210 showed difference between sex and treatment (P<0.001, F=25,990; Figure 3A). Female F_0 group exposure to hypoxia increased 2.4-fold the embryos gene expression after hypoxia (P<0.001), while male F_0 exposed group showed no changes (P=1.37). Both groups embryos also showed difference in hypoxia (P<0.001). Let-7d expression showed difference between both groups and treatments (P=0.005, F=14.317). The female F_0 exposed to hypoxia embryos increased 1.9-fold the expression after hypoxia exposure (P=0.001), while the male F_0 group embryos increased 2.9-fold (P<0.001). The groups also showed difference after hypoxia exposure (P<0.001). miR-21 showed differences between normoxia and hypoxia (P=0.017, F=8.96). Female F_0 exposed to hypoxia embryos increased miR-21 expression 1.6-fold in hypoxia (P=0.016), while the male F_0 group embryos showed no difference between treatments (P=0.271). miR-29a expression increased after the F_0 hypoxia acclimation in F_1 embryos (P<0.001, F=25.860). Both male F_0 hypoxia group embryos (P=0.004) and female F_0 hypoxia embryos group (P=0.014) increased gene expression (5.4-fold female, 6.7-fold male) after hypoxia acclimation. miR-1 expression showed difference between groups and treatments (P=0.039, F=6.042). Female F_0 exposed to hypoxia decreased 3.3-fold embryos F_1 gene expression in hypoxia (P=0.007) and was lower than the male F_0 group embryos (P=0.005), which showed no difference between treatments (P=0.924). miR-181c showed no difference between groups and treatments (P=0.194, F=2.007). Female F_0 exposed to hypoxia embryos increased 1.8-fold the gene expression in hypoxia (P=0.043) and was higher than male F_0 exposed to hypoxia

embryos (P=0.030), which showed no difference between normoxia and hypoxia (P=0.700). miR-204 expression showed no difference between groups and treatments (P=0.257, F=1.49). miR-206 expression showed no difference between groups and treatments (P=0.931, F=0.0134).

MicroRNA target analysis

The miRNA that changed the expression (Let-7d, miR-210, -21, -29a, -181c) after the hypoxia treatment were used for mRNA target prediction. For miRanda analysis, the miRNAs were divided in two groups, the ones altered in F_1 after female F_0 was exposed to hypoxia, and the ones that showed difference in F_1 after male F_0 was exposed to hypoxia. Resulting from miRanda algorithm, let-7d targeted 868, miR-210 targeted 569, miR-21 targeted 665, miR-29a targeted 458, and miR-181c targeted 1029 mRNA. Figures 4 and 5 show the most affected pathways (>10%) by both groups, and some pathways related to hypoxic stress, using the KEGG pathway analysis.

DAVID enrichment analysis for the miRNA group from F_1 embryos from female F_0 exposed to hypoxia showed that the transcripts targeted are mostly related to binding lipids and proteins, cytoskeleton organization, epidermal growth factor-like domain, citrate cycle, guanylate kinase, transcription factors, and metabolic pathways. Meanwhile, the same analysis, using the miRNA group from the F_1 embryos from male F_0 exposed to hypoxia, showed these miRNAs target mainly transcripts related to RNA recognition motif, DNA-binding transcription factor activity, epidermal growth factor, cytoskeleton organization, histone H3-K27 demethylation, and metabolic pathways. All this groups showed an enrichment score less than or equal to -1.3 (equivalent to p 0.05).

Discussion

This study aimed to identify differences in mRNA and miRNA expression in F_1 of zebrafish (*Danio rerio*) after either F_0 male or female fish were exposed to 2 weeks of hypoxia. In general, F_1 embryos showed differences in mRNA and miRNAs expression related to the stressor and to the sex of F_0 being exposed to hypoxia. The pathway analyses revealed some phenotypes that might be affected by the changes in miRNA expression.

mRNA expression

F_0 hypoxia exposure resulted in changes in mRNA expression in 1hpf F_1 fish, and some mRNA showed differences related to the gender exposed to this stressor in F_0. *Hif-1α* expression increased in F_1 embryos 1hpf in the groups that the males were exposed to hypoxia in F_0. HIF-1 is a protein that regulates most of metabolic changes related to hypoxia, having its subunit, *hif-1α*, as a dependent in oxygen availably in order to stabilize the HIF-1 protein (Semenza, 2000). The increase in the expression of *hif-1α* in the embryos from male group exposed to hypoxia might be related to the different tolerance between the genders. Rees et al (2001) demonstrated that after hypoxia exposure, male zebrafish were more tolerant to hypoxia than the females. Other studies have shown that the *hif-1α* capacity to activate anaerobic metabolism, a critical response to hypoxia, might be related to the species hypoxia tolerance (Baptista et al., 2016; Dzhalilova and Makarova, 2020; Heinrichs-Caldas et al., 2019). Mandic et al., (2020) showed that the knockout of *hif-1α* paralogues decreased the hypoxia tolerance in zebrafish larvae and adults, reflecting in the decrease of larvae PO_{2crit} and adult time to loss of equilibrium in hypoxia (LOE), showing that for this species, tolerance to hypoxia is dependent on *hif-1α* expression. Taking together, we hypothesize that the increase *hif-1α* expression in the embryos from the F_0 exposed male group might be

related to the increased tolerance of males, when comparing to females, although this requires further investigation.

Hif-1α expression, and the stabilization of HIF-1, control the activation of several pathways related to hypoxia, including the expression of *hsp70* and *vegf*. In this study, *hsp70* expression increased in relation to hypoxia pre-exposure of F_0 for both groups, male and female F_0 exposed. *Hsp70* is an anti-apoptotic gene that promotes cell survival under several stressors, including hypoxia (Benjamin and McMillan, 1998), and its increase is related to hypoxia tolerance (Jain et al., 2014). *Vegf* is a gene responsible for angiogenesis and its expression results in new blood vessels, which facilitates increased blood flow, the capacity of blood to carry oxygen (Liu et al., 1995), and, under hypoxia, it influences additional aspects of angiogenesis, like vessel patterning, maturation, and function (Krock et al., 2011). Here, *vegf* expression increased post-hypoxia exposure, an adaptation observed in other fishes species such as the hypoxia tolerant species *Astronotus ocellatus* (Baptista et al., 2016), in juveniles of *Epinephelus coioides* (Yu et al., 2008), and *Notothenia coriiceps* (Borley et al., 2010). Functionally in zebrafish, its expression influences blood cell formation during development (Liang et al., 2001).

As the genes discussed above, *hif-1α* also plays a role in animal development (Minet et al., 2000). For example, the knockout of *hif-1α* in mice causes developmental defects, resulting in embryonic or perinatal death (Iyer et al., 1998). In zebrafish, the *hif-1* family is involved in embryonic and larval tissue differentiation and organ formation, and the gene expression pattern observed in this work was influenced by hypoxia exposure, affecting mainly the HIF-1α protein expression (Köblitz et al., 2015). These results show that the increase in *hif-1α* expression in embryos from the male group exposed to hypoxia may also influence embryo development, and furthermore, early life history tolerance to hypoxia.

The increase in the expression of *hif-1α*, *hsp70* and *vegf* indicates an increase in the responses related to hypoxia tolerance in the F_1 embryos after the F_0 was exposed to this stressor. This shows that the exposure to hypoxia in the previous generation influences the responses of the next, and these responses might be related to the hypoxia tolerance of this species.

MicroRNA expression

MiRNA expression was altered in F_1 fish after the F_0 fish were exposed to hypoxia, with different trends related to the F_0 sex exposed to hypoxia. Mir-210 expression increased in the embryos from females exposed to hypoxia, while the male group showed no changes. Mir-210 is a miRNA known for its pivotal role in regulating hypoxia responses (Ivan and Huang, 2014), such as cell proliferation, repression of mitochondrial respiration, DNA repair, vascular biology, and angiogenesis (Chan et al., 2012). Mir-210 expression increased in serum of olive flounder (*Paralichthys olivaceus*) in response to hypoxia, which was accompanied by the increase in *hif-1α* expression (Abdellaoui et al., 2020). In ovarian follicular cells of marine medaka (*Oryzias melastigma*), the increase in miR-210 expression acts as a protective measure against ovarian cell apoptosis, which verifies this miRNA function as anti-apoptotic (Tse et al., 2015). Mir-210 increase has also been shown to facilitate physiological adaptation to hypoxia in *Litopenaeus vannamei*, with a drastic induction of responses to hypoxia in various cell types (Wang et al., 2019). Taken all this data together, the increase in miR-210 observed in our work show a protective and adaptative function for the F_1 fish after F_0 hypoxia exposure.

In the present work, let-7d expression increased in both groups of embryos after the F_0 female and male exposure to hypoxia. Even though its function remains poorly understood, the let-7 miRNA family, the most abundant miRNA family, functions in

developmental and hypoxia responses, with differing expression patterns depending on the cell-type being analyzed (Kolenda et al., 2014; Kulshreshtha et al., 2008). During development, let-7d expression increased in embryos of *Amphilophus citrinellus*, resulting in the downregulation of its target, the *igdcc3*, a gene that affects motor coordination (Franchini et al., 2019). Additionally, the let-7 family overexpression regulates aerobic glycolysis, showing a metabolic regulation role for this miRNA (Zhu et al., 2011).

MiR-21 expression increased in F_1 embryos from female F_0 exposed to hypoxia. This miRNA presents several functions in regulating genes the are related to hypoxia adaptation, such as mitochondrial function, apoptosis, cell cycle and cell survival (Nallamshetty et al., 2013). Xu et al., (2019) showed that the increase in miR-21 expression regulates inflammatory responses in a teleost fish, *Vibrio anguillarum*, by targeting IL-1 receptor-associated kinase 4 (IRAK4), while Liu et al., (2011) showed that miR-21 increases induces angiogenesis, alongside *hif-1α* and *vegf* gene expression. The increased expression of miR-21 that was measured, combined with the functions of other miRNAs, might suggest an increase in angiogenesis in F_1 zebrafish embryos after F_0 exposure to hypoxia.

Mir-29a expression increased in F_1 embryos from both treatments, showing that the previous hypoxia exposure of F_0 effects the F_1 expression, regardless of the sex exposed to this stressor. The miR-29 family acts as an antifibrotic in the heart, kidney, and other organs, also showing to be proapoptotic and having control over cell differentiation (Heid et al., 2017; Kriegel et al., 2012), suggesting a protective effect in the embryos.

Mir-181c acts in the regulation of bioenergetics pathways and is also known as a mitoMir, since it is involved in mitochondrial regulation (Das et al., 2014; Macgregor-Das and Das, 2018). Our results indicate that after the female F_0 exposure to hypoxia,

had an increase in miR-181c expression in the F_1 embryos. This miRNA expression combined with others miRNAs works in the increase of angiogenesis function (Kulshreshtha et al., 2008), which we saw directly in our results with *vegf* expression.

Mir-1 is the only miRNA that had its expression decreased in F_1 after the female F_0 was exposed to hypoxia. This miRNA plays a pivotal role in the development and health of mammals, acting in skeletal and cardiac muscles, nerves, adipose tissue, and even immunological cells (Mitchelson and Qin, 2015). A study investigating studying the interaction between miR-1 and -206 during zebrafish development showed that the expression of these two miRNAs negatively regulates angiogenesis by reducing the levels of *vegfa* (Stahlhut et al., 2012). Taken together with the increased in expression in *vegf*, which seems to be associated with the decrease in miR-1 expression. However, we measured no difference in miR-206 expression in the present study, therefore the individual roles of miR-1 and miR-206 in regulating *vegf* expression can be further studied.

MiR-204 and miR-206 showed no differences between treatments in our present work. Both miRNAs were chosen for this study based on their protective role in hypoxia adaptation. MiR-204 responds to hypoxia in order to regulate autophagy and apoptosis (Qiu et al., 2018; Wang et al., 2016) whereas miR-206 stimulates the cell-cycle, enhances cardiac regeneration, and has respiratory adaptative function in mammals (Zhang et al., 2013, 2016). Some of the functions of these miRNAs are shared by some others studied in the present work. Our results show that different miRNAs that regulate the same pathway, like angiogenesis and apoptosis, show different patterns in expression. MiRNA expression was more impacted in F_1 embryos resulting from the F_0 female exposed to hypoxia, which would result in phenotype changes in the next generation.

Target predictions and hypoxia responses

The results for target predictions are divided in two groups, the miRNAs that increased in F_1 after the F_0 female was exposed to hypoxia (let-7, miR-210, -21, -29a, -181), and the miRNAs that increased after the F_0 male was exposed to hypoxia (miR-29a, let-7).

The results from KEGG and DAVID showed that both groups had their anaerobic/aerobic metabolic pathways, angiogenesis, apoptosis, autophagy and genetic mechanisms affected by the changes in miRNA expression. The embryos from female F_0 exposed to hypoxia changed specific metabolic pathways that are related to hypoxia adaptations, such as the TCA cycle, pyruvate metabolism, insulin signaling pathways, and glycolysis/gluconeogenesis, which are involved in the cellular metabolic switch from aerobic to anaerobic, a crucial feature that allow the fishes to endure and tolerate hypoxia exposure (Hochachka and Somero, 2002, Storey, 2004). In relation to metabolic responses to hypoxia, the embryos from male F_0 exposed to hypoxia also changed its responses, but to a lower degree (%), which might be related to the changes in expression of only miR-29a and let-7.

Another important pathway related to hypoxia is angiogenesis. In this work, both sexes predicted that angiogenesis would be regulated by the miRNA changes, by regulating the VEGF signaling pathway. As discussed before, several fish species regulate *vegf* expression, the same as observed in this work, showing this gene's function in development and hypoxia tolerance (list a couple of your previous refs here). The autophagy and endocytosis pathways were also predicted to be affected by both male and female groups. Autophagy and endocytosis in hypoxia are known to be a protective mechanism, in order to recycle the cellular components (Naves et al., 2013). For example, the inhibition of autophagy increases apoptosis and cell death under hypoxia (Naves et al., 2013). All these *in silico* predictions point to hypoxic adaptation in both

sexes, with higher degrees in the F_1 embryos from F_0 female exposed to hypoxia. This shows that miRNA responses are related to the zebrafish sex and its tolerance to hypoxia.

Conclusion

The present worked showed an increase in most of the studied miRNAs after the F_0 zebrafish were exposed to hypoxia. This work provides evidence of the transgenerational effects of miRNAs and how they act differently when one sex experiences the stressor. This exposure sheds light on the sex-specific parental influence on passing on adaptive phenotypes for zebrafish survival under hypoxia. Future work should focus on how long the changes in miRNA expression remain through generations and how they affect the development of this species. Furthermore, investigation can determine if the changes predicted *in silico* are reflected in genetic, physiological and biochemical responses, by analyzing the respiration and cellular adaptations following hypoxia exposure.

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Tables

Table 1. MiRNA and mRNA primers sequence used for Real-Time PCR, followed by its efficiency.

Target	Foward primer sequence	Reverse primer sequence	Efficiency%
dre-let-7-7d-5p	UGAGGUAGUUGGUUGUAUGGUU	Qiagen Universal Primer	99.5
dre-miR-181c-5p	AACAUUCAACGCUGUCGGUGAGU		97.4
dre-miR-1	UGGAAUGUAAAGAAGUAUGUAU		104.4
dre-miR-21	UAGCUUAUCAGACUGGUGUUGGC		101.2
dre-miR-29a	UAGCACCAUUUGAAAUCGGUUA		99.0
dre-miR-210-5p	AGCCACUGACUAACGCACAUUG		97.8
dre-miR-204-5p	TTCCCTTTGTCATCCTATGCCT		99.5
dre-miR-206-3p	TGGAATGTAAGGAAGTGTGTGG		101.4
dre-hsp70	AAAGCACTGAGGGACGCTAA	TGTTCAGTTCTCTGCCGTTG	103.9
dre-hif-1α	AGACACAGGGAGTGGGTTTG	ATCAAGCCCTGAACATGGAC	95.5
dre-vegfa	CAGCTTTCCTTCCGCCATTC	AGATGCGTGTTGCACATGGT	95.2
dre-18S	ATGGCCGTTCTTAGTTGGTG	GAACGCCACTTGTCCCTCTA	98.2
dre-eif1α	CAAGGAAGTCAGCGCATACA	GAACGCCACTTGTCCCTCTA	102

Figures

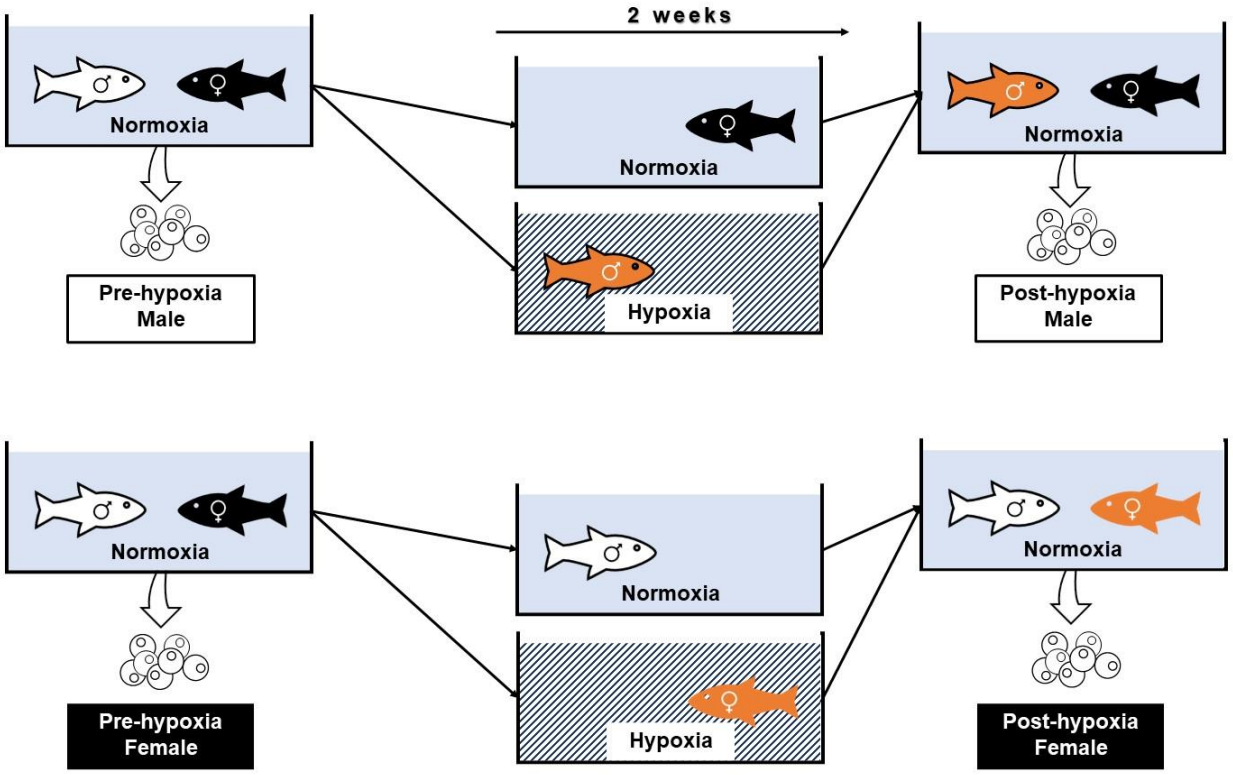


Figure 1. Experimental design. Breeding pairs produced embryos which were collected one-hour post fertilization (hpf) as the pre-hypoxia controls. These were separated as embryos from breeding pairs where the male was to be exposed to hypoxia (pre-hypoxia male embryos) and where the female was to be exposed to hypoxia (pre-hypoxia female embryos). The breeding pairs were separated and either the male or female fish were exposed to hypoxia for 2 weeks. Subsequently, the same breeding pairs were returned to normoxic conditions and produced either the post-hypoxia male embryos or post-hypoxia female embryos, which were collected 1 hpf.

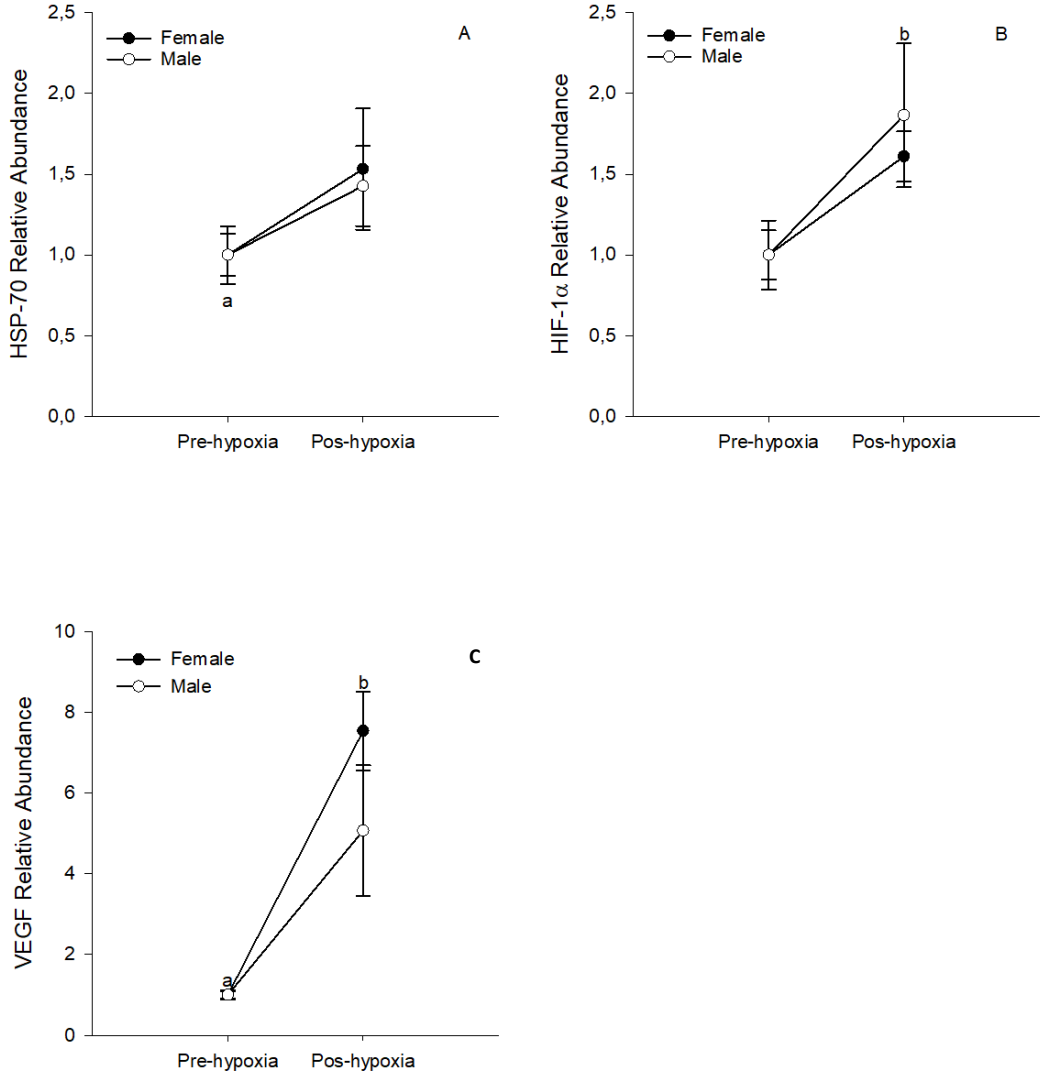
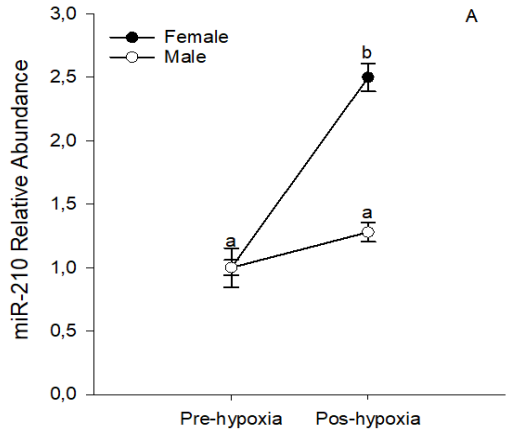
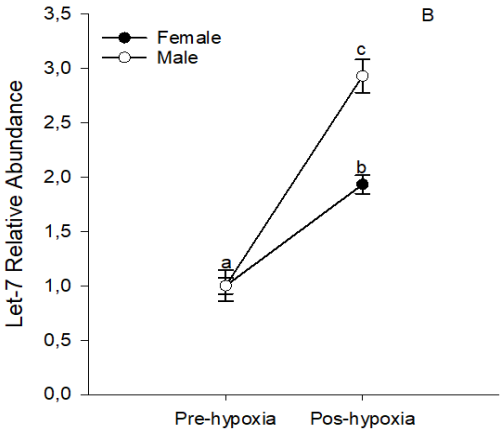
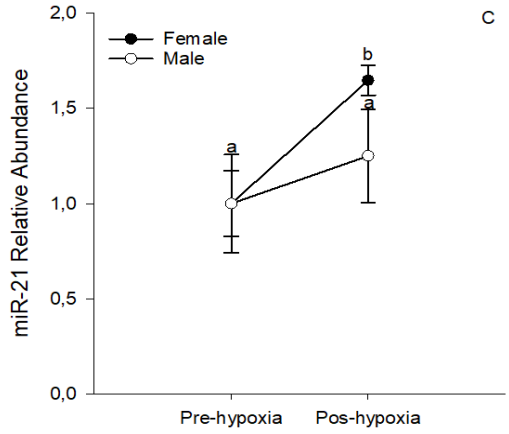
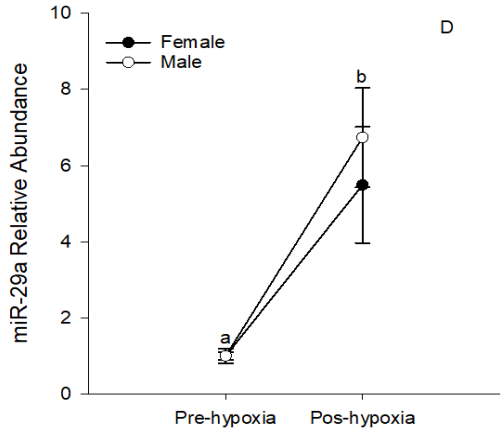


Figure 2. Relative expression of *hsp70* (A), *hif-1α* (B) and *vegf* (C) in zebrafish embryos pre- and post- 2 weeks of hypoxia exposure. The data is labeled based on which of the breeding pair (male or female) was exposed to hypoxia. The statistical significance was analyzed using a Repeated Measure Two-Way ANOVA with Treatment and Sex as factors. Dots with different letters indicate differences between groups and treatments ($P<0.05$). Deviation bars represent the standard error of the mean.


A
Female
Male
miR-210 Relative Abundance
3,0
2,5
2,0
1,5
1,0
0,5
0,0
a
b
a
Pre-hypoxia
Pos-hypoxia


B
Female
Male
Let-7 Relative Abundance
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
a
c
b
Pre-hypoxia
Pos-hypoxia


C
Female
Male
miR-21 Relative Abundance
2,0
1,5
1,0
0,5
0,0
a
b
a
Pre-hypoxia
Pos-hypoxia


D
Female
Male
miR-29a Relative Abundance
10
8
6
4
2
0
a
b
Pre-hypoxia
Pos-hypoxia

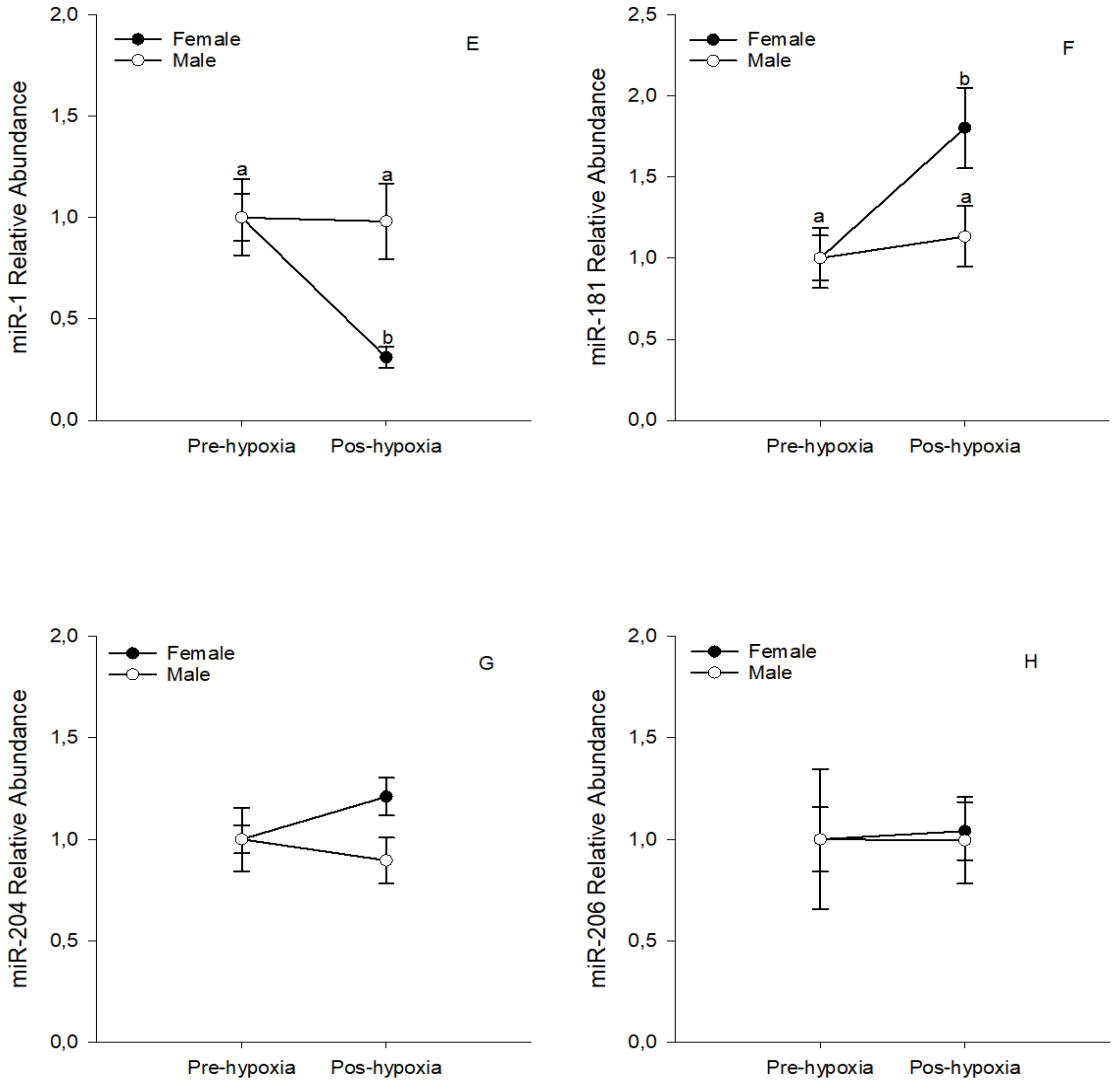


Figure 3. Relative expression of miR-210 (A), let-7 (B), miR-21 (C) miR-29a (D), miR-1 (E), miR-181 (F), miR-204 (G) and miR-206 (H) in embryos of zebrafish pre- and post- 2 weeks of 45% hypoxia exposure. The data is labeled based on which of the breeding pair (male or female) was exposed to hypoxia. The statistical significance was analyzed using a Repeated Measure Two-Way ANOVA. Dots with different letters indicate differences between groups and treatments ($P<0.05$). Deviation bars represent the standard error of the mean.

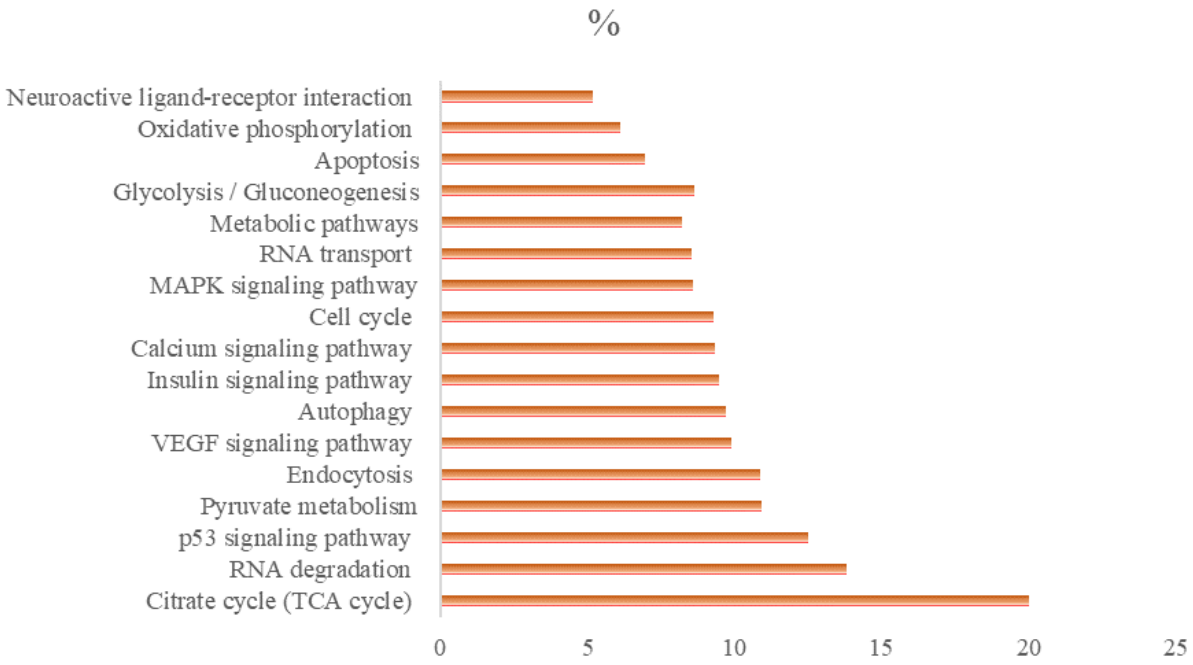


Figure 4. Key KEGG pathways targeted in F_1 embryos from F_0 females exposed to hypoxia. Percentages of the pathways targeted by Let-7, miR-210, -21, -29a, -181 are shown. This table shows the most affected pathways (greater than 10%) and relevant pathways related to hypoxia stress.

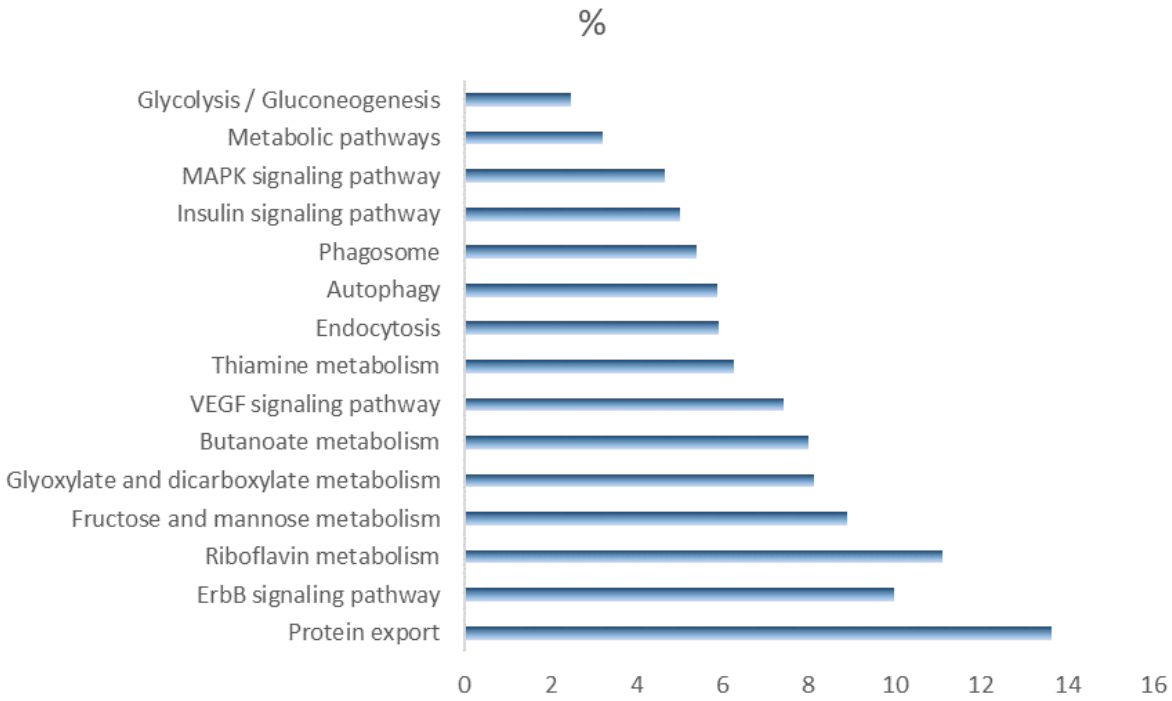


Figure 5. Key KEGG pathways targeted in F_1 embryos from F_0 males exposed to

hypoxia. Percentages of the pathways targeted by miR-29a andlet-7 are shown. This table shows the most affected pathways (greater than 10%) and some relevant pathways related to hypoxia stress.

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CONCLUSÃO GERAL E PERSPECTIVAS

De forma geral, os resultados dessa tese demonstraram, de diferentes formas, a influência da hipóxia sobre a fisiologia, respiração, bioquímica, genética e epigenética de espécies de peixes. O primeiro capítulo demonstrou que ambientes com drásticas variações naturais na concentração de oxigênio influenciam a tolerância à hipóxia das espécies que ali habitam. Além disso, ao se comparar espécies filogeneticamente relacionadas, foi possível observar que os níveis de tolerâncias estão altamente relacionados ao uso do oxigênio quando essas espécies estão expostas a esse estressor. Os presentes resultados mostram que o *Mesonauta festivus*, animal com maior tolerância neste trabalho, deprime o consumo de oxigênio e ativa o metabolismo anaeróbico, enquanto o *Aequidens pallidus* não ativa o metabolismo anaeróbico e apresenta uma dependência da respiração mitocondrial, aumentando sua capacidade catalítica e diminuindo sua P_{50}, o que mostra que, em relação à exposição a hipóxia, a dependência exclusiva do metabolismo aeróbico não é uma boa estratégia. Além disso, esse capítulo expôs a vulnerabilidade do *Aequidens pallidus* e também de outras espécies que habitam os ambientes aquáticos de terra firme, um ambiente em ameaça de surgimento de episódios hipóxicos repentinos, algo predito como resultado das mudanças climáticas e desflorestamento.

O segundo capítulo dessa tese teve como objetivo avaliar a possível função dos microRNAs sobre a tolerância à hipóxia. Como demonstrado no capítulo anterior, a tolerância à hipóxia está ligada às mais diversas estratégias metabólicas, demonstrando ser um traço espécie-específico com forte influência do ambiente que os animais habitam. Neste capítulo foi demonstrada, pela primeira vez, a influência dos microRNAs sobre a tolerância à hipóxia: O *Aequidens pallidus*, animal menos tolerante, alterou a expressão do miR-181c e miR-210 no fígado quando exposto à hipóxia,

microRNAs responsáveis por modificações na respiração mitocondrial e angiogênese, dois traços que se mostraram fundamentais na estratégia dessa espécie para tolerar hipóxia. Por outro lado, quando exposto à hipóxia, o *Mesonauta festivus* diminuiu a expressão do miR-181c e aumentou a expressão do let-7, demonstrando o envolvimento desses microRNAs na mudança do metabolismo celular de aeróbico para anaeróbico no fígado, estratégia determinante para a alta tolerância dessa espécie. Esses resultados demonstram que os microRNAs possuem influência na regulação da maquinaria gênica e que suas respostas estão intimamente relacionadas à tolerância à hipóxia dessas duas espécies.

No terceiro capítulo, seguindo a investigação da função dos microRNAs sobre as respostas à hipóxia, foi avaliada a sua função transgeracional e o quanto isso está relacionado ao sexo exposto a esse estressor. Neste capítulo foi observado um aumento na maioria dos microRNAs estudados na F_1 de *Danio rerio* após a exposição da F_0 à hipóxia. Esses resultados evidenciam os efeitos transgeracionais dos microRNAs e como eles agem diferentemente quando um dos sexos da F_0 é exposto a esse estressor, além de evidenciar que há uma influência do sexo do animal em transmitir fenótipos adaptativos que permeiam a sobrevivência a baixos níveis de oxigênio.

A conclusão deste trabalho trouxe novas questões e perspectivas em relação ao que foi estudado. No primeiro capítulo, a discussão a respeito da tolerância do Cichlidae *Aequidens pallidus* evidenciou a plasticidade fenotípica reduzida dessa espécie e o quanto isso a torna vulnerável frente às previsões de aquecimento global. Testes futuros com transplantes desses indivíduos para regiões de lagos, onde há uma forte variação nos níveis de oxigênio dissolvido, podem evidenciar se, mesmo com a plasticidade fenotípica reduzida, esse animal possui a capacidade de se aclimatar a outros ambientes, fator que pode ser determinado pelo tempo e intensidade da exposição a um estressor.

Em relação à expressão intergeracional dos microRNAs, estudos em diferentes tempos de exposição e diferentes concentrações de oxigênio podem esclarecer melhor o papel destes RNAs na tolerância à hipóxia, combinando essas características com a expressão destes RNAs em diferentes tecidos, uma vez que a literatura apresenta que diferentes tecidos possuem diferentes estratégias metabólicas em relação à hipóxia, além de já estar determinado que a resposta dos microRNAs é tecido-específica.

Por fim, ao se avaliar os efeitos transgeracionais dos microRNAs, permaneceu em aberto os efeitos diretos desses microRNAs nas gerações seguintes e ainda por quantas gerações essas alterações nos microRNAs permanecem. Ainda, estudando o mecanismo de transmissão desses microRNAs para a geração seguinte, o presente trabalho abre questões a respeito da transmissão de microRNAs mitocondriais, por parte das fêmeas, para a geração seguinte.

Este estudo trouxe novidades no que diz respeito à relação entre organismos aquáticos e as variações na concentração de oxigênio, bem como abre diversos questionamentos e caminhos que podem ser explorados em pesquisas futuras, o que ainda hoje evidência e corrobora com o que foi dito por Peter Hochachka, que em relação à tolerância à hipóxia, os peixes da Amazônia permanecem como uma “under-explored goldmine”.