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

# Os peixes amazônicos vivem perto dos seus limites térmicos? O efeito das mudanças climáticas sobre a ecofisiologia de peixes de Igarapé da Amazônia Central

**Manaus-AM**
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Derek Felipe de Campos

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**Orientadora: Vera Maria Fonseca de Almeida e Val, Dra.**

Tese apresentada ao Programa de Pós-Graduação em Biologia Água Doce e Pesca Interior do Instituto Nacional de Pesquisas da Amazônia como parte dos requisitos para obtenção do título de Doutor em Biologia de Água Doce e Pesca Interior.

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**“Arrasamos as selvas e implantamos selvas de cimento. Enfrentamos o sedentarismo com esteiras, a insônia com remédios. E pensamos que somos felizes ao deixar o humano”.**

**José Mujica**

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**Resumo**

Desde a revolução industrial, a terra vem enfrentando aumentos na concentração de CO2 que, consequentemente, têm levado ao aumento das temperaturas médias globais. Dos últimos 23 anos 22 tiveram temperaturas acima da média global. Temperatura é o principal fator que afeta a vida das espécies ectotérmicas. Além disso, espera-se que espécies tropicais sejam especialmente vulneráveis a aumentos de temperatura, uma vez que muitas delas parecem ter uma faixa de tolerância térmica mais estreita e vivem mais perto de seus limites térmicos. No entanto, até o presente momento estudos que avaliem a vulnerabilidade das espécies Amazônicas são inexistentes. Dentre estas espécies, os peixes de Igarapé parecem ser especialmente vulneráveis, uma vez que ocorrem em um ambiente térmico extremamente estável. Portanto, a presente tese tem como objetivo avaliar a capacidade de adaptação frente ao aumente de temperatura e $CO_2$ sobre os parâmetros fisiológicos em espécies de peixes de Igarapé. No capitulo I nossos resultados mostraram que espécies ativas apresentam alta demanda energética a fim de suprir os custos de manutenção para uma alta atividade e, por isso, apresentam uma reduzida tolerância térmica. Ainda, no capitulo II, *Hyphessobrychon melazonatus*, espécie ativa, aclimatada ao cenário de mudanças climáticas extremo apresentou grandes distúrbios osmorregulatórios, que podem ter importantes impactos na capacidade de sobrevivência ao longo prazo Na realidade, no capitulo III nós observamos que todas as três espécies apresentam alterações metabólicas e danos celulares aclimatas as condições climáticas futuras, no entanto, *H. melazonatus*, foi a espécie que apresentou os maiores índices de mortalidade, danos lipídios e redução na janela térmica. No capitulo IV nós mostramos que *Pyrrhullina brevis* aclimatadas por 180 dias no cenário extremo apresenta um redução no tamanho corporal, de acordo com a terceira resposta universal a temperatura. No entanto, ao contrário da hipótese

OCLTT, a redução no tamanho não está ligada a uma incapacidade de suprimento de oxigênio aos tecidos, mas sim parece estar relacionado a um aumento nos níveis de danos celulares que impedem o crescimento de acordo com a life-history trade-off theory. Nossos resultados ressaltam a importância de políticas públicas voltadas para a diminuição dos agentes causadores das mudanças climáticas e para a preservação das áreas de floresta que têm papel fundamental na manutenção da temperatura dos Igarapés.

# 1. INTRODUÇÃO

## 1.1 O que são as mudanças climáticas?

Durante o período de evolução geológica houve mudanças históricas no clima que envolveu alterações na temperatura da terra. Por exemplo, no Eoceno (≈55 milhões de anos) ocorreu um aumento na temperatura global de 5-7°C e níveis atmosféricos de dióxido de carbono ($CO_2$) 8-16 vezes maiores que os valores atuais (Kerr, 2011). Atualmente, as temperaturas globais estão previstas para aumentar em média 3°C até 2050 (Rowlands et al., 2012). Embora essas condições pareçam ser menos extremas em relação às do passado, as atuais alterações climáticas são únicas porque estão sendo atribuídas em grande parte as atividades antrópicas e em uma velocidade muito mais rápida do que aquelas observadas anteriormente.

Desde a revolução industrial, as emissões globais e a acumulação na atmosfera dos chamados gases do efeito estufa, principalmente dióxido de carbono ($CO_2$), aumentaram dramaticamente. Como consequência, os níveis de $CO_2$passaram de 280 μatm para os atuais 400μatm (IPCC 2013), a uma taxa de 1% a 3,4% ao ano (e Quere et al. 2009). Os climatologistas preveem que os níveis futuros possam atingir 1000 μatm até o final do século caso as emissões permaneçam dentro das mesmas taxas(Caldeira e Wickett 2003, IPCC 2013). O impacto das mudanças climáticas é um dos desafios ambientais mais importantes que o mundo enfrenta hoje e é principalmente impulsionado pelo aumento da concentração de $CO_2$ oriundos das atividades antrópicas (IPCC 2013).

O processo de aquecimento da terra ocorre devido ao aumento de $CO_2$ e outros gases relevantes do efeito estufa atrapalhar a radiação de calor na atmosfera, evitando a liberação do mesmo da estratosfera e assim aquecendo a Terra (Haywood et al., 2009). As temperaturas globais da superfície aumentaram 0,74 ± 0,18°C durante o ultimo século (Solomon et al., 2007). Enquanto os futuros cenários de mudanças climáticas dependem principalmente de como as sociedades irão lidar com futuras emissões de carbono o aquecimento global é atualmente a preocupação ambiental mais importante.

Os sistemas aquáticos desempenham um papel fundamental na mitigação das mudanças climáticas, participando do sequestro de calor e carbono da atmosfera. Como

consequência, os oceanos vêm se tornando mais quentes a uma taxa de aproximadamente 0,1 ° C nas últimas décadas (IPCC 2013). Especialistas em clima preveem para a temperatura dos oceanos aumentarem em 4° C (IPCC 2013). Embora para os sistemas aquáticos continentais as variações térmicas sejam dependentes de suas histórias naturais, para os ambientes Amazônicos, dado o seu tamanho e posição geográfica, também é esperado expressivos aumentos nas temperaturas médias, bem como secas extremas e ondas de calor.

Infelizmente, as consequências do aumento das emissões de $CO_2$ não estão restritas ao aquecimento dos sistemas aquáticos, a absorção contínua de $CO_2$ mudará a química da água. O dióxido de carbono quando combinado com água forma ácido carbônico ($H_2CO_3$), que se dissocia em bicarbonato ($HCO_3^-$) e carbonato ($CO_3^{2-}$) libera íons de hidrogênio ($H^+$). O aumento das concentrações de $H^+$ reduzirá o pH das águas, um processo conhecido como Acidificação. Este processo tem sido mais bem estudado nos ambientes oceânicos, onde estudos apontam que desde o período pré-industriais uma redução em média de 0,1 unidades de pH (Meehl et al., 2007). Ainda que este valor pareça ser negligenciável, ele representa um aumento de 30% nos íons $H^+$ nas águas oceânicas, e prevê-se ainda que se o processo de emissões de $CO_2$ continuar poderá levar a uma diminuição adicional de 0.4-0.5 unidades (Caldeira e Wickett 2005). Apesar de nenhum estudo ainda se tenha verificado os efeitos do aumento das concentrações de $CO_2$ sobre a acidificação das águas amazônicas, é esperado que estes efeitos sejam ainda mais pronunciados uma vez que alguns tipos de águas amazônicas apresentam características únicas, tais como baixa quantidade de íons e ambientes já extremamente acidificados.

Particularmente, ainda existe um entendimento limitado sobre as consequências dos efeitos sinérgicos de múltiplos estressores climáticos sobre a vulnerabilidade das espécies amazônicas. Portanto, entender como esses desafios impostos pelas mudanças climáticas irão afetar as espécies amazônicas se faz urgente, para tomada de medidas a fim de conservar a região mais com a maior diversidade de peixes dulcícolas do planeta.

## 1.2 O impacto das mudanças climáticas sobre a fisiologia dos organismos ectotérmicos

Os organismos ectotérmicos são aqueles que têm a sua temperatura corpórea regulada pela temperatura ambiental e, portanto, todas as suas funções biológicas são diretamente influenciadas pela temperatura (Pörtner et al., 2006; Brierley e Kingsford

2009). A extensão do impacto do aumento da temperatura é variável e depende da janela térmica das espécies. As mudanças climáticas devem favorecer organismos com amplas faixas térmicas (euritérmicos) e que, consequentemente, são capazes de tolerar maiores variações na temperatura (Stillman e Somero 2000; Calosi et al. 2008; Tewksbury et al. 2008; Schulte, 2016). Estes organismos são comumente atribuídos a ambientes temperados onde grandes variações de temperatura ocorrem sazonalmente, portanto, é provável que estas espécies tenham uma maior capacidade de adaptação as mudanças climáticas (Stillman, 2003). Por outro lado, espécies tropicais têm se mostrado extremamente suscetíveis aos efeitos do aquecimento global uma vez que vivem em um ambiente térmico homogêneo e perto dos seus limites térmicos. Deste modo, é esperado que espécies amazônicas apresentem pouca capacidade de se adaptar as mudanças climáticas (Tewksbury et al. 2008, Madeira et al., 2016, Campos et al., 2017).

Com a elevação da temperatura ocorre um aumento na velocidade das reações, portanto, um aumento na demanda energética, e consequentemente, na taxa metabólica. As espécies podem se aclimatar e se adaptar dentro de uma amplitude de temperatura onde conseguem manter seu desempenho. A habilidade dos organismos em manter o desempenho ótimo em um ambiente mais quente tem sido relacionada com a capacidade energética. A hipótese de tolerância térmica limitada pela capacidade de oxigênio (oxygen and capacity-limited thermal tolerance, OCLTT hypothesis em inglês) sugere que a capacidade das espécies em suprir oxigênio aos tecidos torna-se limitadas nas temperaturas criticas (Pörtner, 2001, 2010; Pörtner & Farrell,2008). A fundamentação desta hipótese é que em altas temperaturas um aumento nas necessidades básicas de oxigênio, a taxa metabólica basal, TMB; (Chabot *et al.*, 2016) leva a uma redução no seu escopo aeróbico. A redução do escopo aeróbico levará progressivamente a um modo anaeróbico de produção de energia, menos eficiente que gera produtos secundários danosos e que só suporta a sobrevivência durante períodos curtos.

Uma elevação da temperatura para além do ótimo fisiológico pode levar a uma perda na integridade das estruturas moleculares, que ativam progressivamente a defesa antioxidante e a resposta de choque térmico (Pörtner etal. 2006; Pörtner 2010). Nestas temperaturas, muitosprocessos biológicos, como metabolismo, crescimento, comportamento, alimentação emecanismos bioquímicos e de reprodução são afetados negativamente. Essas restrições podemcomprometer a aptidão geral, a sobrevivência, a

distribuição e a abundância das espécies (por exemplo, Roessig et al. 2004; Hoegh-Guldberg et al., 2007; Pörtner 2010; Byrne 2011; Rosa et al. 2012; Rosa et al. 2013; Rosa et al. 2014a; Vasseur et al. 2014). Por exemplo, Portner and Knust (2007) mostraram que a redução na capacidade cardíaca em altas temperaturas levava a uma restrição do escopo aeróbico e um aumento na mortalidade destas espécies. Neste trabalho os autores relacionaram a diminuição do escopo aeróbico com a redução da abundancias de *zoacer viviparus* em anos de temperaturas elevadas. No entanto, a generalidade desta teoria tem sido contestada (Clark et al., 2013, Norin and Clark, 2016).

Um aspecto comum e inevitável do metabolismo aeróbico é a formação das espécies reativas de oxigênio (EROS). As EROS são moléculas altamente reativas capazes de causar diversos danos celulares. Por isso, os organismos têm evoluído um número de estratégias para mitigar os efeitos das EROS. A concentração de EROS é controlada pelos sistemas antioxidantes enzimáticos e não-enzimáticos. Contudo, altas temperaturas perturbam este equilíbrio aumentando a produção de radicais livre. O desequilíbrio entre os sistemas antioxidante e a produção de EROS pode acarretar na superprodução de espécies reativas que causam danos a diversos componentes celulares, tais como lipídeos, proteínas e DNA.

Recentemente, têm-se sugerido que os acúmulos de danos resultados do estresse oxidativo podem levar a uma diminuição na aptidão e função fisiológica adequada (Sohal, 2002); uma situação que provavelmente ocorrerá com a temperatura prevista de aquecimento dos sistemas aquáticos. De fato, diversos trabalhos têm mostrado que espécies tropicais aclimatadas a um aumento de temperatura entre 1,5 - 4°C apresentam altos índices de danos celulares (Vinagre et al., 2012; Madeira et al., 2014; Madeira et al., 2016; Rosa et al., 2017). No entanto, estudos que avaliem a capacidade de aclimatação aos cenários climáticos, tanto do ponto de vista energético e de estresse oxidativo, em espécies amazônicas são insuficientes.

Assim como a temperatura, altas concentrações de $CO_2$ têm profundos efeitos sobre a fisiologia que restringe o desempenho animal. Os futuros aumentos previstos de $CO_2$ podem causar uma ampla gama de efeitos sub-letais em uma variedade de espécies de peixes de água doce (revisado por Hasler et al., 2018).

Os principais impactos da hipercania nos peixes de água doce estão relacionados à regulação osmótica e do mecanismo acido-base. Estes mecanismos reguladores tipicamente envolvem a obtenção e retenção de $HCO_3^-$ e excreção do $H^+$ acumulado

(por exemplo, Cameron, 1978; Heisler et al., 1982; Brauner& Baker, 2009; Hannan et al., 2016a). A regulação acido-base e osmótica dos teleósteos ocorrem principalmente através das brânquias por transportadores específicos de íons que demandam altas quantidades de energia (ATP) (Sullivan et al., 1996; Claiborne et al. 1999; Edwards et al. 2001; Perry et al. 2003; Choe et al. 2004; Tresguerres et al.2005).

Além disso, as mudanças no pH do plasma podem restringir a capacidade e fornecimento de oxigênio, uma vez que este altera a afinidade da hemoglonina (Pörtner et al., 2004). Portanto, ocustos energéticos associados à manutenção da homeostase em situações de exposição crônica a acidificação pode alterar os orçamentos de energia finitos de animais de água doce, e consequentemente, resultar em redução nas taxas de crescimento, calcificação e reprodução (por exemplo, Fivelstad et al., 2003, 2007; Hosfeld et al.,2008; Good et al., 2010;Abbey-Lambertz et al., 2014; Fivelstad et al., 2015).

Do mesmo modo, a acumulação simultânea de $HCO_3$ e redução de $Cl^-$ resultante do desequilíbrio ácido-base podem interferir nos receptores GABA-A, alterando as funções dos neurotransmissores cerebrais. Seus efeitos incluem ainda mudanças nas respostas olfativas a sinais de predadores, presas e substratos, alterações no comportamento de lateralização e maior ansiedade. De fato, diversos trabalhos têm observados distúrbios comportamentais em peixes, incluindo mudanças na personalidade (Jutfelt et al.,2013; Ou et al., 2015), alteração no padrão de movimentação (Hasler et al., 2016b), e padrões de atividade alterada(Regan et al., 2016). Valores de pH reportados queinduziu as mudanças comportamentais acima variaram de5.8 a 7.7, e é provável que estes comportamentosas mudanças se originam de respostas fisiológicas à acidificação (Ou et al., 2015; Regan et al., 2016).

As projeções dos efeitos das mudanças climáticas sobre os organismos vêm se baseando em como o desempenho fisiológico é alterado frente aos desafios térmicos e de hipercapnia. A capacidade das espécies em se adaptar às mudanças climáticas pode determinar a sua sobrevivência no futuro ambiente. Recentes trabalhos indicam que mudanças climáticas podem ter efeitos adversos sobre os processos fisiológicos e bioquímicos dos peixes, o que pode resultar em mudanças na distribuição, fenologia e tamanho das espécies (Parmesan & Yohe 2003; Root et al. 2003; Perry et al. 2005; Kleypas et al., 2006; Gardner et al. 2011; Sheridan & Bickford 2011;)

## 1.3 O ambiente Amazônico e suas peculiaridades

A bacia Amazônica compreende um notável sistema hidrológico que suporta mais de um milhão de $km^2$ de ecossistemas aquáticos (Castello et al., 2013). Este sistema abrange uma grande diversidade de ambientes aquáticos, tais como rios, paranás, igapós, várzeas e Igarapés. Cada ambiente possuem características físicas e químicas únicas e dinâmicas próprias, o que confere pressões evolutivas distintas sobre suas biotas. Os grandes rios e suas áreas de inundação têm sido bem estudo e três tipos gerais de agua ocorrem na bacia Amazônica: rios de água branca, rios de agua clara e rios de água preta (Sioli, 1984).

Embora estes três tipos de água sejam os mais comumente reconhecidos e estudados, sob a densa floresta amazônica se esconde uma vasta rede de pequenos corpos d'água, denominados de Igarapés. Os Igarapés representam uma grande porção dos sistemas aquáticos amazônicos onde suas superfícies somadas são várias vezes superiores à do rio Amazonas e sua extensão combinada é mais de mil vezes maior do que o Amazonas (Fittkau, 1967). Ao contrário dos ambientes abertos das planícies de inundação dos grandes rios, os pequenos riachos da Amazônia são recobertos por uma densa e exuberante floresta que fornece proteção à entrada dos raios solares e cria um ambiente térmico extremamente estável sofrendo pouca ou nenhuma variação anual (25-26°C) (Espirito-Santo et al., 2018). Estes ambientes são influenciados diretamente pelo regime de chuva local onde ocorre a expansão dos ambientes aquáticos fornecendo novos habitats à comunidade de peixes.

Os diferentes ambientes aquáticos amazônicos fornecem distintas pressões sobre a adaptação e evolução das espécies de peixes da região amazônica. Por exemplo, enquanto espécies que habitam as várzeas e igapós precisam lidar com variações diárias e sazonais na concentração de oxigênio, dióxido de carbono e temperatura, as espécies de peixes de igarapé sobrevivem dentro de um ambiente menos variável (Pires et al., 2018). Portanto, sabendo-se que a capacidade de lidar com temperaturas extremas depende da história térmica vivida pelas espécies podemos esperar que as populações que habitam os riachos sejam mais susceptíveis ao aumento da temperatura e $CO_2$ projetados pelas mudanças climáticas, apesar disso, o grau de vulnerabilidade para estas espécies ainda é desconhecido.

O clima na região Amazônica já vem experimentando aumentos na temperatura de 0,5 a 0,8°C na última década do século XX (Pabón, 1995a; Pabón et al., 1999; Quintana-Gomez, 1999). Além disso, a precipitação na Amazônia têm apresentado

variações atípicas (Marengo et al., 2000). Os períodos 1950-1976 foram regionalmente chuvoso no norte da Amazônia, e desde 1977 essa região têm sido exposta a períodos mais intensos de seca (IPCC 2001), sugerindo um efeito de variabilidade climática em longo prazo. Alguns modelos climáticos sugerem que a bacia amazônica está em risco especial para as mudanças climáticas. Aumento de temperatura e diminuição da precipitação podem causar cada vez mais ondas de secas com mudanças substanciais na sazonalidade da precipitação (Marengo et al., 2000). Acoplado com mudanças no uso da terra, essas alterações podem levar a impactos devastadores na biodiversidade aquática, principalmente dos ecossistemas de igarapé que são ambientes extremamente dependentes das chuvas regionais e que sofrem maior pressão do uso da terra (Ilha et al. 2018).

Uma vez que o aquecimento global irá impactar substancialmente as espécies ectotérmicas, como descrito anteriormente. A tolerância térmica desempenha um papel importante nos limites de distribuição local e global dos peixes de água doce (Campos et al., 2017). As distribuições de espécies aquáticas provavelmente mudarão, uma vez que algumas espécies invadem habitats de alta altitude ou desaparecem dos seus limites inferiores de suas distribuições. No entanto, peixes de igarapé ocorrem em ambientes únicos com temperatura relativamente constante e barreiras físicas e químicas que, em muitas vezes, impedem a dispersão destas espécies para ambientes de melhores confortos térmicos. Além disso, temperaturas elevadas têm efeitos adversos imediatos sobre os parâmetros fisiológicos aumentando as demandas metabólicas e, portanto, a quantidade de energia (Carpenter et al., 1992). O aumento da temperatura da água e a redução da precipitação também podem reduzir o habitat adequado durante os meses de verão secos e quentes. Portanto, se faz extremamente necessário investigar os limites térmicos e os efeitos da temperatura e $CO_2$ sobre a fisiologia dos organismos amazônicos, a fim de se entender os possíveis efeitos das mudanças climáticas sobre a comunidade de peixes da porção mais estável da Amazônia.

## 1.4 A Ictiofauna de Igarapés da Amazônia Central

A Ictiofauna de Igarapés da Amazônia Central A Bacia do Rio Amazonas têm a maior diversidade de espécies de peixes que qualquer outra região do mundo; são mais de 2200 espécies descritas (Reis et al., 2003), muitas delas endêmicas e que habitam pequenos cursos d’água denominados igarapés, esses pequenos riachos são

caracterizados por águas ácidas devido à presença de ácidos húmicos e fúlvicos, pobres em nutrientes, pois a cobertura vegetal prejudica a penetração de luz, e por isso, plantas aquáticas são praticamente inexistentes (Junk e Furch, 1985; Walker, 1995); e apresentam temperatura relativamente constante durante todo o ano (Mendonça et al., 2005). Igarapés são ambientes oligotróficos e suas cadeias alimentares são dependentes de material alóctone, tal como folhas, galhos e pequenos insetos (Goulding et al., 1988; Walker, 1991). Contudo, uma grande variedade de pequenos peixes é frequentemente abundante, e de 20 a 50 espécies podem ocorrer num único canal, com diferentes papéis na intricada cadeia metabólica destes ambientes (Lowe-McConnell, 1999; Sabino& Zuanon, 1998; Araújo-Lima et al, 1995; Castro, 1999). Grande parte desta alta diversidade é devido à complexidade estrutural encontrada nesses ambientes (Mendonça et al., 2005). Os igarapés são um mosaico de diferentes habitats e microhabitats determinados pela resposta de fatores espaço-temporais tais como entrada da água, velocidade de fluxo, profundidade, tipo de sedimento e detritos que influenciam diretamente a estrutura biótica (Mendonça et al., 2005; Sabino & Zuanon, 1998). A distribuição das espécies de peixes de igarapé mostra uma marcada estratificação vertical e horizontal. Segundo Sabino e Zuanon (1998) das 29 espécies amostradas em um igarapé da Amazônia central 65% estão associados com o fundo (explorando o substrato arenoso, troncos caídos ou vegetação submersa). Essas espécies, conhecidas como bênticas, incluem os Loricaridae (*Ancistrus* sp. *Rineloricaria heteroptera* e *Farlowella* sp.), que são principalmente encontrados associados a troncos caídos ou enterrados na areia, e alguns Ciclidae (*Aequidens* aff. *pallidus* e *Satanoperca daemon*) que podem ser encontrados forrageando perto do fundo ou em tocas próximas a troncos caídos. A superfície da água é principalmente explorada por Lebisianidae (*Nannostomus eques*, *Copella* sp. e *Pyrrhulina* aff *brevis*), que usa somente a região marginal no meio da vegetação submersa, onde a correnteza é mais fraca. Já os Characidae (Hyphessobrychon spp e *Moenkhausia colleti*) são observados nadando ativamente à meia água em áreas de correnteza (Fig. 1). Tais características ambientais fornecem diferentes pressões sobre a evolução das espécies (Val e Almeida-Val, 2006). Por isso, espécies que ocupam habitats distintos apresentam adaptações diferentes ligadas ao estilo de vida de cada uma. Espécies de ambientes de corredeiras, com fluxo rápido, têm adaptações morfológicas e fisiológicas para se manter em constante natação na coluna d'água, e para isso apresentam uma maior demanda energética e capacidade cardíaca. Tais adaptações são distintas das espécies que ocupam áreas de baixo fluxo de

corrente, como poças, ou que são encontradas associadas ao substrato, que pouco se movem durante o seu ciclo de vida, e por isso apresentam baixas necessidades energéticas, sendo muitas vezes dependentes do metabolismo anaeróbico (Driedzic & Almeida-Val, 1996, Almeida-Val et al., 2000). Vários grupos de peixes têm adaptações fisiológicas para sobreviver em condições de mosaicos estruturais e isso pode explicar a diversidade das assembleias de espécies (Val & Almeida-Val., 1999). Possivelmente, as diferentes espécies podem ser afetadas diferentemente pelo aumento da temperatura, mas isso exige um maior detalhamento nos estudos ecológicos e fisiológicos para determinar respostas individuais.

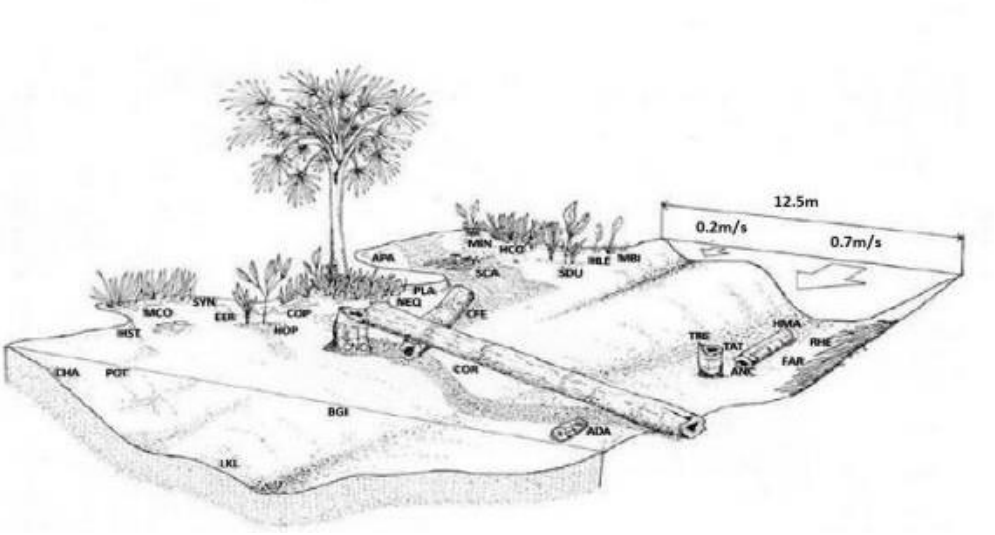


Figura 1. Representação diagramática da distribuição espacial de peixes no sítio de estudo do igarapé do guaraná por Sabino & Zuanon (1998). ADA, *Auchenipterichties dantei*; ANC, *Ancistrus* sp.; APA, *Aequidens* aff. *pallidus*; BGI, *Bryconops giacopini*; CHA, *Characidium* sp; CNO, *Crenicicla notophthalmus*; COP, *Copella* sp.; COR, *Cichla orinocensis*, CRE, *Crenicicla* sp.; EER, *Erythrinus erythrinus*; FAR, *Farlowella* sp.; HCO, *Hypselecara coryphaenoides*; HLE, *Hypopygus* aff. *lepturus*; HMA, *Hemicetopsis macilentus*; HOP, *Hoplias* aff. *malabaricus*; HST, *Hemigramus stictus*; LKL, *Leporinus klausenwitzi*; MBI, *Microsternarchus bilineatus*; MCO, *Moenkhausia colletti*; MIN, *Mesonauta insignis*; NEQ, *Nannostomus eques*; PLA, *Pyrrhulina* aff. *laeta*; POT, *Potamorrhaphis* sp.; RHE, *Rineloricaria heteroptera*; SDA, *Satanoperca daemon*; DSU, *Steatogenys duidae*; SYN, *Synbranchus* sp.; TAT, *Tatia* sp.; TRE, *Tatia reticulata*. Adaptado de Sabino & Zuanon (1998).

Os peixes evoluíram conjuntamente com as condições hidrológicas e micro clima local. Em particular, a conformidade térmica dos ectotermos os torna especialmente susceptíveis a mudanças nas temperaturas ambientais (Huey, 1982; Atkinson, 1994). O aumento de temperatura é uma grande preocupação nos tempos

atuais já que as condições térmicas ambientais podem chegar perto das condições sub-ótimas para muitos peixes de riachos que estão adaptados a uma pequena variação de temperatura. Mesmo pequenos aumentos na temperatura (de pouco mais de 1 ou 2°C) podem ser suficientes e apresentar grandes efeitos sobre a fisiologia de peixes tropicais (Calosi et al., 2011; Tewksbury et al., 2008). Fisiologicamente, a temperatura pode afetar o desempenho e a resistência natatória (Ojanguren e Branta, 2000), a necessidade energética (Fulton et al., 2010) e a frequência cardíaca (Solokova e Portner, 2002), que altera todo o fittness (aptidão) de uma espécie. Para enfrentar as mudanças climáticas, essas populações devem apresentar adaptações ditadas em grande parte pelo aumento do custo energético, caso contrário pode-se esperar uma diminuição na capacidade reprodutiva e, como consequência, na abundância dessas espécies, alterando toda a intricada relação ecológica dos pequenos sistemas aquáticos. Entender os limites térmicos e a capacidade de adaptação à temperatura nas espécies com diferentes estilos de vida da região de maior diversidade de peixes do mundo é extremamente importante para tomada de decisões em planos de conservação e preservação frente aos possíveis efeitos das mudanças climáticas em curso. A maioria dos estudos fisiológicos realizados na Amazônia têm se concentrado nos grandes rios e em espécies comercialmente importantes (por exemplo, Almeida-Val et al., 2000; Val e Almeida-Val, 1995; Val et al., 2006). Poucos estudos abordaram fatores ecológicos em grupos de espécies não comerciais (Chipari-Gomes et al., 2000; Almeida-Val et al., 2000). Apenas recentemente Duarte et al. (2013) verificou os efeitos do pH sobre a regulação iônica de espécies de igarapé. No presente trabalho, pretendemos investigar os efeitos da temperatura sobre a capacidade cardíaca de peixes de igarapés da Amazônia. O principal objetivo é aprofundar o conhecimento sobre os mecanismos de adaptação bioquímicos e fisiológicos à temperatura das espécies amazônicas e verificar os possíveis efeitos das mudanças climáticas sobre eles. Para tanto, iremos verificar a capacidade de tolerância térmica e aclimatação de diferentes espécies de peixes de igarapé e relacionar com seu estilo de vida e evolução das espécies.

## 1.5 Objetivos da tese

As mudanças climáticas são uma das principais preocupações para os conservacionistas, principalmente na região com maior biodiversidade do mundo. Espécies tropicais têm sido consideradas extremamente suscetíveis ao aumento de

temperatura, uma vez que ocorrem perto dos seus limites térmicos e apresentam baixa capacidade de aclimatação. No contexto amazônico, as espécies de peixes de Igarapés parecem ser as mais ameaçadas pelas alterações climáticas uma vez que ocorrem em um habitat térmico homogêneo. No entanto, até o presente momento estudos que avaliem os limites térmicos e a capacidade de aclimatação destas espécies são inexistentes. O principal objetivo desta tese é fornecer informações e ganhar poder preditivo para caracterizar e quantificar o grau de suscetibilidade das espécies de peixe Igarapé frente às alterações climáticas previstas para o ano de 2100, bem como descrever os mecanismos fisiológicos de adaptação das espécies. A tese está composta por 4 capítulos formatados em artigos científicos, publicados ou a serem publicados, de acordo com as normas das revistas. Especificamente, os principais objetivos dos capítulos são:

Capitulo I – Investigar o efeito do estilo de vida sobre o metabolismo energético e determinar sua influência na tolerância térmica das espécies de peixes de Igarapé;

Capitulo II – Investigar o efeito da aclimatação ao cenário climático extremo (4,5°C e 900pmm de $CO_2$, acima dos níveis atuais) sobre os mecanismos osmorregulatórios em três espécies de peixes de Igarapé;

Capitulo III – Determinar a suscetibilidade de três espécies de peixes de Igarapé ao cenário climático extremo (4,5°C e 900pmm de $CO_2$, acima dos níveis atuais);

Capitulo IV – Entender os mecanismos fisiológicos que levam a redução no tamanho de peixes aclimatados ao cenário climático extremo.

**Capitulo I**

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# The influence of lifestyle and swimming behavior on metabolic rate and thermal tolerance of twelve Amazon forest stream fish species

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# The influence of lifestyle and swimming behavior on metabolic rate and thermal tolerance of twelve Amazon forest stream fish species

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ABSTRACT

The metabolism of fishes is profoundly affected by environmental factors such as temperature, oxygen concentration, and pH levels. Also, biotic elements, for instance, activity levels of species, have been suggested to affect the energy demand, driving their capacity to support environmental challenges. The present work aims to investigate the effects of the lifestyle and swimming activities levels of fishes living in Amazon forest stream on the aerobic metabolism and thermal tolerance. Intermittent flow respirometry was used to measure routine metabolic rate and thermal maximum metabolic rate with a thermal ramp methodology. Critical thermal tolerance, thermal aerobic scope, and thermal factorial aerobic scope were calculated for twelve species belonging to different families. Our findings showed a correlation between routine and thermal maximum metabolic rate and, between metabolic rate and activity levels. Species belonging to Characidae and Crenuchidae families have high resting metabolic rates, which decrease their factorial aerobic scope and reduce their abilities to cope with warming events. Therefore, these species have low thermal tolerance. Instead, species from families Rivulidae and Cichlidae showed opposite metabolic results and larger thermal windows. We hypothesize that these responses are related to an evolutionary trade-off between lifestyle and energetic requirements and warming will favor species with low activity performance.

## 1. Introduction

Aerobic plasticity is particularly important to animal performance with potential implications for growth rate, reproduction and survival (Clarke, 2004; Killen et al., 2010; Metcalfe et al., 2016). The aerobic metabolism of ectothermic animals is affected by environmental factors such as temperature, oxygen concentration, and pH levels (Clarke and Fraser, 2004; Clarke and Pörtner, 2010; Kochhann et al., 2015). In Addition, biotic factors have been suggested to affect the routine and maximum metabolic rate, driving animal abilities to face environ- mental challenges. For instance, fishes living in the deep ocean have lower resting metabolic rates than those living in more shallow waters, possibly because they have lower energetic requirements to avoid predation (Clarke and Johnston, 1999; Seibel and Drazen, 2007). Thus, more active species tend to have a higher routine and maximum me- tabolic rates; these traits allow a greater absolute aerobic scope, and hence more active lifestyles (Clarke, 2004). These metabolic differences may be caused by variation in mitochondrial concentration or mem- brane proliferation, but also by higher costs of cardiovascular work or muscle tonus that improve athletic performance (Morris and North, 1984; Zimmerman and Hubold, 1998). A higher metabolic rate may also allow a rapid response to an environmental challenge. However, if this is an evolutionary advantage, it remains in a great discussion, since an elevated aerobic capacity demands important energy costs, which may cause a limitation of factorial aerobic scope (Killen et al., 2010; Stoffels, 2015).

Limitations on aerobic scope have been pointed out as a factor that constrains the ability of an organism to perform various ecological functions, what led to the hypothesis that fishes living at the boundaries of their thermal limits are unable to maintain sufficient oxygen supply for their routine metabolism due to the temperature-induced cardiorespiratory constraints (Pörtner and Knust, 2007; Farrell, 2007; Eliason et al., 2011). According to the so-called OCLTT (oxygen- and capacity-limited thermal tolerance) hypothesis, the aerobic scope is limited by insufficient oxygen supply at both sides of the thermal window and sets the performance in animals, with an optimum close to the upper pejus temperature (Tp) (e.g., Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Pörtner et al., 2010). Therefore, it provides access to understanding the physiological mechanisms that limit the performance, integrating whole-organism and tissue levels. Thermal limitation results from insufficient capacity reflected in a decrease in systemic oxygen levels (hypoxemia) and, finally, a transition to anaerobic metabolism.

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Consequently, the organismal thermal tolerance is determined by the animal ability to extract oxygen and to efficiently deliver it to tissues. Accordingly, an optimization of species oxygen supply mechanisms that can be translated in high aerobic scope would bring it a broader thermal tolerance window (Pörtner, 2001; Claireaux and Lefrançois, 2007; Pörtner and Farrell, 2008; Farrell et al., 2008; Cucco et al., 2012). However, the potential relationship between lifestyle and aerobic plasticity, which might set the thermal tolerance in stable habitats, has not been studied.

Thermal limits have been considered one approach to compare the physiological mechanism of adaptation between species and has been extensively applied to investigate the thermal ecology in congeneric species inhabiting thermal gradients (Hochachka and Somero, 2002; Gilman et al., 2006; Helmuth et al., 2006; Helmuth, 2009; Campos et al., 2017). It is well known that fishes experiencing high seasonal thermal variations in their habitats show higher metabolic plasticity and a wider thermal window (Magozzi and Calosi, 2014). Unlike seasonal environments, Amazonian forest stream areas form complex hydrological networks (Junk, 1983) with the vast majority running through the dense forest and presenting high oxygen concentration and small temperature variation (range between 25 and 28 °C) (Espírito-Santo et al., 2009; Costa et al., 2015). Temperature is predicted to increase up to 4 °C due to climate change and may reach a 7 °C increase in some areas of the Earth Planet (IPCC et al., 2014). As described to other tropical fishes (Tewksbury et al., 2008) we might expect that Amazonian species will be vulnerable to such changes, once they evolved in a relatively stable thermal environment, and supposedly live close to the thermal maxima.

Amazon forest streams are stable thermal habitats that contain one of the greatest diversity of fish species in the world with different lifestyles which results in different energy plasticity. Such ecological difference in energy demands may influence their oxygen supply and delivery capacities, playing a pivotal role in determining their thermal limits. In the present work, we investigated the potential effects of lifestyle and swimming behavior on metabolic rate and thermal tolerance of Amazon stream fishes, a group that presents great ecological niche diversity. These characteristics make these fish species ideal for examining the constraints of ecological influences on metabolic rate and thermal tolerance. Herein, we considered the difference in routine metabolic rate and thermal maximum metabolic rate of twelve fish species of first- and second-order streams and correlated this parameter to species' swimming activities levels and their consequence to thermal limits. The present work aimed to determine the critical thermal maximum of fish of Amazon forest streams, and verify if the effects of metabolic rate on their thermal tolerance are related to their lifestyles and swimming behavior.

## 2. Materials and methods

### 2.1. Collection and maintenance of fish

The study was conducted in first- and second-order streams (sensu Petts, 1994) at Reserva Ducke (02°53'S, 59°58'W), a protected area located in the central Brazilian Amazon near the confluence of the Negro and Solimões rivers and bordering Manaus. Fish were collected by hand and seine nets (2-mm mesh); the stream extent (1 m ± 0,5) was closed at its margins with block nets (5-mm mesh) to prevent fish movements into or out of the reach; the operators then moved upstream along the range attempting to cover all areas systematically within 1-h period, fishes were also collected in ponds with hand nets (Mendonça et al., 2005). Captured fish were maintained in a container with stream water and aeration. Then they were transferred to 150-L tanks (Fortlev®) with constant aeration until experiments started. Half of the water was replaced every 12 h. The fish were maintained in this resting tank for at least 48 h, to avoid measuring metabolic rate during the digestion phase. The temperature was 25 ± 0.5 °C. All experimental setups were conducted with stream water in the Reserva Ducke laboratories. The experimental sets are in accordance with CONCEA Brazilian Guide for Animal Use and Care and were authorized by INPA's Council for Ethics in Animal Use (CEUA - protocol number 027/2015).

### 2.2. Metabolic rate in a thermal ramp

We measured routine metabolic rate in a thermal ramp with the use of Intermittent-flow respirometry in an automated apparatus DAQ-M (Loligo Systems, Tjele, Denmark) in individuals (n = 8–6) of each of the twelve species collected. This apparatus consists of a recirculating circuit with three phases: flush, wait, and measurement. The time phases were 180 s flush, followed by 120 s wait, and 600 s measurement. Thus, the duration of an entire 'loop' (flush + wait + measurement) was 15 min. The fall of oxygen inside the chamber never dropped more than 1 mg $l^{-1}$ at each loop. The experimental sets were made for each single species, where fishes were individually placed in a 70-mL respirometer chamber immersed in a larger water bath containing a heater with digital thermostat to control temperature (TIC-17RGT, FullGauge, ± 0.01accuracy) and left for six hours to handily recovery at the natural temperature of streams (25 °C). Subsequently, the metabolic rate was measured for four loops (routine metabolic rate) and the thermal ramp was initiated with an increase of 0.25 °C at each loop (15 min) of recirculating circuit reaching 1 °C $h^{-1}$ following the protocol suggested by Vinagre et al. (2014) and tested by Campos et al. (2016) to tropical fishes. Thermal limits were estimated using critical thermal methodology (CTM) (Lutterschmidt and Hutchison, 1997). The critical thermal maximum was defined as the temperature at which 50% of fish presented a final loss of equilibrium, or LOE (inability to maintain dorsal-ventral orientation for at least 1 min, Beitinger et al., 2000). After the thermal ramp, fish were allowed to recover in rest tank with streams temperature water and, after 24 h, all fish were returned to their environment.

In the present work, we refer to routine metabolic rate (mean values at 25 °C) measured at the acclimatization temperatures of the Amazonian streams (25 °C) during the thermal ramp assay as RMR. *T*MMR refers to the maximum metabolic rates observed during this thermal ramp, which, in this work, occurred before thermal limits. The difference between *T*MMR and RMR is termed as *T*scope based on former work of Jayasundara and Somero (2013). Factorial aerobic scope (FAS) was calculated as the ratio of *T*MMR to RMR (*T*MMR: RMR) for each individual. According to these authors, this approach assumes that the increase in oxygen consumption is exclusively due to the effects of temperature variations on metabolic processes and it is useful to test thermal limitations based on oxygen supply.

### 2.3. Measuring swimming activity levels on Amazonian streams fishes (lifestyles and swimming behavior)

Morphology data have been extensively used as a proxy to predict fundamental niche of fish species in streams; caudal fin morphology has been specially applied in studies investigating swimming behavior (or capacity). Caudal fin aspect ratio describes the shape of the tail, which is used to propel fish while swimming and is a correlate of average activity level across fish's species (*sensu* Pauly, 1989). It is calculated as

$$A\ h^2/s$$

where A is the aspect ratio, h is the height of the caudalfin, and s is the square area of the caudal fin. Caudal fin aspect ratio is directly correlated with activity levels in fish species and has been used as a powerful index of lifestyle and swimming behavior, where high values indicate high swimming activity levels (Pauly, 1989).

### 2.4. Statistical analyses

Statistical analyses were performed through Sigma Stat software

Table 1
Critical thermal maximum (CTmax), resting metabolic rate (RMR) and thermal maximum metabolic rate (*T*MMR) of Central Amazon fishes from Igarapés located at Reserva Ducke (02°53'S, 59°58'W), nearby Manaus. Data were obtained with the acute increase in thermal ramp methodology at 1 °C $h^{-1}$ (see Section 2 for complete description). Diff erent letters indicate significant diff erences between species ($p < 0.05$).

| Order | Family | Species | Weight (g) | CTmax (°C) | RMR ($mgO_2.kg^{-1}$ $h^{-1}$) | *T*MMR ($mgO_2.kg^{-1}$ $h^{-1}$) |
|---|---|---|---|---|---|---|
| Characiformes | Characidae | *Hyphessobrycon MELAZONATUS* | 0.64 ± 0.12 | 32.60 ± 1.28$^{a}$ | 285.1 ± 42.3$^{a}$ | 583.7 ± 65.6$^{a}$ |
| | | *HEMIGRAMUS* cf. *geisleri* | 0.35 ± 0.15 | 32.37 ± 0.85$^{a}$ | 331.1 ± 27.9$^{a}$ | 615.5 ± 46.2$^{a}$ |
| | | *IGUANODECTS geisleri* | 1.55 ± 0.2 | 30.87 ± 0.63$^{a}$ | 308.7 ± 28.2$^{a}$ | 598.4 ± 40.0$^{a}$ |
| | Crenuchidae | *CHARACIDIUM pteroides* | 1.12 ± 0.22 | 35.16 ± 0.51$^{abc}$ | 198.3 ± 31.5$^{b}$ | 493.9 ± 51.4$^{b}$ |
| | | *Crenuchus spilurus* | 0.92 ± 0.26 | 34.71 ± 0.5$^{ab}$ | 211.8 ± 17.4$^{b}$ | 443.5 ± 62.9$^{b}$ |
| | | *MICROCHARACIDIUM eleotrioides* | 1.12 ± 0.20 | 35.21 ± 0.48 $^{abc}$ | 236.5 ± 18.8$^{b}$ | 567.7 ± 45.7$^{b}$ |
| | Lebisianidae | *NANNOSTOMUS BACKFORDI* | 0.55 ± 0.05 | 35.50 ± 0.36 $^{abc}$ | 226.2 ± 22.4$^{b}$ | 531.0 ± 40.5$^{b}$ |
| | | *NANNOSTOMUS MARGINATUS* | 0.49 ± 0.05 | 35.67 ± 1.07$^{bc}$ | 207.1 ± 28.8$^{bc}$ | 514.2 ± 107.4$^{b}$ |
| | | *PYRRHULINA Aff brevis* | 2.16 ± 0.7 | 36.21 ± 0.69$^{bc}$ | 188.5 ± 24.8$^{bc}$ | 529.1 ± 52.2$^{b}$ |
| Cypriniformes | Rivulidae | *ANABLEPSOIDES micropus* | 1.07 ± 0.44 | 37.07 ± 0.37 $^{cd}$ | 129.57 ± 23.6$^{c}$ | 402.2 ± 73.2$^{c}$ |
| Perciformes | Cichlidae | *Aequidens PALLIDUS* | 1.22 ± 0.97 | 38.38 ± 0.92$^{d}$ | 156.5 ± 22.5$^{c}$ | 494.4 ± 47.7$^{c}$ |
| | | *APISTOGRAMMA HIPPOLYTAE* | 2.02 ± 1.16 | 38.62 ± 0.37$^{d}$ | 165.1 ± 17.4$^{c}$ | 427.9 ± 66.2$^{c}$ |

(v.3.5), and graphs were built with Sigma Plot software (v.11.0). The data are presented as the mean ± SD (standard deviation, n = 8). In all cases, the levels of significance were 95% ($p < 0.05$). The normality and variance homogeneity was checked before testing. CTMax, RMR, and *T*MMR are compared between families by one-way ANOVA, and diff erences between groups are tested by Tukey posthoc. Regression analyses were tested to verify the correlation between RMR and *T*MMR; Caudalfin aspect ratio and RMR; *T*FAS and CTMax.

## 3. Results

We performed a thermal ramp of twelve species belonging to five families of three orders, collected in riffles streams, low-flow streams, ponds and adjacent pools. The comparison of species within the same family revealed no diff erences for the analyzed parameters. However, diff erences were observed among species belonging to diff erent families. Therefore, the results were grouped in fish families (Table 1).

### 3.1. CRITICAL THERMAL TOLERANCE

We observed diff erences when compared critical thermal maxima between the studied families. Characidae presented the lowest thermal tolerance, followed by Crenuchidae and, then, by Lebisianidae and Rivulidae. The highest tolerance was observed in species belonging to the Cichlidae family ($F = 58$, $p < 0001$)(Fig. 1).

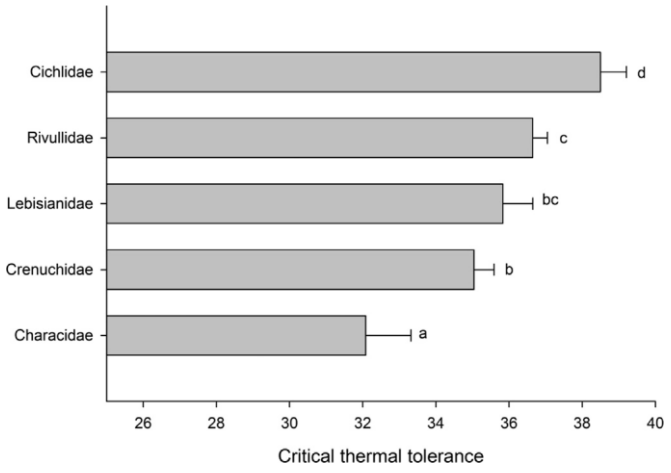


Fig. 1. Critical thermal maximum (CTmax) in diff erent families of Amazon fishes from Reserve Adolpho Ducke. The data were obtained with a thermal ramp methodology (increasing of 1 °C $h^{-1}$). Diff erent letters indicate significant diff erences among the ana- lyzed fish families ($p < 0.05$).

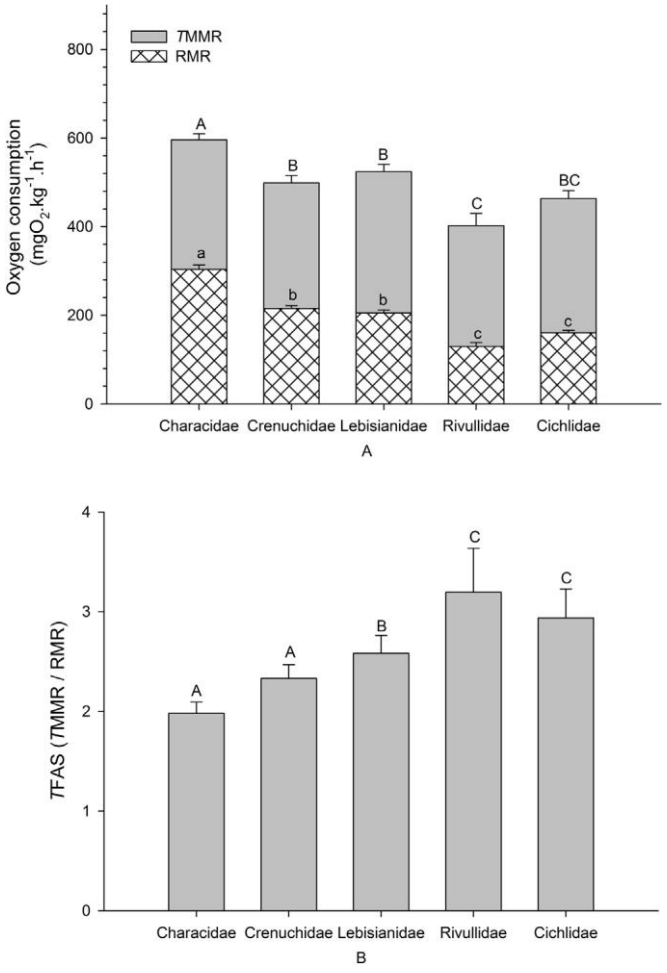


Fig. 2. A) Routine metabolic rate RMR (shaded bars) and thermal maximum metabolic rates (*T*MMR) (grey bars) of the diff erent studied fish families. RMR was measured at the respective acclimatization temperatures prior to the heat ramp. *T*MMR is the maximum oxygen consumption rate recorded during the heat ramp, which represents acute exposure to increasing temperature in fish acclimated to ambient temperature (25 °C). Diff erent letters indicate significant diff erences in RMR (lowercase) and *T*MMR (uppercase) across analyzed fish belonging to indicated families(one-way repeated-measures ANOVA, $P < 0.05$). B) Thermal factorial aerobic scope, calculated as *T*MMR/RMR, of the diff erent studied fish families. Diff erent letters indicate significant diff erences between families ($P < 0.05$).

### 3.2. Effects of THERMAL RAMP in METABOLIC RATE of AMAZON STREAM fishes

The comparisons of metabolic rates (RMR and *T*MMR) indicated differences between families (Fig. 2A). Species from Characidae family presented higher metabolic rates compared to all others species from all studied families; species from the families Crenuchidae and Lebisianidae showed intermediate values, and species from Rivulidae as well as from Cichlidae families exhibited the lowest values for these parameters (RMR $F = 62.27$, $p < 0.001$; *T*MMR $F = 12.34$, $p < 0.001$). Notwithstanding, species from Cichlidae and Rivulidae families showed higher factorial aerobic scope (FAS) compared to fishes belonging to the other families (Fig. 2B) ($F = 13.16$, $p < 0.001$), there was no difference in *T*scope.

### 3.3. Effects of swimming ACTIVITY levels on METABOLIC RATE AND THERMAL TOLERANCE

There was no correlation between weight and routine metabolic rate between ($p < 0.01$, $R^2 = 0.32$) and within (Characidae: $p = 0.039$, $R^2 = 0.05$; Crenuchidae: $p = 0.06$, $R^2 = 0.36$; Lebisianidae: $p = 0.01$, $R^2 = 0.31$; Rivulidae: $p = 0.45$, $R^2 = 0.1$; Cichlidae: $p = 0.39$, $R^2 = 0.068$) the families. Although between families p-value is lower than 0.05, the low correlation factor value ($R^2 = 0.32$) does not support this correlation. Therefore, this data suggest that weight has no effects on oxygen consumption in the species studied herein (Supplementary material). A positive correlation between RMR and *T*MMR was observed ($p < 0.0001$, $R^2 = 0.689$). Therefore, species with elevated RMR also exhibited an increase in *T*MMR (Fig. 3); furthermore, there was a positive correlation between FAS and CTMax (Fig. 4) ($p < 0.0001$, $R^2 = 0.57$), and a relationship between RMR and Caudal fin aspect ratio (Fig. 5) was found ($p < 0.0002$, $R^2 = 0.596$). All this data suggest that species with higher swimming activity levels present higher RMR and *T*MMR, and a limitation of FAS that sets its thermal limits.

In summary, Characidae and Crenuchidae were the families whose species presented higher swimming activity levels, demonstrating higher Avalues of caudal fin aspect ratio and higher metabolic rates with lower thermal limits, in opposite to the results observed for slow species belonging to the Rivulidae and Cichlidae families.

## 4. Discussion

Based on our results, we may state that lifestyle and swimming behavior influence the routine metabolic rate and thermal maximum

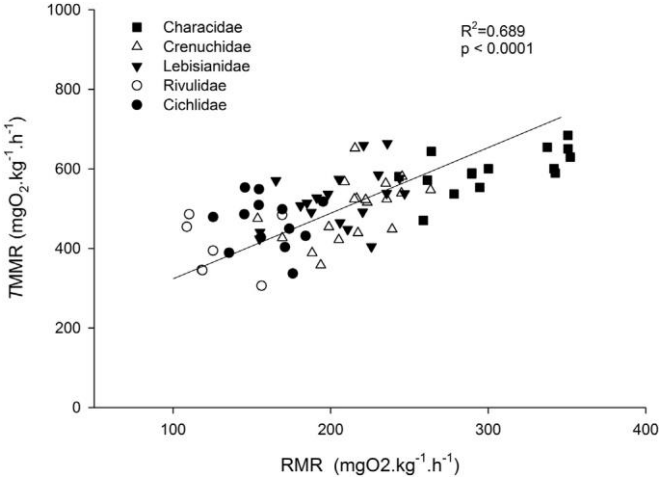


Fig. 3. Correlation analyses between individual routine metabolic rate (RMR) and thermal maximum metabolic rate (*T*MMR), measured under an acute increase in temperature (change of 1 °C $h^{-1}$), of Amazon forest stream fishes.

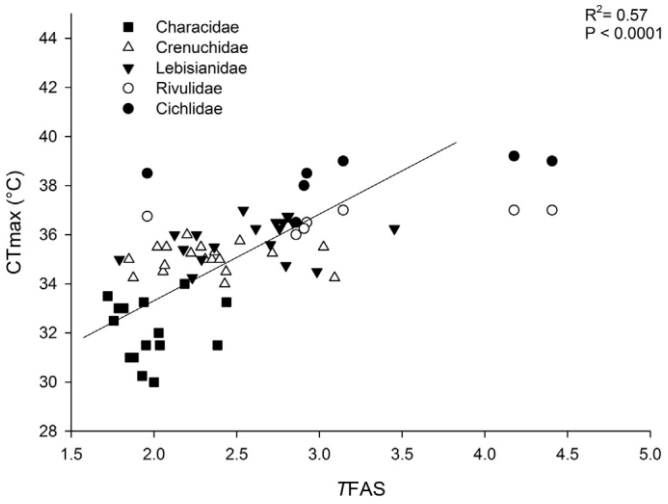


Fig. 4. Correlation analyses between individual factorial aerobic scope (FAS = *T*MMR/RMR) and critical thermal maximum (CTMax) of Amazon forest stream fishes.

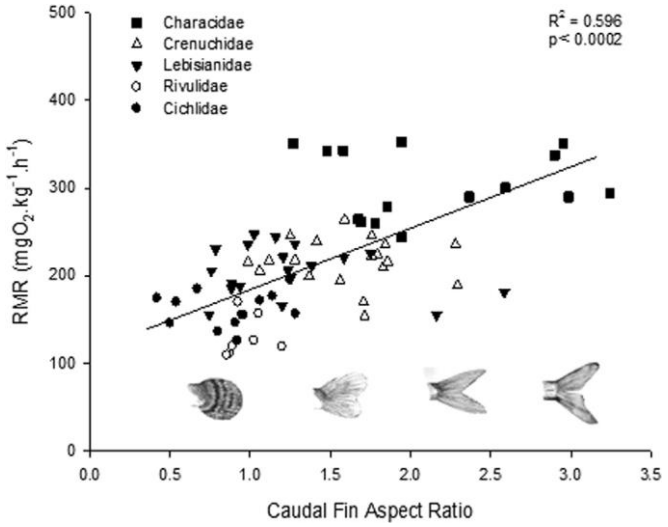


Fig. 5. Correlation analyses between individual routine metabolic rate (RMR) and caudal fin aspect ratio (morphology approach to indirect measure of activity levels in fishes) of Amazon forest stream fishes. Cichlidae/Rivulidae, Lebisianidae, Crenuchidae and Characidae caudal fins (left to right).

metabolic rate of Amazon forest stream fishes, revealing differences in the factorial limitation in metabolic demand, herein measured as FAS. The distinct aerobic plasticity could explain the 6 °C of variation in thermal tolerance between families. This is well supported by the strong correlation between factorial aerobic scope and critical thermal limits.

The evolution of fish lifestyle has brought them to develop several ecological adaptive traits that may determine their resilience or resistance (Pörtner and Knust, 2007; Killen et al., 2010; Eliason et al., 2011; Stoffels, 2015). Particularly, metabolic traits are correlated with lifestyle, such that fast species have higher metabolic rates than sedentary ones. In the present work, we observed that species presenting higher activity levels, like Characidae and Crenuchidae, showed higher routine and higher thermal maximum metabolic rate. High metabolic energy in continuous swimmers is a consequence of a higher mitochondrial concentration, higher cardiovascular work or higher muscle tonus that improves athletic performance (Clark, 2004). So, increasing metabolic demands allow a more active lifestyle; once continuous swimmers depend on high ATP supply to muscle and cardiac work.

Killen et al. (2016) hypothesized that there is a metabolic

continuum resulting from natural selection under ecological demands where species that exhibit a high level of activities appears to favor higher resting and maximum metabolic rates. On the other side of this continuum, the selection of sedentary lifestyles might reduce the resting and maximum metabolic rates. For example, benthic fish species present lower routine metabolic rates compared to those species living in the pelagic layer of the water column, possibly a result of different swimming behavior (Killen et al., 2010). Our results corroborate this hypothesis since they show a positive correlation between activity levels (based on personal observations and caudal fin aspect ratio) and routine metabolic rate, suggesting that natural selection of continuous swimming lifestyle has favored increases in the routine and maximum metabolic rates. Furthermore, Killen et al. (2016) proposed that species ecology traits are crucial for explaining the interspecific variation in metabolic rates.

Indeed, the evolutionary trade-off hypothesis pointed out that closely related species tend to have similar ecology characteristics and lifestyles than do more distantly related organisms (Clarke, 2004). Additionally, closely related species present an inter-dependent relationship between lifestyle and metabolic requirements. Therefore, in stable thermal habitats, like tropical rainforest streams, we might expect that lifestyle trait (e.g., swimming behavior, reproductive events, and growth rate) may determine the aerobic respiration ability, which has a vital role in setting the thermal limits of species.

Physiological and life-history traits influence how species respond to abiotic factors, and so it is assumed that lifestyle may be a trait that contains much information about how species will respond to environmental change (Clarke, 2004; Burton et al., 2011; Stofeels, 2015; Killen et al., 2016). In fact, we found a strong correlation (Figs. 3 and 5) between swimming performance, RMR and caudal fin morphology, which differs from sedentary fish, round fin format (Cichlidae and Rivulidae), throughout high swimming fish families, that have the V (or forked) type fin format in Characidae and Chrenuchidae. Thus, we could roughly predict the factorial aerobic scope of a species using their fin morphology, with potential effects on metabolic plasticity and thermal tolerance. Indeed, these data encourage further studies using a post-hoc test comparing all existing literature data including the present work. Although this will require statistical expertise and a literature review, this data would be an important further step towards achieving a general theory of how physiological trade-offs are correlated with lifestyle.

As mentioned above, species living in lotic conditions demand a high athletic performance, and typically require increase cardiac output (Farrell, 2007), respiratory surface area (Muir and Hughes 1969), and muscle mitochondrial density (Johnston et al., 1998); which supports the high respiration rates and ATP production required for an active lifestyle, all these traits contribute to increasing maintenance costs. This better metabolic machinery allows higher sustained energy throughout, thus enabling greater assimilation of energy. However, these traits may reduce physiological tolerance, once high metabolic rates may increase critical oxygen tension.Wells (2009) has pointed that fast fishes present low oxygen affinity hemoglobins, high Hill's coefficient, and a large Bohr effect, promoting the offloading of oxygen to muscle and supporting an active lifestyle. However, these traits reduce the capacity to extract oxygen from the water. Throughout these traits, thermal limitation results from a decrease in systemic oxygen levels (hypoxemia), which consequently is determined by the animal ability to extract oxygen and to efficiently deliver it to tissues. So, we may expect a trade-off between lifestyle and thermal tolerance.

*T*FAS represents the factor by which organism increase its metabolic rate above routine levels, and is thus an animal's capacity to support oxygen-consuming physiological functions related to thermal stress. A physiological increase in maintenance metabolic demands reflects in a factorial limitation in the ability of oxygen supply, which should also be translated as low factorial aerobic scope varying within a relatively narrow thermal range (Nilsson et al., 2009; Donelson and Munday, 2015). In fact, our results showed a correlation between *T*FAS and CTMax across families, suggesting that families presenting limited factorial aerobic capacity have low ability to face warming events, and, therefore, reduced thermal limits. In a former work, Stoffels (2015) suggested a physiological trade-off along a fast-slow lifestyle continuum in fishes. In that work, he proposed that sedentary species present low oxygen requirement and are more resistance to hypoxia. Tolerance to hypoxia and temperature are strongly related, due to the metabolic effects on oxygen supply and demand. Consequently, a factorial aerobic limitation should be the reason why athletic species present smaller thermal windows. However, *T*FAS is not related to CTMAx within fa- milies, once there is a great influence of taxonomic levels in the data. We observed that metabolic rate is more variable within families compared to CTMax. The data suggest that CTMax is a trait more conserved between families and, others factors than factorial aerobic scope should be important to determine thermal limitation within fa- milies.

It is important to mention that an advantage of the current approach used in this work is that the ability to increase oxygen consumption with warming is solely due to the effects of temperature on metabolic processes (Jayasundara and Somero, 2013). Hence, *T*MMR might reflect a compensatory response of energetic limitations induced by a cellular stress response during an acute heat stress and, consequently, we may expect that a higher capacity to increase aerobic scope may be the cause of a higher thermal tolerance.

In fact, *APPISTOGRAMMA HYPOLLITAE* and *Aequidens PALLIDUS*, from family Cichlidae, and *ANABLEPSOIDES micropus*, from family Rivulidae, exhibited lower activity levels and metabolic rates presenting high levels of thermal tolerance. Adaptation of species with lower metabolic rate favors the increase in oxygen extraction capacity and transport efficiency by modulating hemoglobin-oxygen affinity as showed by Nilsson and Renshaw (2004) for *Hemiscyllium OCELLATUM*; and Dhillon et al. (2013) and Fu et al. (2014) for *CARASSIUS AURATUS*. Species of Cichlidae and Rivulidae have, as well, a variety of respiratory responses to environmental challenges (Almeida-Val et al., 2000; Anderson and Podrabsky, 2014). For instance, Kochhann et al. (2015) observed regulation of metabolic rates to balance ATP supply and demand in the cichlid *APPISTOGRAMA AGASSIZII*, increasing efficiency of energy production when exposed to acute high temperature. Moreover, these works revealed that *A. AGASSIZII* down-regulated metabolic rate during hypoxia, indicating significantly higher capacity for metabolic reduction. The thermal effects on killifishes have been extensively studied and, in general, present similar metabolic regulation compared to Cichlidae species, such as increase of metabolic efficiency to ATP supply at higher temperatures. Moreover, Anderson and Podrabsky (2014) verified that late embryos of *Austrofundulus LIMNAEUS*, living at pounds in Maracaibo basin from Venezuela, present no increased heart rates (an indirect metabolic rate measurement), and became less sensitive to warming incubation during development, indicating a temperature insensitivity when acclimated to 30 °C. Therefore, adaptation to slow life-style species have increased oxygen extraction abilities which permit low metabolic rate and high factorial aerobic scope that sets high thermal tolerance in sedentary species.

Clark et al. (2017) proposed that warming climate favor low-performance phenotypes of *Plectropomus LEOPARDUS* with potential effects on predator-prey interactions and community dynamics. In Amazon forest streams, sedentary species have low capacities to migrate between habitats. Therefore, we may speculate that warming, affecting high swimming performance species should alter community ecology with potential impacts on a metabolic continuum of streams since a little quantity of organic material should be transported down streams by sedentary species.

Summarizing, we found a strong correlation between swimming lifestyle and metabolic traits, the families Characidae and Crenuchidae studied in the present work showed high resting metabolic rates and thermal maximum metabolic rates. They also presented higher activity

levels related to their living habitats, indirectly measured by their forked (V type) caudal fin aspect ratios;furthermore, species with higher metabolic rates presented lower thermal tolerance. Thus, we hypothesize that these higher resting energetic demands in athletic fishes decrease the factorial aerobic scope, and retain the oxygen-processing ability to face warming waters. Therefore, these groups will probably be the most affected ones by the future climate changes, especially to the wave heats that increase the temperature in a short time. Characidae and Crenucidae species are predicted to be more sensitive to global warming because they appear to have a narrow thermal tolerance range. However, evaluation of the acclimation capacity and potential epigenetic effects must be tested before any predictions are made.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jtherbio.2018.02.002.

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**Capitulo II**

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## Gill physiology and morphology of three Amazon forest stream fishes under an extreme climate change scenario

**In prep to: Frontiers in physiology**

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Abstract

Climate change is one of the major threatened for an organism that inhabits tropical environment due to their adaptation to low thermal variability. Temperature and high pCO2 in the water affect both respiration and osmoregulation, therefore, understanding the gills physiology is vital to predicting the effects of climate change since it plays a central role in gas exchange and ion regulation. In the present study, we exposed three different species (*A. agassizii*, *P. brevis* and *H. melazonatus*) to two distinct climate scenario, the current and the extreme (plus +4.5C and 900ppm CO2, above current scenario) and investigate their effects on osmorespiratory compromise. We evaluated the whole O2 consumption, net ionic flux rates ($Na^+$, $K^+$ ,$Cl^-$ and total ammonia fluxes), mitochondrial physiology, and morphological changes in the gills. Our findings showed that all species increased oxygen consumption. The mitochondrial respiration meets the supply ATP demand to functional activities of ATPases. In addition, all species presented alterations on gills morphology increasing the respiratory surface area to improve oxygen exchange. However, only *A. agassizii* and *H. melazonatus* increased ion loss. Our results suggest specie-specific alterations on osmorespiratory compromise under climate change scenario.

## 1. Introduction

Understanding the physiological impacts of interacting temperature and $CO_2$ is critical for predicting how animals will respond to human-induced climate change (Rudd, 2014; Todgham and Stillman, 2013; McBryan et al., 2013). Elevated temperature induces effects on chemical and biochemical reactions, increasing the oxygen demand, cardiac output and blood flow in ectothermics. These alterations have detrimental effects on ionic balance, since respiration and osmoregulation represent a

trade-off between gas exchange and ionic/osmotic fluxes, such that the latter become greater when the need for the former increases (Randall et al., 1972; Nilsson, 1986). Moreover, high $pCO_2$ disrupts the expensive acid-base balance of fish, since they are able to adjust their internal acid–base by adjusting plasma $HCO_3^-$ levels through the differential regulation of $H^+$ and $HCO_3^-$ effluxes, which are coupled to, respectively, the influx of $Na^+$ and $Cl^-$. Therefore, hypercapnia enhancing the physiological consequences of the osmorespiratory compromise when fish species face warming events (Rosa et al., 2016).

In this sense, understanding the gills physiology is vital to predicting the effects of climate change, since gills plays a central role in gas exchange and ion regulation.Accordingly, there are important trade-offs in gill design and physiology where increased processes to improve gas exchange have detrimental effects on ion regulation and, consequently, to acid-base balance. To date, the osmorespiratory compromise is well studied in the context of swimming activity (Robertson et al., 2015), low oxygen concentration (Iftikar et al. 2010; Robertson et al. 2015b) and elevated temperature (Mitrovic and Perry, 2009). However, little is known about theinteraction between temperature and hypercapnia (Kreisset al., 2015), which would be critical to understand the effects of climate change.

Acclimation to elevated temperature is known to alter a variety of gill physiological processes associated with oxygen supply and demand in fish, and can alter the capacity for oxygen uptake by changing gill surface area through regression of the interlamellar cell mass (ILCM) (Sollid et al., 2005; Mitrovic and Perry, 2009). Besides, high levels of $CO_2$ result in the toxic accumulation of protons, which promotes respiratory acidosis. Physiological mechanisms linked to $CO_2$ excretion are increasesin the ventilation rate, $HCO_3^-$ regulation and activation of H- ATPase (Perry and Gilmour,

2006). Therefore, both warming and high pCO2 promotes disturbance on the osmorespiratory compromise. Maintaining ion homeostasis is an expensive mechanism that demands high ATP supply to the v-type $H^+$ and $Na^+K^+$ATPases and, therefore, mitochondria should play an important role to energy supply in gills (Kreiss et al., 2015). However, as far we know, there is no studies evaluating the role of mitochondrial physiology on the osmorespiratory compromise.

Amazon forest stream is a thermal stable habitat that presents high fish diversity and endemism; Climate change is one of the major threatened for an organism that inhabits tropical environment due to their adaptation to low thermal variability (Stillman, 2003; Tewksbury et al., 2008). In addition, riparian deforestation is increasing in the Amazon region and has increase temperature of small streams up to 5°C (Macedo et al., 2013). However, little is known about the effects of climate change scenario or temperature on osmorespiratory physiology on Amazon fishes. Therefore, in the present study, we investigated the osmorespiraroty compromise on three abundant species of Amazon forest stream. The species were choose because they present a distinct lifestyle and, as we showed previously (Campos et al., 2018), these have pronounced differences in metabolic rate what could affect the osmorespiratory compromise in different ways.

In the present study, our primary objective was to evaluate gill physiological and morphological adaptations during exposure to two different climate change scenarios. Our main goal was to understand how climate change would affect the osmorespiratory compromise. For that purpose we evaluated the O2 consumption, ionic net flux rates ($Na^+$ , $K^+$ , $Cl^-$ and ammonia fluxes), mitochondrial physiology, and morphological changes in the gills. We hypothesized that elevated temperature and pCO2, would affect distinctly the osmorrespiratory compromise of the species, since these species

have different metabolic demand related to their lifestyle. In accordance with our previously report, the fast swimming lifestyle species (*Hyphessobrychon melazonatus*) should present the greatest physiological alterations compared to sedentary ones (*Apistogramma agassizii* and *Pyrhulina brevis*).

## 2. Material and Methods

### 2.1. Fishes collection and Maintenance

Fishes were collected in small order streams at a protected reserve located in the central Brazilian Amazon bordering Manaus named the Reserva Florestal Adolpho Ducke (02°53'S, 59°58'W). Amazon forest streams are stable thermal habitats with very low seasonal and daily temperature variation (24-26°C) and high oxygen concentration (5-8 $mgO_2$. $L^{-1}$). Fish were collected by hand and seine nets (2-mm mesh) (see Mendonça et al., 2005 for details). After capture, fishes were held in a 50-L aquarium with constant aeration and transferred to LEEM – Laboratory of Ecophysiology and Molecular Evolution at Brazilian National Institute for Research of the Amazon. At LEEM facilities, the species were individually held at 150-L tank (Fortlev®) for two-weeks before experimental setup start. Half of the water was replaced every 24 hours. During this period, fish were fed *ad libittum* daily with TetraMin® Flakes. The outdoor temperature was 26±1.0°C. All housing and experimental sets are in accordance with CONCEA Brazilian Guide for Animal Use and Care and were authorized by INPA's Council for Ethics in Animal Use (CEUA - protocol number 027/2015).

### 2.2 Experimental scenario exposure

The species, *Apistogramma agassizii*(1.2 ±0.3g), *Pyrrhullina brevis*(4.3 ±0.5g) and *Hyphessobrychon melazonatus*(1.0±0.2g)were exposed to two distinct climate

scenarios inclimate rooms. These systems consist of climatic rooms with temperature and $CO_2$ computer controlled. Sensors measured temperature and $CO_2$ at Reserve Ducke every other minute and transmit the data to laboratory computers that control environmental rooms according to the climate scenarios. The climate scenarios provided by the Assessment Report of the IPCC (2014) for the year 2100 were simulated in the current scenario room (current temperature and $CO_2$ levels) and extreme scenario room (4.5°C and 850 ppm $CO_2$ above current levels). Room temperature and $CO_2$data are available in supplementary material (SM1).

Adults of the three species were transferred to 10-L PVC tanks in three replicates per species containing six individuals per tank for each scenario, N=36 per species. The fish were maintained in each climate room scenario for 30 days. To avoid ammonia accumulation, 30% of the water was replaced every day using environmentally stabilized room water. The pH, $O_2$, $CO_2$ and temperature levels of the water were measured daily (Table 1). The fish were fed *ad libitum* once a day using commercial TetraMin® Flakes and fish were unfed 48 hours before experimental set-ups.

*2.2.1 Oxygen consumption*

Firstly, we measured fish oxygen consumption in a closed chamber system to concomitantly measurethe net ion flux rates, as described below. For that purpose, individuals were transferred to a 150mL PVC chamber for *A. agassizii* and *H. melazonatus* and a 500ml PVC chamber for *P. brevis*, maintaining the approximately 100:1 ratio of water volume/fish weight (n=6), the temperature and $CO_2$ in the chamber did not differ of the acclimation aquarium in the climate room. Fish were permitted to recover from handly for 2 hours in fully oxygenated water. After this period, aeration

was stopped, and the fish oxygen consumption was monitored by 15 min using an Oxy-4 oximeter (Loligo system). After this period, the oxygen was turn-on for 45 min. Also, background respiration was measured concomitantly. The oxygen consumption was measured three times to each individual, so we calculated the mean of the replicate of oxygen consumption following: $MO_2 = -\Delta O.Vresp.\ B^{-1}$,

Where ΔO is the rate of change in oxygen concentration ($mgO_2\ h^{-1}$), V resp is the volume of the respirometry chamber, and B is the mass of the individual (kg). There was not oxygen consumption in the background respiration. After that, fishes were euthanized by concussion and euthanized by medullar section. Gills were removed to physiological and morphological analyses as described below. In addition, 10mL of water was collected before the oxygen consumption start and at the end of 3 hours of oxygen consumption analyses to measure ions flux rates.

2.2.2. Ions flux

In water, major ions components ($Na^+$ and $K^+$) concentrations were determined by flame emission spectrometry (Analyser), using an atomic spectroscopy curve standard (Perkin Elmer Pure) as reference. Total chloride ($Cl^-$) was measured by the colorimetric assay described by Zall et al., 1956. Aqueous ammonia levels were measured using the colorimetric assay developed by Verdouw et al., (1978).

*2.2.3.Mitochondrial respiration*

Gills were dissected and the branchial arch from the right-side was separated to measure mitochondrial respiration. The gills filaments were separated and immersed in ice-cold relaxing buffer (BIOPS, 2.8 mM $CaK_2EGTA$, 7.2 mM $K_2EGTA$, 5.8 mM $Na_2ATP$, 6.6 mM $MgCl_2 \cdot 6H_2O$, 20 mM taurine, 20 mM imidazole, 0.5 mM dithiothreitol, 50 mM K-MES, 15 mM Naphosphocreatine and 50 mM sucrose, pH 7.2).

The gills were teased into fiber blocks using a dissecting microscope and placed in 1 ml ice-cold BIOPSalong with 50 μg/ml. After 30 minutes, fiberswere washed three times for 10min in 2ml of modified mitochondrial respiratory medium (MiRO5, 0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6H_2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM $KH_2PO4$, 20 mM HEPES, 160 mM sucrose and 1 g/l BSA, essentially free fatty acid, pH 7.2 at 25 °C; Gnaiger et al. (2000)). The fibers were blotted dry on filter paper and weighed into 5–10 mg bundles for respiration assays in 2mL of MIR05. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

*2.2.4. Mitochondrial respiration assay*

Mitochondrial respiration was measured in ahigh resolution respirometer, Oroboros Oxygraph-2k™ (Oroboros Instruments, Innsbruck, Austria) utilizing the SUIT protocol (Braz-Mota et al., 2018) at mean acclimation scenario temperature (26°C for Current scenario and 30°C for extreme climate scenario). Oxygenwasmaintained above saturation to ensure saturationat respective assay temperatures tomaintain steady state oxygen flux, the SUIT protocol is following.

The Complex I (CI) substrates (2 mM malate,10 mM pyruvate and 10 mM glutamate) were added to measure state II respirationin the absence of ADP (denoted Leak). Excess ADP(2.0mM) to stimulate oxidative phosphorylation was added to saturate CI. Cytochrome c (10 μM) tested outermembrane integrity. Phosphorylating respiration with CI and CII substrates(OXP-I, II, state III respiration) was attained by addition of succinate(10mM). Followed by titrationsof carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone (CCCP, 0.5 μmol/l) to uncouple mitochondria (ETS). By the additionof rotenone (0.5 μM), malonate (15mM) and antimycin (1 μM),CI, II and III were inhibited, respectively. The residual flux following theaddition

of these inhibitors was attributed to background respiration.Cytochrome c-oxidase (CCO, CIV) was measured by the addition ofthe electron donor couple N,N,N′,N′-tetramethyl-p-phenylenediamine(TMPD, 0.5 mM) and ascorbate (2 mM). All the chemicals were purchase from Sigma Aldrich.

2.3. Ionoregulatory enzymes

Gills were homogenized (1:10 w/v) in SEI buffer (pH 7.5) containing (in mM): sucrose 150, imidazole 50, EDTA 10 and deoxycholic acid 2.5, and centrifuged at 2,000 ×g for 7 min at 4 °C.

The activities of both NKA and v-type $H^+$-ATPase were determined by NADH oxidation in an enzymatic reaction coupled to the hydrolysis of ATP (Kültz and Somero, 1995). The assay is based on the inhibition of NKA activity by oubain (2 mM), and v-type $H^+$-ATPase by Nethylmaleimide (NEM, 2 mM). Supernatants were added to a reaction mixture containing (in mM): imidazole 30, NaCl 45, KCl 15, MgCl2 3.0, KCN 0.4, ATP 1.0, NADH 0.2, fructose-1,6-bisphosphate 0.1, PEP 2.0, with 3 U $mL^{-1}$ pyruvate kinase and 2 U $mL^{-1}$ lactate dehydrogenase. Samples were run with and without ouabain or NEM. Absorbance was followed over 10 min at 340 nm. NKA and $H^+$ATPase activities were calculated by the differences between total activity and activities with ouabain and NEM inhibitors, respectively.

Carbonic anydrase activity was quantified according to the assay described by Vitale et al., (1999), based on Henry, (1991). Gills were homogenized (1:10 w/v) in phosphate buffer (10 mM, pH 7.4) and centrifuged at 2000g for 5 min at 4 °C. Supernatants (50 µL) were added to 7.5 mL ofreaction buffer, pH 7.4 containing (in mM): mannitol 225, sucrose 5, and tris-phosphate 10 and 1 ml of cold distilled water saturated with $CO_2$. Immediately after the addition of $CO_2$-saturated water, the

reduction in pH was followed for 20 s, with pH readings every 4 s. Carbonic anhydrase specific activity (CAA) was calculated as: CAA = $[(CR/ NCR)^{-1}]$ $mg^{-1}$ total protein in the sample (CR: Catalyzed rate and NCR: non-catalyzed rate).

### 2.4. Gill morphology

Second gill arches from the left side were fixed in 4% neutralized formalinfor 24 h and subsequently dehydrated in ethanol (70%, 80% and 96%). Samples were embedded in Paraplast Plus (Sigma Aldrich), and serial sections of 3-μm thickness were prepared on glass slides, which were stained with hematoxylin and eosin, and then contrasted with Periodic acid–Schiff (PAS stain). Samples were analyzed at 40×magnification in a light microscope [Laica Stereomicroscope DM 2000, 10× zoom; image capture using cellSens Software (standard)] to determine lamellar distance, lamellar height and basal lamellar width (Wegner, 2011). Images were digitized using ImageJ (v1.48). We measured lamellar height and basal length for five consecutively lamellae from each filament. We estimated lamellar frequency based on the distance between five lamellae at a randomly chosen point along the filament. Lamellar surface area was calculated as two times the product of lamellar height and lamellar length.

### 2.5. Statistical Analyses

The data are presented as mean ± SEM (n = 6). All statistical analyses were performed in SigmaStat 3.1 using a significance level of 5% Parametric ANOVA or non-parametric Kruskal-Wallis test (depending on whether the data met the assumptions of normality and homoscedasticity) to detect significant differences in the gills physiology and morphology among different climate change scenarios and species. *Post hoc* tests were carried out using Tukey's HSD. The results of mitochondrial respiration

are presented as the difference between the phosphorylate complex (CI and ETS) and Leak respiration to denote the amount of phosphorylation coupling to ATP synthesize.

## 3. Results

### 3.1. The effects of climate change scenario exposure on osmorespiratory compromise of Amazon fishes

The oxygen consumption varied significantlyamong species and between scenarios ($F=16.38$; $p<0.0001$). *Hyphessobrychon melazonatus* presented highe rmetabolic rate at current and extreme scenario compared to *Apistogramma agassizii* and *Pyrhullina brevis*. Besides, all species present higher metabolic rates at the extreme scenario compared to current. The metabolic rate increased by 1.78 times for *H. melazonatus*, 1.72 times for *P. brevis* and 2.18 for *A. agassizii* (Fig 1).

The ions flux rate were different between species and scenarios (table 2). The higher total ammonia excretion was observed in *H. melazonatus,* while the lower in *A. agassizii* at current scenario, despite, there were no difference between species at extreme climate change scenario. In addition, *H. melazonatus* and *P. brevis* did not change $NH_4^+$ excretion at extreme compare to current scenario. On the other hand, *A. agassizii* increased $NH_4^+$ excretion at extreme scenario compare to current ($F= 19.86$; $p<0.0001$).

Besides, among the studied species, *H. melazonatus* presented higher $Na^+$ excretion at both current and extreme scenario compared to *A. agassizii* and *P. brevis* and also presented higher $Na^+$ excretion at extreme scenario when compared to current ($F=11.75$; $p<0.0001$). The species *A. agassizii* and *P. brevis* did not changed the Na+ excretion.

The $K^+$ excretion was higher in *H. melazonatus* at both the current and extreme scenario when compared to the other species. There was no change in this species at extreme scenarios when compared to current ($F=44.83$; $p<0.0001$). Besides, *A. agassizii* increased $K^+$in the extreme climate scenario compare to the current. Amongst species, *H. melazonatus* presented higher $Cl^-$ excretion in current scenario. In addition, *P. brevis* presented the lower excretion in the extreme scenario compare to the others species. Amongst scenarios *A. agassizii* increased $Cl^-$ excretion while *H. melazonatus* and *P. brevis* decreased $Cl^-$ excretion at extreme scenario when compare to current.

There were no exposure effects of neither the climate change scenario nor the species on branchial $Na^+/K^+$-ATPase activity in all species (Fig. 3A). In *P. brevis*, gill v-type $H^+$-ATPase activity was stimulated compared to current scenario after 30 days of exposure (Fig. 3B). In contrast, *H. melazonatus* and *A. agassizii* did not change $H^+$-ATPase activity (Fig. 3B). *H. melazonatus* exposed to extreme climate scenario had its CA activity inhibited (Fig. 3C). Conversely, CA activity in *A. agassizii* and *P. brevis* showed no alterations (Fig. 3C).

3.2. The effects of climate change on gills mitochondrial metabolism of Amazon fishes

Mitochondrial respiration differs between species. Leak respiration is higher in *H. melazonatus* and lower in *A. agassizii* in the current scenario. At the extreme climate change scenario, *P. brevis* increased leak respiration compared to the current ($F=11.99$; $p< 0.001$; Fig. 2A). Thephosphorylate oxidation of complex I is higher in *A. agassizii* and *P. brevis*in the current scenario and increased in *P. brevis* at the extreme climate change scenario when compared to current ($F=13.61$; $p< 0.0001$; Fig 2B). Among species, *P. brevis* presented higher ETS respiration at current scenario while increased at extreme climate change scenario compared to the current ($F=12.17$; $p<0.0001$; Fig.

2E). The cytochrome C oxidase activity (CIV) is higher in *P. brevis* compared to *A. agassizii* at current scenario. However, *A. agassizii* increased CCO respiration in theextreme scenario compared to the current and, therefore, did not differ with*P. brevis* at extreme climate scenario ($F=3.88$; $p<0.001$; Fig 2F).

3.3 Gills remodelling under climate change exposure

Regarding gill morphology, *A. agassizii* and *H. melazonatus* presented similar responses increasing lamellar height, width and distance in the extreme scenario compared to the current one. In opposite, *P. brevis* showed different trends increasing only lamellar width (table 3; Figure 5). The morphometrics calculus indicates that *A. agassizii* increased lamellar frequency and all species increased respiratory surface area.

4. Discussion

Understanding the physiological mechanisms of species to cope with climate change scenarios is critical to determine species` adaptation and vulnerability, especially regarding gas exchange and ion balance. Here, we observed that the three studied species present distinct gillsregulatorymechanisms;therefore, warming and high $CO_2$ levels will influence Amazon fish species in different ways. In general, *H. melazonatus* showed the greatest alterations in ionoregulation, increasing $Na^+$ efflux and decreasing carbonic anhydrase activities, indicating an ionic and acid-base imbalance. On the other hand, *P. brevis* adjust its mitochondrial physiology, increasing leak respiration and oxidative phosphorylation to regulate ATP demand these response could be related to an increase in $H^+$ ATPase in order to maintain iono balance. While, *A. agassizii* increased cytochrome c oxidase activity at mitochondria levels, and $NH_4^+$ and $Cl^+$ excretion,all this data should be related to its high metabolic demand and gill remodelation needs when facing global warming.

4.1. The osmoregulatory compromise in Amazon fishes under climate change exposure

Consistent with former works,our results showed that acclimation to elevated temperature and $pCO_2$increases routine metabolic rate (McBryan et al., 2015; Peck et al., 2010; Schulte, 2015; Campos et al., 2017). Elevated metabolic rate with warmingis related to the effects on the rate of reactions, the expression of new proteins (Schulte, 2004), the remodeling of cell membranes (McBrayan et al., 2015), and the operation of molecular chaperones (Madeira et al., 2018). Meanwhile, high $pCO_2$ increase oxygen consumption by altering the expensive ion and acid–base regulatory mechanisms through ATPases activities and cardiac output (for review see Heuer and Grossel, 2014). However, our findings showed that functional capacities of Na+/K+ ATPase remained unaffected by high Temperature and pCO2 (Fig. 3A). In accordance, previous studies showed branchial $Na^+/K^+$ ATPasecapacities were either found reduced or also unchanged under pCO2 expected by climate change scenario (Esbaugh et al., 2012; Kreiss et al., 2015). On the other hand, *P. brevis* increased functional capacities of branchial $H^+$ ATPase acclimated at extreme climate change indicating an activation to maintain internal pH caused by high pCO2 levels at extreme climate change. In constrast, $H^+$ ATPase were thermallycompensated (i.e. down-regulated) in cod acclimated to 18°C at hypercapnia (Michael., 2015).

Our results showed that *H. melazonatus* increased $Na^+$ efflux, while *A. agassizii* increased total ammonia and, $Cl^-$ and $K^+$ efflux related to theirincreased metabolic rate at the extreme scenario. Although we observed that these species increased ion loss, they showed those increase for different ions, suggesting a specie-specific ion and acid-base regulatory mechanism.

Herein, we observed that *H. melazonatus* presented iono disruption that should be linked to the inhibition of carbonic anhydrase, sinceWood et al., (2015) studying *Paracheirodon axelrodi* (Characidae) proposed a link between $Na^+$ uptake, Ammonia excretion and the supply of $H^+$ mediated by carbonic anhydrase. The authors suggested that internally generated $H^+$ ions provided by the catalyzed hydration of $CO_2$ are integrated to the $Na^+$ uptake mechanismof the cardinal tetra. Our data for *H. melazonatus* (Characidae) corroborates theses hypothesessince under warming and high $pCO_2$this speciesincreased $Na^+$ loss and decreased both $NH_4^+$ excretion, not significantly, and carbonic anhydrase.Therefore, we propose that climate change scenario will disrupt carbonic anhydrase affecting $NH_4^+$ excretion and $Na^+$ uptake. Downregulation of CA have been reported in the gulf toadfish exposed to hypercapnia for 8 –72 h (Esbaugh et al., 2012)and, has also been demonstrated in embryos and larvae of medaka exposed (Tseng et al., 2013). The authors suggest that the downregulation seen supports the observation that the compensatory response to elevated $pCO_2$ involves $HCO_3$ uptake rather than $H^+$ excretion.

Studies from lebisianidae species are scarce, however, the single study (Rafael Duarte Thesis) from this family (*Nannostomus marginatus*) provides evidence that increases in $Na^+$ influx rates were accomplished by the $H^+$-ATPase enhance when the animal are exposed to low pH. Our data corroborates these findings since we observed an increase in $H^+$ATPase for *P.brevis* under extreme scenario exposure, even though this species showed no differences for Na+ netflux. Lin & Randall (1993) first reported the presence of an $H^+$-ATPase in gills of teleost fish, also showing an increase of $H^+$-ATPase activity in fish acclimated to low $Na^+$ levels or under respiratory acidosis. Recent studies using a molecular approach have demonstrated the involvement of gill $H^+$-ATPase in $Na^+$ uptake, and their linkage with metabolic acid secretion in freshwater

acclimated fish (Evans 2011; Hwang et al 2011). Therefore, to maintain ion regulation these species increase $H^+$ATPase to $Na^+$ uptake in exchange for an acidic equivalent ($H^+$). In addition, the lower $Cl^-$ excretion levels suggest a decrease of exchange with$HCO_3^-$ to maintain internal pH (Gilmour and Perry, 2008). Again, although no significant, theslightly increase in net $Na^+$could be related to branchial permeability that under warming and acidic conditions improve ions efflux (Gonzalez & Wilson, 2001; Gonzalez et al., 2002).

In contrast, the Cichlidae *A. agassizii*, increased total ammonia and $K^+$ excretion these seems to be related to the lower intrinsic gill permeability for $Na^+$ in cichlids, as proposed by Gonzalez et al., (2002). Therefore, under the extreme climate change scenario these species showed an increase in $Na^+/NH_4$ and $Na^{+/}K$ transporters, which probably increased the excretion of $NH_4$ and $K^+$. Randall et al. (1999) suggested that $NH^+_4$ is transported from the blood into the ionocyte by the basolateral Na+/K+-ATPase with $NH_4^+$substituting for $K^+$, and from the ionocyte to the external medium by the apical $Na^+/H^+$ ($NH^+_4$) exchanger.Although no significant changes were observed in $Na^+K^+$ATPase activity that could support this idea, the increase in both excretion $K^+$ and $NH^+_4$ suggest the involvement of these mechanisms.

## 4.2 The role of mitochondrial respiration on ionoregulation under climate change

The iono regulation in fishes is an expensive mechanismthatoccurs through the two major cell types present in the gill epithelium: the mitochondrion-rich chloride cell (CC) and the pavement cell (PVC). Therefore, energy supply plays important role in the regulation of ATP levels in the cellsfor ionic and acid-base balance in gills. According to Kreisset al (2015) Functional capacities of ATP-synthase were closely correlated

with Na+/K+ ATPase and, to a lesser extent, with H+ ATPase capacities in Atlantic cod (*Gadus morhua*) acclimated to warming and high pCO2 levels. Therefore, branchial aerobic ATP supply was coordinated by ATP demanding components of ion and acid-base regulation.

Overall, our findings showed high mitochondrial acclimation capacities in all species, because the levels of oxidative phosphorylationhave conserved between acclimation treatments.In accordance, no changes in functional activity of NAK ATPases were observed in all species. In addition, *P. brevis* increased $H^+$ATPase activity, this was supplied by the increase in the oxidative phosphorylation that improve ATP demanding (Fig 2 B and C).

Although *P. brevis* increased leak respiration, which could decrease ATP supply, they also increased oxidative phosphorylation at the same rate. Therefore, there was no energetic impairment. Warming depresses OXP coupling and extreme heat stress causes irreversible changes in mitochondrial inner membrane integrity in the fish heart (Iftikar et al., 2014), this acts through increased inner mitochondrial membrane proton leakage, and therefore decreased OXP efficiency. However, proton leak can also occur through the activation of uncoupling proteins (UCP) which is not related to impairments at the mitochondrial membrane and could maintain ATP synthase.

The mitochondrial efficiency are dependent on the components of oxidative phosphorylation (OXP) being intact (Brand and Nicholls, 2011). In hearts, the decrease in oxidative phosphorylation has been identified as a factor that limits cardiorespiratory systems in stenothermal fishes at warming (Iftikar et al., 2014). Iftikar and colleagues showed that tropical reef fish (*Thalassoma lunare*) the increase in leak respiration was coupled to mitochondria membrane disruption that impaired tissue energy supply and

decreased cardiac output at warming acclimation. Our findings suggest that the alterations in ion exchange of the Amazon forest streams species was not related to mitochondrial capacity dysfunction, since no changes inoxidative phosphorylation were observed and, consequently, the increase in ion loss must be related to branchial permeability (discussed below)or protein disruption, as observed by inhibition of carbonic anhydrase in *H. melazonatus*.

While Leak respiration is indicative of uncoupling ATP production, the CCO respiration is an indicator of maximal aerobic capacity for ATP production.The activity of CCO in ectotherms is typically highest in tissue that has high metabolic demands (Ludwig et al., 2001). Herein, we observed that CCO is associated with high oxygen flux compared to ETS respiration this indicates that the studied species are capable of increasing oxidative phosphorylation under events of high metabolic demand. Interestingly, *A. agassizii* acclimated to extreme climate scenario increased CCO respiration indicating that when necessary this species can increase oxidative phosphorylation to improve ATP supply in gills to maintain iono-regulatory and acid-base balance.

4.3. Gills remodeling and consequences for the osmorespiratory compromise

Gills mechanisms to deal with high oxygen demand events, like hypoxia, swimming and temperature involve the lamellar morphological plasticity (for review see Gilmour and Perry, 2018). Consistent with former works, our findings corroborates that acclimation climate change scenario induce gills remodelling (Mitrovic and Perry, 2009; McBryan et al., 2015). Herein, we observed that all species increased respiratory surface area by altering gill morphology in the extreme climate scenario acclimation (table 3). Mitrovic and Perry (2009) suggest that increases in the lamellar surface area

seem in warming conditions are related to the regression of the interlamellar cell mass (ILCM) that increases the lamellar height. In fact, this has been shown to occur in a variety of fish species (Sollid et al., 2003, 2005; Nilsson, 2007; Matey et al., 2008; Dhillon et al., 2013; Johannsson et al., 2014; Anttila et al., 2015). The reversible gill remodeling trough the retraction of the ILCM has been found to be correlated to the $O_2$ demand. The main support for this comes from a study that revealed a significant relationship between hypoxia tolerance and the capacity for gill remodeling across a range of carp species (Dhillon et al., 2013). In accordance, we observed an increase of lamellar height that may have related to the regression in the ILCM in *A. agassizii* and *H. melazontus* related to the improvement of oxygen uptake (Figure 5).

In fishes, oxygen supply capacity have been correlated with thermal performance, therefore, species must meet the oxygen demand at warming to improve survival (Johansen et al., 2015; Pörtner and Knust, 2007). In fact, McBryan et al., 2015 related that warming acclimation improves hypoxia tolerance by increase oxygen consumption through gills remodelling. In accordance, our findings showed that all species increased the respiratory surface area to increase oxygen to meet tissue oxygen demand; however, it comes with the cost of ion losses in *H. melazonatus* and *A. agassizii*. However, *P. brevis* showed distinct gills remodeling mechanism and did not presented differences in ion losses, therefore, the mechanism used by this species should be more efficient to increase oxygen consumption without increase ion loss.

## 5. Conclusion

In summary, our findings showed that acclimation to elevated temperature and pCO2 induces gills physiologic and morphologic alterations. However, our results

showed distinct physiological mechanisms to deal with extreme climate scenario in the species studied.

The alteration in gills morphology that increased respiratory surface area was an important mechanism to improve oxygen consumption, even though it increased the salt loss in *A. agassizii* and *P. brevis*. Besides, *P. brevis* and *A. agassizii* showed important mitochondrial plasticity that helped to maintain ATP production to deal with iono-regulation. The *H. melazonatus* presented the greatest physiological and morphological alterations, for instance, increased oxygen consumption, $Na^+$ loss and respiratory surface area while decreasing carbonic anhydrase.

Therefore, we concluded that under extreme scenario Amazon forest stream fishes will exhibit a disruption of the osmorespiratory compromise. Although, some species are able to metabolically compensate these alterations.

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Table 1. Summary of the results of abiotic factors (Temperature, $O_2$, $CO_2$, pH) in the water and in the air of experimental climatic controlled room; The room is real-time computer controlled simulating current levels and extreme climate scenario (A2, plus 4.5°C and 900ppm $CO_2$) proposed by IPCC 2014.

| | Water | | | | Air | |
|---|---|---|---|---|---|---|
| Scenario | Temperature °C | $O_2$ (mg.$L^{-1}$) | $CO_2$ (ppm) | pH | Temperature °C | $CO_2$ (ppm) |
| Current | 26.5 ± 1.0 | 6.5 ± 0.5 | 11±2 | 6.2 ± 0.3 | 27.5 ±1.7 | 476 ± 15 |
| A2 | 30.5 ± 1.0 | 6.3 ± 0.4 | 28 ±2 | 5.8 ± 0.5 | 32.0 ± 1.8 | 1294± 16 |

Table 2. Ions excretion (µmol. $kg^{-1}.h^{-1}$) of three Amazon forest streams fishes acclimated for 30 days to current and extreme (+4.5°C + 900 ppmCO2, above current levels). Different letters indicate statistical difference by Two-way ANOVA and tukey`s post hoc test ($p<0.05$).

| | *A. agassizii* | | *P.brevis* | | *H. melazonatus* | |
|---|---|---|---|---|---|---|
| | Current | Extreme | Current | Extreme | Current | Extreme |
| $Na^+$ | -233.27 ±60.4$^a$ | -287.19 ± 62.2$^a$ | -315.4 ± 76.4$^a$ | -523.9 ± 37.5$^a$ | -429.1± 77.06$^a$ | -1061.0 ± 164.3$^b$ |
| $K^+$ | -63.2 ± 23.1$^a$ | -158.4± 49.4$^b$ | -95.7± 28.9$^a$ | -93.56 ± 26.2$^a$ | -608.9± 46.9$^c$ | -673.8± 61.8$^c$ |
| $Cl^-$ | -178.3 ± 62.5$^a$ | -745.7± 86.3$^b$ | -441.7±296.5$^b$ | -144.4± 126.6$^a$ | -1769.2± 455.6$^c$ | -885.4± 284.8$^b$ |
| $NH_4$ | -178.36 ± 87.5$^a$ | 745.7 ± 152.2$^b$ | -784.4 ± 68.26$^b$ | -636.28 ± 53.8$^b$ | -1434.8± 186.2$^c$ | -992.7 ± 191.8$^{cb}$ |

Table 3. Effects of climate scenario acclimation on gills morphology in Three Amazon forest streams fishes. Different letters indicate statistical difference by Two-way ANOVA and tukey`s post hoc test ($p<0.05$).

| | *A. agassizii* | | *P. brevis* | | *H. melozonatus* | |
|---|---|---|---|---|---|---|
| | Current | Extreme | Current | Extreme | Current | Extreme |
| Lamellar height (mm) | $0.637\pm0.047^{a}$ | $0.727\pm0.030^{b}$ | $0.920\pm0.0328^{c}$ | $0.844\pm0.030^{c}$ | $0.878\pm0.060^{c}$ | $1.071\pm0.045^{d}$ |
| Lamellar width (mm) | $0.053\pm0.003^{a}$ | $0.100\pm0.006^{b}$ | $0.092\pm0.005^{b}$ | $0.223\pm0.012^{d}$ | $0.0837\pm0.002^{b}$ | $0.114\pm0.012^{c}$ |
| Lamellar distance (mm) | $0.208\pm0.021^{a}$ | $0.289\pm0.014^{b}$ | $0.301\pm0.006^{c}$ | $0.278\pm0.020^{bc}$ | $0.204\pm0.004^{a}$ | $0.219\pm0.011^{a}$ |
| Filament width (mm) | $0.227\pm0.062^{a}$ | $0.304\pm0.053^{a}$ | $0.225\pm0.066^{a}$ | $0.325\pm0.051^{a}$ | $0.163\pm0.023^{b}$ | $0.229\pm0.010^{a}$ |
| Lamellar frequency ($mm^{-1}$) | $4.91\pm0.52^{a}$ | $2.49\pm0.15^{b}$ | $2.56+0.28^{b}$ | $2.19+0.20^{b}$ | $3.80+0.08^{c}$ | $3.28+0.16^{b}$ |
| Respiratory surface area ($mm^{2}$) | $0.062\pm0.007^{a}$ | $0.15\pm0.011^{b}$ | $0.086+0.006^{a}$ | $0.185+0.022^{b}$ | $0.073+0.006^{a}$ | $0.120+0.025^{b}$ |

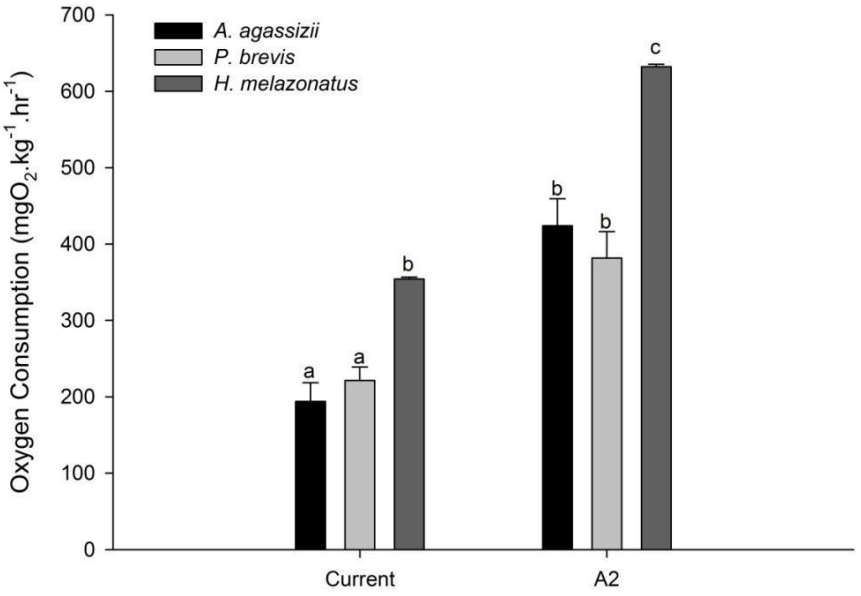



Figure 1. Oxygen consumption of three Amazon forest stream fishes acclimated for 30 days at climate change scenarios (IPCC 2014). Different letters indicate statistical difference by Two-way ANOVA and tukey`s post hoc test ($p<0.05$).

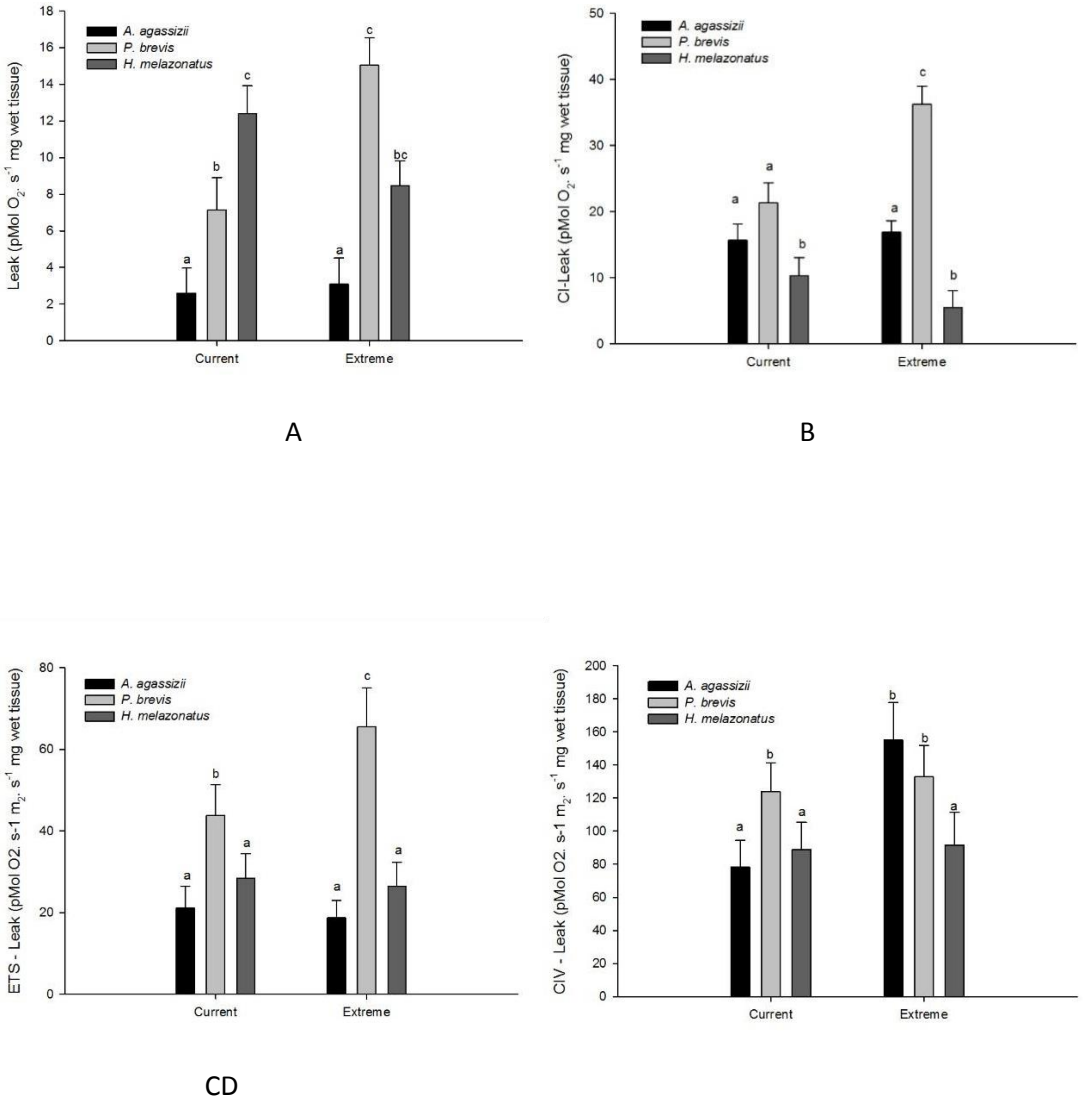
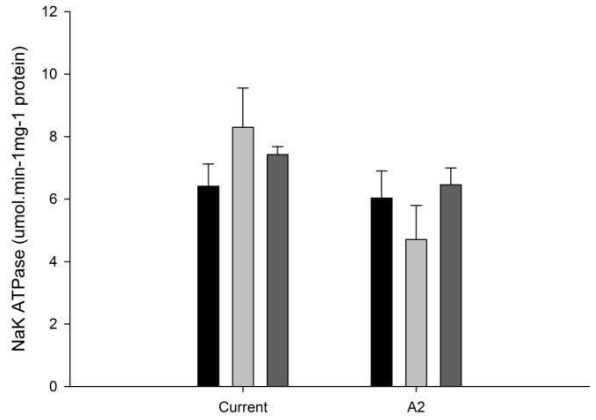
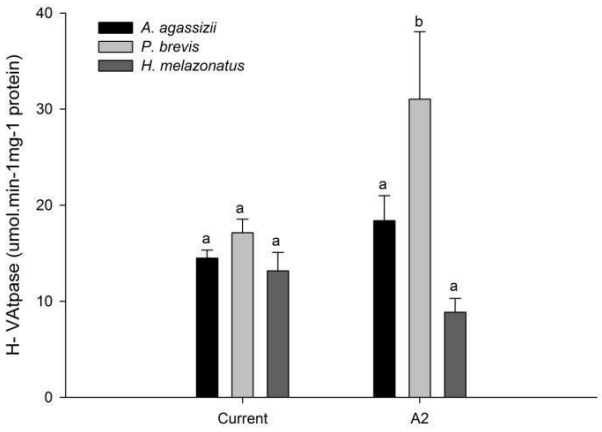


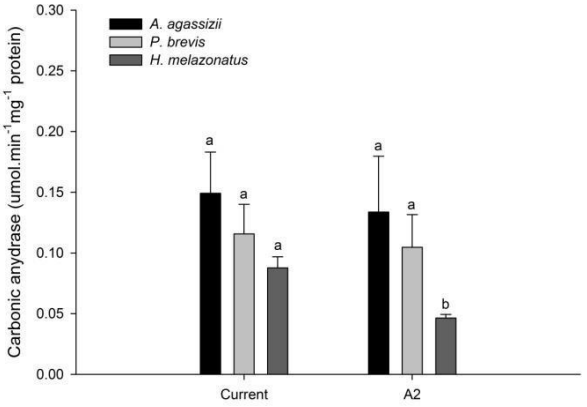

Figure 2. Gills mitochondrial physiology of three Amazon forest stream fishes acclimated for 30 days at climate change scenarios (IPCC 2014). A) Leak State, B) Complex I, C) Complex ETS, D) Cytochrome C oxidase activity. Different letters indicate statistical difference by Two-way ANOVA and tukey`s post hoc test ($p<0.05$).




A




B




C

Figure 4. Effects of climate change scenario (IPCC 2014) on A) NaK ATPase, B) $H^+$ ATPase and C) Carbonic anydrase of three Amazon forest stream fishes acclimated for 30 days. Different letters indicate statistical difference by Two-way ANOVA and tukey`s post hoc test ($p<0.05$)

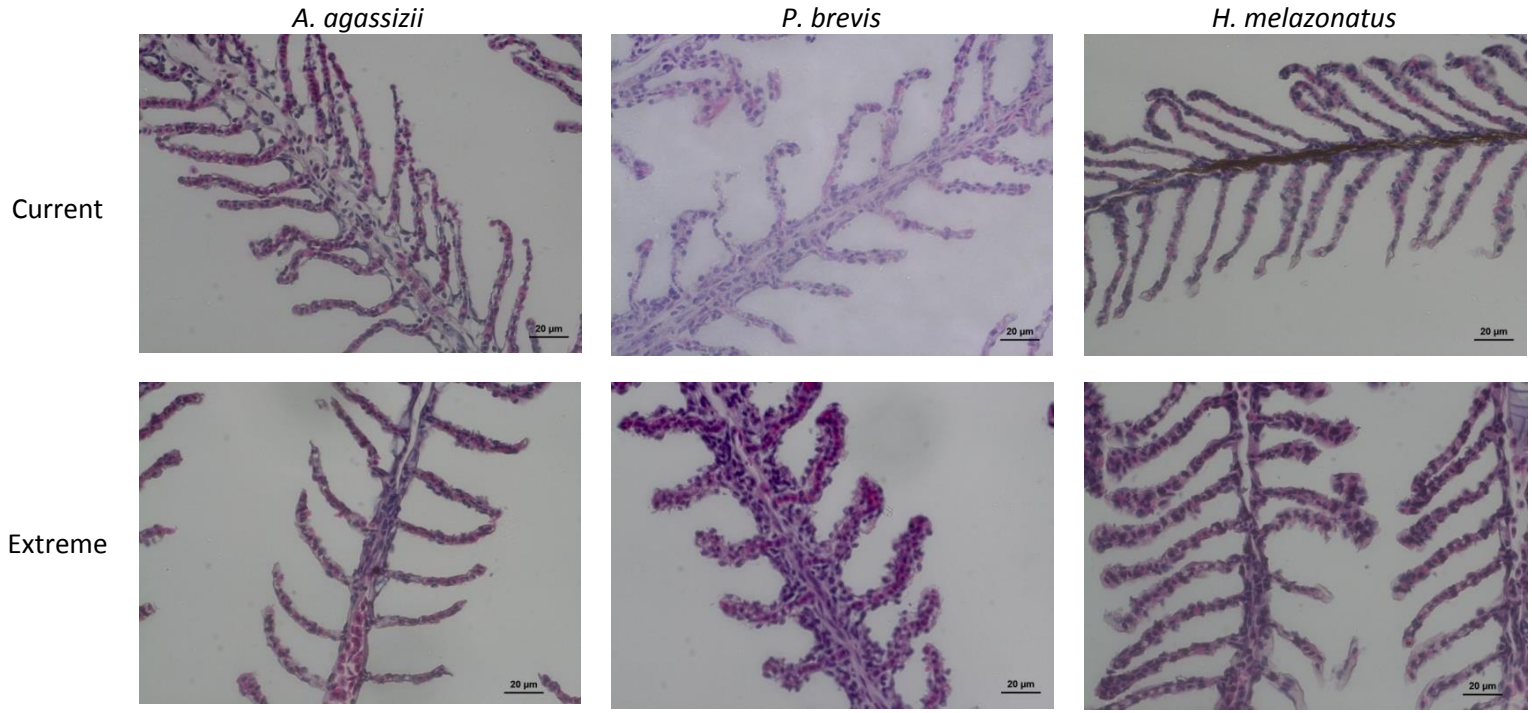



Figure 5. Gills morphology of *Appistogramma agassizii*, *Pyrhullina brevis* and *Hyphessobrychon melazonatus* acclimated for 30 days at current and extreme climate change scenario (A2, plus 4.5°C and 900ppm $CO_2$) proposed by IPCC 2014.

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# Predicting thermal sensitivity of three Amazon fishes exposed to climate change scenarios

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ABSTRACT

Increased temperature and $CO_2$ levels are predicts for the next decades. Tropical species are expected to be especially vulnerable to these alterations once many of them appear to have a narrower thermal tolerance range compared to subtropical and temperate species; and live closer to their thermal limits. Herein, we experimentally investigated the effects of climate change scenario on metabolic and oxidative stress in three ornamental fishes of Amazon forest streams. *Apistogramma agassizii*, *Pyrhullina brevis* and *Hyphessobrychon melazonatus* were exposed to current and extreme climate scenarios (4.5 °C and 900 ppm $CO_2$ above current levels) and had respiratory profile, antioxidant enzymes and neurotransmitter responses evaluated. The integrated biomarkers response index (IBR) was calculated to examine specie's acclimation abilities. After 30 days of exposure, we observed distinct physiological mechanisms to cope with climate change. Overall, Amazonian fish species are susceptible to climate change since they showed increase in metabolic rate and oxidative stress. Yet, sedentary ones *A. agassizii* and *P. brevis*, appeared to be less impacted by climate change than *H. melazonatus*, once they presented high survival rates, thermal tolerance and low IBR values. In contrast, *H. melazonatus*, an athletic species, low survival rates, lipid peroxidation, lower thermal tolerance and high IBR. This study provides evidence that future climate changes will affect energy supply and promote species-specific damages in metabolic pathways, with consequent physiological impairments, which may have detrimental effects at population and ecosystem levels.

## 1. Introduction

Several models of climate changes predicts an increase in tropical water surface temperatures up to 3 °C for the next century (Lough, 2007; Meehl et al., 2007; Ganachaud et al., 2011; IPCC, 2014). Some models predict a temperature increase of 3–5 °C during this period for the Amazon region (Marengo, 2009, PBMC, 2013). Besides climate change, riparian deforestation is increasing in the Amazon region and has increase temperature of small streams up to 5 °C (Macedo et al., 2013). Temperature is one of the most important environmental factors affecting the performance and distribution of organisms (Madeira et al., 2016a). However, little information exists concerning the Amazon stream fish physiology responses to changes in temperature.

Temperature has profound impacts on chemical and biochemical reactions, influencing the whole ectothermic aerobic metabolism. Accordingly, warming increases endogenous Reactive Oxygen Species (ROS) in ectothermic organisms, with potential impacts to energy supply and performance (Madeira et al., 2012). Moreover, atmospheric $CO_2$ levels are expected to reach values up to 940 ppm by the end of the twenty-first century (IPCC, 2014). $CO_2$ reacts with water resulting in an increase of $H^+$ and $HCO_3^-$ concentrations and lower $CO_3^{2-}$ levels. This process, named water acidification is expected to acidify the surface water by 0.14–0.42 units by the end of this century (Pörtner et al., 2014). Physiologically, water acidification disrupts the acid-base balance of fish, with consequences to metabolic performance (Rosa et al., 2016). Also, elevated $CO_2$ concentrations induce oxidative stress due to the parallel increase of $H^+$ ions (extra and intracellular acidosis) (Feder and Hofmann, 1999; Dean, 2010; Sampaio et al., 2018).

Tropical species are expected to be especially vulnerable to warming because many of them appear to have a narrower thermal range compared to subtropical and temperate species, and tend to live closer to their thermal limits (Pörtner and Farrell, 2008). Therefore, even small increases could lead to declines in individual and populations performance (Stillman, 2003; Tewksbury et al., 2008). Actually, Campos et al. (2018) showed that active species have lower thermal tolerance compared to sedentary ones when analysing fish inhabiting forest streams of the Amazon; however, their acclimation abilities has not been studied yet. Besides, Campos et al. (2017) studying palm

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swamp species showed that the congeneric species, *PARACHEIRON AXELRODI* and *P. SIMULANS*, have low acclimation abilities and decreased thermal safety margin at high acclimation temperature. Species vulnerability to warming has been associated with loss of aerobic capacity and oxidative damage, in particular, to the constraints of decreased oxygen supply and increased demand. According to the OCLTT (oxygen- and capacity limited thermal tolerance) hypothesis, the aerobic capacity is limited by insufficient oxygen supply and sets the performance in animals (e.g. Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Pörtner et al., 2010).

The ability of fish to respond to environmental stress depends on the management of internal components and processes to maintain physiological homeostasis (Somero, 2010). Mechanisms involving physiological homeostasis are mainly related to oxidative defense capacity and the mechanisms of alterations of general energetic metabolism (Farrell, 2007; Pörtner and Farrell, 2008; Somero, 2010; Madeira et al., 2016a). Warming and high $pCO_2$ induces cellular redox changes and have been reported to activate reactive oxygen species (ROS) scavengers (such as catalase, glutathione-S-transferase and peroxidases) to reduce the flux of ROS produced during oxidative metabolism (Somero, 2010). However, when the production of ROS exceeds levels of antioxidant defense, the oxidative stress occurs (Vinagre et al., 2012). Overproduction of ROS can damage important biomolecules, such as DNA, proteins, and lipids, and initiate a cascade of events, bringing about impaired cellular function and even apoptosis events (Iftikar and Hickey, 2013). Rosa et al. (2016) reported a substantial vulnerability of tropical shark (*Chiloscyllium PUNCTATUM*) exposure to climate change scenario (+4 °C and pH 7.5) related to disruption of the brain neurotransmitter, increases in oxidative stress and consequent decrease in aerobic capacities. However, some species demonstrate physiological mechanisms that allow overcoming the negative consequences of thermal stress, which includes the avoidance of free radicals accumulation, protein degradation and energy depletion (Hofmann and Somero, 1995; Tomanek, 2010). For instance, Lopes et al. (2018) suggest that ROS-scavenging molecules provide an effective defense mechanism in dealing with ROS formation in newly hatched sharks (*Chiloscyllium PLAGIOSUM*) allowing this species overcoming the impacts of upcoming climate change scenario.

The Amazon forest streams are a thermo-stable environment that support high fish diversity with a variety of lifestyles. The species have important ecological roles, such as energy cycling and transfer between land and aquatic environments. In addition, fish species from Amazon forest stream have an important economic role since most of them are used in ornamental trades. Even though they are not considered at risk by conservational programs, climate change can affect their survival. Therefore, understanding the acclimation abilities of these species are essential to comprehend the climate change effects and use these information for conservation programs.

In this sense, the capacity of species to deal with free radicals and to maintain aerobic capacity and thermal safety margin can be a critical point for defining their vulnerabilities to near-future climate change scenarios (Deschaseaux et al., 2010). These biological responses can be used as stress biomarkers providing early warning signals of physiological stress, and could indicate occurrences of adverse ecological consequences in fish communities (Wu et al., 2005). Therefore, in this study, we experimentally investigated the effects of two climate change scenarios on metabolic and oxidative stress in three Amazon forest stream fishes. In particular, the main goals were: (i) to investigate the effects of two climate scenarios on the metabolic profile of three spe- cies; (ii) to perform a comparative assessment of extreme climate change scenario at a molecular biomarker of oxidative effects in white muscle tissue; and (iii) to analyze the acclimation capacities and thermal limits in which the physiological responses are modulated by climate change scenarios. We hypothesize that exposure to extreme climate change scenario (4.5 °C and 900 ppm CO2 higher than current scenario) Amazon forest stream fishes will increase metabolic demand and oxidative stress indicating low acclimation abilities.

## 2. Material and methods

### 2.1. Collection AND MAINTENANCE of fish

We selected three species based on their distinct life-styles and swimming behavior, which according to our previous work (Campos et al., 2018), can be identified by their physiological abilities to deal with warming environment: *APISTOGRAMMA AGASSIZII*, *PYRRHULLINA brevis* and *Hyphessobrychon MELAZONATUS*. Fishes were collected in first- and second-order streams at Reserva Ducke (02°53'S, 59°58'W), a protected area located in the central Brazilian Amazon near the confluence of the Negro and Solimões rivers, bordering Manaus. Fish were collected by hand and seine nets (2-mm mesh) (see Mendonça et al., 2005 for details). Captured fish were maintained in a 50-L aquarium with constant aeration and transferred to LEEM – Laboratory of Ecophysiology and Molecular Evolution at Brazilian National Institute for Research of the Amazon – INPA. Each species was held at one 150-L tanks (Fortlev®) for two-weeks before experiments. Half of the water was replaced every 24 h. During this period, fish were fed once a day until satiation with TetraMin® Flakes. The temperature was maintained close to that of the streams (26 ± 1.0 °C). All housing and experimental procedures were in accordance with CONCEA Brazilian Guide for Animal Use and Care and were authorized by INPA's Council for Ethics in Animal Use (CEUA – protocol number 027/2015).

### 2.2. CLIMATE SCENARIOS exposure

The three species (*A. AGASSIZII*, *P. brevis* and *H. MELAZONATUS*) were exposed to two distinct climate scenarios at controlled environmental rooms. This system consists of environmental rooms with temperature and $CO_2$ controlled by computer according to different scenarios. The climate scenarios provided by the Assessment Report of the IPCC (2014) for the year 2100 were simulated in these environmental rooms: the current scenario (current temperature and $CO_2$ levels) that was set according to data acquisition every two minutes at the natural forest by Fieldlogger 512 k (Novus Produtos eletrônicos LTDA); and A2 room (extreme scenario) that was set at 4.5 °C and 900 ppm $CO_2$ above the current scenario. The sensors at natural forest measured temperature and $CO_2$ every two minutes and transmitted the data to the laboratory computers that control the environmental rooms according to the above scenarios in real time. Data of room's temperature and $CO_2$ are available in Supplementary material (SM1).

Adults of the three chosen species were transferred to 10-L PVC tanks in three replicates per species containing six individual each tank, in both scenarios, N = 36 per species. They were maintained in each climate room for 30 days, during November 2017 (Amazon dry season). To avoid ammonia accumulation, 30% of the water was replaced every day using environmentally stabilized water. The pH, $O_2$, $CO_2$ and temperature levels of the water were daily measured (Table 1). The fish were fed *AD libitum* once a day using commercial TetraMin®Flakes and fish were unfed 48 h before experiments.

### 2.3. BIOMARKERS ASSAYS

#### 2.3.1. Oxygen consumption

After 30 days acclimation we measured fish oxygen consumption in a closed chamber system. For that purpose, individuals were transferred to a 150 mL PVC chamber for *A. AGASSIZII* and *H. MELAZONATUS* and 500 mL PVC chamber for *P. brevis*, maintaining the same proportion of water volume/fish weight. We sampled 8 individuals for *A. AGASSIZII* and *P. brevis*, 3 individuals from two aquariums and 2 from another aquarium in each climate scenario, and for *H. MELAZONATUS* we sampled 2 individuals from each aquarium in each climate scenario. Fish were allowed to handily recover for 2 h in fully oxygenated chamber. After

Table 1
Temperature, $O_2$, $CO_2$, and pH means in the water and in the air of experimental climate rooms; The rooms are real-time computer controlled simulating current levels and extreme climate scenarios (A2, plus 4.5 °C and 900 ppm $CO_2$) proposed by IPCC 2014.

| | Water | | | | Air | |
|---|---|---|---|---|---|---|
| Scenario | Temperature °C | $O_2$ (mg·L$^{-1}$) | $CO_2$ (ppm) | pH | Temperature °C | $CO_2$ (ppm) |
| Current | 26.5 ± 1.0 | 6.5 ± 0.5 | 11 ± 2 | 6.4 ± 0.3 | 27.5 ± 1.7 | 476 ± 15 |
| Extreme | 30.5 ± 1.0 | 6.3 ± 0.4 | 28 ± 2 | 5.8 ± 0.5 | 32.0 ± 1.8 | 1294 ± 16 |

this period, the oxygen of the chamber was shut-off, and the fish oxygen consumption was monitored by 15 min using an Oxy-4 oximeter (Loligo system). After this period, the oxygen was turned-on for 45 min. Also, background respiration (chamber without fish) was measured con- comitantly. The oxygen consumption was measured three times for each individual during 4 h, so we calculated the mean of the replicate of oxygen consumption following: $MO_2$ (μL$O_2$·h$^{-1}$·kg) = −ΔO·Vresp·B$^{-1}$,
where ΔO is the rate of change in oxygen concentration (μL$O_2$·h$^{-1}$), Vresp is the volume of the respirometric chamber, and B is the mass of the individual (kg). After that, fishes were anesthetized by concussion and euthanized by medullar section. Muscle was removed, immersed in liquid $N_2$ and stored in an ultrafreezer at −80 °C until enzyme assays.

### 2.3.2. Metabolic enzymes

For metabolic enzyme assays, lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and citrate synthase (CS), 0.01 g of white muscle was individually homogenized in a imidazole buffer (150 mM imidazole, 1 mM EDTA and TritonX at pH 7.4) followed by centrifugation at 10,000*g* in a Sorvall RC-5B for 15 min at 4 °C. The activities of LDH and MDH were measured following the oxidation of NADH at 340 nm and the oxidation of DTNB (5,5′-Dithiobis(2-nitrobenzoic acid) at 412 nm for CS (for details see Driedzic and Almeida-Val (1996), modified by Campos et al., 2017). The enzyme activities were determined at 25 °C using a SpectramaxPlus 384 (Molecular Device, USA). The protein was quantified by Bradford assay (Bradford, 1976).

The oxidative phosphorylation capacity was measured by electron transport system (ETS) activities. ETS was measured using the method proposed by Packard (1971) and improved by G.-Tóth (1993). The ETS activity of each animal was determined using the procedure described in Simčič and Brancelj (2003, for detail see). ETS activity was measured as the rate of tetrazolium reduction to formazan and converted to equivalent oxygen consumed per mg of wet mass per hour (μL$O_2$·mg$^{-1}$·h$^{-1}$) as described by Kenner and Ahmed (1975).

### 2.3.3. Antioxidant enzymes and LPO levels

The glutation-S-transferase (GST) activity in white muscle was determined using 1-chloro 2,4-dinitrobenzene (CDNB) as substrate, according to Keen et al. (1976). Changes in absorbance were recorded at 340 nm. The enzyme activity was calculated as nmol CDNB conjugate formed per min per mg protein. To determine the catalase (CAT) activity, the inhibition rate of $H_2O_2$ decomposition was monitored at 240 nm (Beutler, 1975), and expressed as μM $H_2O_2$ min$^{-1}$ mg protein$^{-1}$. The lipid peroxidation (LPO) levels were quantified based on the oxidation of the $Fe^{+2}$ to $Fe^{+3}$ by hydroperoxides in acid medium in the presence of ferrous oxidation-xylenol orange, at 560 nm, according to the method described by Jiang et al. (1991).

### 2.3.4. Acetylcholinesterase activity (AChE)

The AChE activity in white muscle was determined by the method of Ellman et al. (1961). Briefly, samples were homogenized in phosphate buffer 1:4 (0.1 M, glycerol 20%, pH 7.5), centrifuged for 20 min at 12,000*g* (4 °C) and the supernatant used for measurement of AChE activity. Acetylthiocholine iodide (ATC) 9 mM was used as a substrate and 5,50-dithio-bis (2-nitrobenzoic) acid (DTNB) as color reagent. Kinetic activity of AChE was measured at 415 nm and the units are μM

### 2.4. IBR calculations

The integrated biomarkers response index (IBR) was calculated according to Beliaeff and Burgeot (2002), as the triangular star plot areas calculated for each two neighboring data. To calculate IBR, the general mean ($m$) and the standard deviation ($s$) of all data regarding a given biomarker was calculated, followed by a standardization to obtain $Y$, where $Y = (X - m)/s$, and $X$ is the mean value for the biomarker at a given temperature. Then, $Z$ was calculated using $Z = -Y$ or $Z = Y$, in the case of a biological effect corresponding respectively to an inhibition or a stimulation. Afterwards, the score ($S$) was calculated by $S = Z + |Min|$, where $S \geq 0$ and $|Min|$ is the absolute value for the minimum value for all calculated $Y$ in a given biomarker at all measurements made. Star plots were then used to display Score results ($S$) and to calculate the integrated biomarker response (IBR) as:

$$IBR = \sum_{t=1}^{n} A_i$$

$$A_i = \frac{S_i \times S_{i+1} \times sin\alpha}{2}$$

where $Si$ and $Si + 1$ are two consecutive clockwise scores (radius coordinates) of a given star plot; $Ai$ corresponds to the area of the connecting two scores; and $\alpha = 2\pi/n$ where $n$ is the number of biomarkers used for calculations. The IBR is obtained by summing up all the $Ai$. The IBR calculations were always performed with the same order of bio- markers for all temperatures taking into account their biological func- tion ($MO_2$, ETS, CS, MDH, LDH, CAT, GST, LPO, Ache).

### 2.5. Critical thermal tolerance

Thermal limits were estimated using critical thermal methodology (CTM) (Lutterschmidt and Hutchison, 1997). After the acclimation period, in each climate change scenario, five individuals of each species were held in 10 L aquaria with a digital thermostat to control tem- perature (TIC-17RGT, FullGauge, ± 0.01 accuracy) and allowed han- dily recover for 24 h. After this period, the thermal ramp was initiated with an increase of 0.3 °C/min according to a review of Lutterschmidt and Hutchison, 1997. The critical thermal maximum was defined as the temperature at the fish presented a final loss of equilibrium, or LOE (inability to maintain dorsal-ventral orientation for at least 1 min, Beitinger and Bennett, 2000).

### 2.6. Statistical analyses

The data are presented as mean ± SD (n = 8–6). All statistical analyses were carried out in SigmaStat 3.1 using a significance level of 5%. Survival rate was measured along the experiment and tested using Using Kaplan-Meier analysis among species. Parametric Test t or non- parametric Mann-Whitney (depending on whether the data met the assumptions of normality and homoscedasticity) were performed to detect significant differences in the biomarkers endpoints and CTMax among different climate change scenarios within each species. Also, we carried out cluster analyses and heatmaps in MetaboAnalyst 4.0 (R package; according to Chong et al., 2018) using glog transformed and auto-scaled data, Cluster analyses followed the criteria (i) cluster rows,

(ii) similarity metrics: Euclidean, iii) clustering method: Ward. The colour scale in heatmaps ranges from red (higher than mean biomarker concentration/activity) to blue (lower than mean biomarker concentration/activity).

## 3. Results

Significant differences in abiotic factors measured between climate change scenarios were observed. The extreme climate scenario presented higher temperature and $CO_2$ levels in the air, and these parameters followed in the water where, in the A2 climate room, water temperature, $CO_2$ were higher and pH was lower (mean of 30.5 °C, 27 ppm $CO_2$, and 5.8 pH) compared to the current scenario (mean of 26.5 °C, 11 ppm $CO_2$, and 6.5 pH) (Table 1). The $CO_2$ reacts with water resulting in an increase of $H^+$ and $HCO_3^-$ concentrations and lower $CO_3^{2-}$ levels, therefore significant part of the $CO_2$ was responsible by the decrease of pH at the extreme scenario (Table 1). It is important to mention that, in natural systems, groundwater could alter the pattern we have observed here.

The survival rate at A2 scenario differed among the three species ($p > 0.001$); *P. brevis* presented 83% of survival along the experiment, while *H. MELAZONATUS* showed 66% of survival rate. We did not observe mortality in the *A. AGASSIZII* along the experiment (Fig. 1). Results of statistical analyses showed distinct adaptation mechanisms to face the climate change scenarios among the studied species (Table 2; Fig. 4). Oxygen consumption was the single parameter that increased in all species (Fig. 2).

*APPISTOGRAMMA AGASSIZII* increased LDH and decreased ETS activities, indicating metabolic depression and an activation of the anaerobic pathway in white muscle (Table 2). In addition, the antioxidant enzyme GST and LPO increased. Also, this species presented an increased activity of the enzyme AChE (Table 2). Although *A. AGASSIZII* increased oxidative stress, the IBR increased by 1.7 times, indicating a high capacity to acclimate to the extreme scenario (Fig. 3). This is well supported by 1.5 °C increase in thermal tolerance to the scenario A2 (current scenario 40 °C and A2 41.5 °C; Fig. 5).

Besides increased oxygen consumption, *P. brevis* increased CAT and AChE activities (Table 2), and increased IBR by 1.9 times at extreme scenario compared to the current scenario (Fig. 3). In addition, this species increased critical thermal maximum by 1 °C in average in thermal tolerance to the extreme scenario (current 39 °C and A2 40 °C; Fig. 5).

In contrast, *H. MELAZONATUS* presented the higher alterations in endpoint biomarkers. This species increased the Krebs' cycle oxidation, indicated by citrate synthase activity and also by the activation of antioxidant defense, observed by the increase in the CAT activity, with a consequent increase in LPO, indicating high levels of oxidative damage (Table 2). Interestingly, this species decreased AChE activity, an opposite trend when compared to the others species. Moreover, *H. MELAZONATUS* presented the highest increase in IBR, i.e., 2.58 times (Fig. 3) and did not change thermal tolerance (36 °C; Fig. 5).

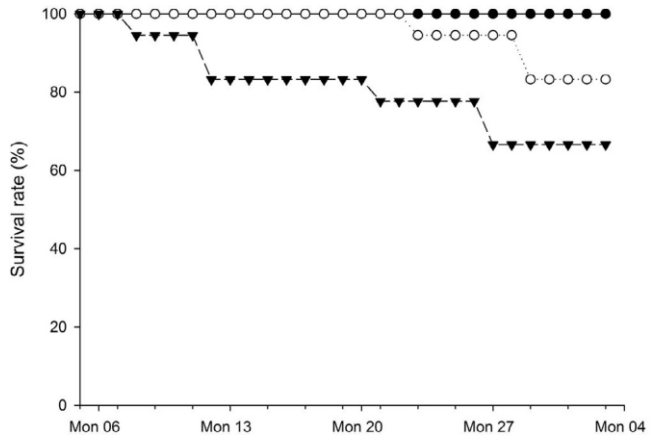


Fig. 1. Survival rate of *APISTOGRAMMA AGASSIZI* ( —●— ), *PYRRHULLINA brevis* ( ····◇··· ), *Hyphessobrychon MELAZONATUS* ( –▼– ) exposed to warming climate change scenario (A2, 4.5 °C and 900 ppm $CO_2$ higher than current scenario) proposed by IPCC, 2014. Using Kaplan-Meieranalysis, we observed differences in survival rate ($p > 0.001$).

The cluster analyses and heatmaps indicate a clear segregation between current and extreme scenario. Exposed to extreme climate change scenario (A2; IPCC, 2014) all species presented an increase in biomarkers levels. In addition, *H. MELAZONATUS* present the large biomarkers increase under climate change scenario (Fig. 4). All these data indicate low acclimation ability and high vulnerability to climate change scenario for this specie.

## 4. Discussion

Warming and high $pCO_2$ levels have negative impacts on ectotherms increasing metabolic demands to deal with physiological stress. The temperature increase affects the rate of reactions and promotes cellular damage, while high $pCO_2$ increases the energy consuming for ion and acid–base regulatory mechanisms. Thus, the energetic costs associated with maintaining homeostasis in situations of chronic exposure to climate change can alter the finite energy budgets resulting in a reduction in growth, reproduction and survival performance (Pörtner and Knust, 2007; Madeira et al., 2016a,b; Rosa et al., 2016; Ilha et al., 2018).

Predictions of climate change impacts on populations and ecosystems require the assessment of physiological capacity to acclimation and tolerance (Semsar-kazerouni and Verberk, 2018). The findings of this study suggest that active life-style fish, such as *H. MELAZONAYUS*, lacks the ability to tolerate or acclimate to a new climate scenario, as opposed to the sedentary ones, *P. brevis* and *A. AGASSIZI*. This outcome is supported by the high mortality rates, increased oxidative stress and loss of neurotransmission capacity endured by *H. MELAZONATUS* at A2 (*sensu* IPCC, 2014) climate scenario. This result confirms that near-future climate scenario will have lower impact on fish species with low activity performances, in accordance with former studies (Stoffels, 2015; Campos et al., 2018). These findings imply that athletic fishes may have less capacity to survive under climate change scenario due to a low acclimation capacity. In contrast, sedentary species may have physiological traits that increase resistance and facilitate local persistence. The energetic cost for activity in fast species, combined with energetic cost of homeostasis should decrease the energy amount to deal with further energetic demand at warming, overwhelming their energy capacity.

### 4.1. *BIOMARKERS response to CLIMATE CHANGE exposure*

Acclimation capacities to warming and hypercapnia have been related to aerobic compensation that improves tolerance and maintains homeostasis. In the present work, a consistent result with previous studies (Peck et al., 2012; reviewed by Schulte, 2015). For Amazon species, Campos et al. (2017) also found that two congeneric ornamental fishes increased their routine metabolic rates at warming acclimation. In a recent review, Peck et al. (2012) found that a 10 °C increase in temperature was accompanied by a 1.2- to 4.3-fold increase in oxygen consumption ($Q10 = 1.2–4.3$) across 15 families of marine fish. Increased energetic costs under chronic exposure to warming can decrease the energy budgets resulting in a reduction in growth, reproduction and survival performance (Pörtner and Knust, 2007; Madeira et al., 2016a,b; Rosa et al., 2016). In fact, Ilha et al. (2018) observed that deforestation causes stream warming and decrease body size of Amazonian fishes.

Although all species increased oxygen consumption, such increase was not related to oxygen demands in the muscle. For instance, *A. AGASSIZII* activated its anaerobic power (LDH activities) and decreased

Table 2
Lactate dehydrogenase (LDH) activity (uMol·min$^{-1}$·mg$^{-1}$ protein), Malate dehydrogenase activity (MDH) (μMol·min$^{-1}$·mg$^{-1}$ protein), Citrate synthase activity (CS) (μMol·min$^{-1}$·mg$^{-1}$ protein), Electron transport system activity (ETS) (μLO2 mg$^{-1}$ h$^{-1}$), glutathione-S-transferase activity (GST) (μMol·min$^{-1}$·mg$^{-1}$ protein), Catalase activity (CAT) (μMol $H_2O_2$·min$^{-1}$·mg$^{-1}$ protein), Levels of lipoperoxidaton (LPO) (μMol cumeme hydroperoxide mg. protein) and Acetilcolinestarase activity (Ache) (μMol·min$^{-1}$·mg$^{-1}$ protein) measured in white muscle of *APPISTOGRAMMA AGASSIZII*, *PYRRHULLINA brevis* and *Hyphessobrychon MELAZONATUS* exposed to current scenario and extreme climate change scenario (A2; 4.5 °C and 900 ppm $CO_2$ above current levels). * indicates significant diff erent from current scenario using *t*-test ($p < 0.05$).

| | | LDH | MDH | CS | ETS | GST | CAT | LPO | Ache |
|---|---|---|---|---|---|---|---|---|---|
| *A. AGASSIZII* | Current | 1.17 ± 0.3 | 1.33 ± 0.54 | 0.08 ± 0.04 | 10,112.9 ± 2070.0 | 22.52 ± 5.7 | 0.029 ± 0.02 | 15.2 ± 6.9 | 8.12 ± 1.97 |
| | Extreme | 1.98 ± 0.7* | 1.05 ± 0.34 | 0.08 ± 0.03 | 7130.0 ± 980.2* | 45.40 ± 9.9* | 0.026 ± 0.01 | 23.8 ± 5.4* | 12.14 ± 4.52* |
| *P. brevis* | Current | 1.51 ± 0.2 | 1.15 ± 0.24 | 0.09 ± 0.02 | 5215.2 ± 1888.2 | 56.98 ± 14.3 | 0.005 ± 0.003 | 16.38 ± 4.3 | 7.76 ± 1.44 |
| | Extreme | 1.72 ± 0.4 | 1.17 ± 0.23 | 0.10 ± 0.01 | 5389.0 ± 1340.0 | 60.25 ± 21.1 | 0.015 ± 0.008* | 16.05 ± 5.9 | 11.75 ± 3.65* |
| *H. MELAZONATUS* | Current | 1.38 ± 0.4 | 1.04 ± 0.24 | 0.10 ± 0.01 | 8489.5 ± 3527.9 | 50.75 ± 15.0 | 0.005 ± 0.002 | 13.72 ± 1.6 | 7.57 ± 1.26 |
| | Extreme | 1.67 ± 0.4 | 1.24 ± 0.34 | 0.15 ± 0.03* | 7736.4 ± 2099.0 | 50.70 ± 12.1 | 0.010 ± 0.004* | 21.33 ± 3.8* | 5.00 ± 1.20* |

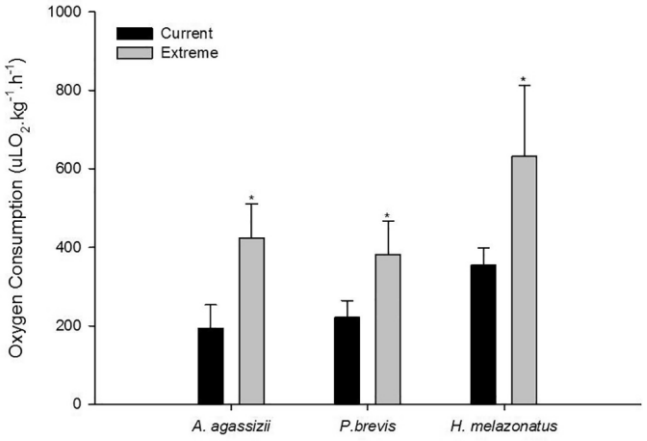


Fig. 2. Oxygen consumption of three Amazon forest stream fishes (*APPISTOGRAMMA AGASSIZII*, *PYRRHULLINA brevis* and *Hyphessobrychon MELAZONATUS*) acclimated for 30 days at climate change scenarios (IPCC 2014). * indicates significant diff erent from current scenario using *t*-test ($p < 0.05$).

oxidative phosphorylation (ETS activities), while *H. MELAZONATUS* increased aerobic oxidation (CS). These species-divergent responses seem to be strongly related to fishes' life-style and swimming behavior. For instance, *A. AGASSIZII* is a sedentary species living in pounds and pools, swimming throughout burst propulsion, which is related to anaerobic power. Therefore, decreasing oxidative phosphorylation does not represent an energetic impairment of movement for this species, once it appears that this species increases its glycolytic power to supply muscle energy. Instead, *H. MELAZONATUS* increased substrate oxidation in the Krebs cycle to support the increase of energy demand. Moreover, this species presented no increase in oxidative phosphorylation (ETS activity), what could mean impairment of electron transport chain, leading to important energy impacts and compromising its swimming performance.

Respiration represents a delicate trade-off , once cellular oxygen increase is both an essential component for aerobic energy generation as well as a toxic component, forming oxygen radicals in the mitochondria in an oxygen-dependent manner. Thus, increasing oxygen consumption to meet energy supply may have an odd cellular cost (Madeira et al., 2016a,b). In the present work, all species increased antioxidant systems in extreme climate change scenario, *A. AGASSIZII* increased GST, while *P. brevis* and *H. MELAZONATUS* increased CAT. These results suggest that an inherent thermal sensitivity of the cell lead to suffi cient thermal damage that required the activation of protective processes through activation of ROS scavengers (antioxidants) (Jesus et al., 2017; Sampaio et al., 2018). These results indicate a response to increased oxygen consumption demand, which leads to accelerated mitochondrial respiration uncoupled to ATP generation and, consequently, the generation of oxygen free radicals within tissues caused by warming and high $CO_2$ levels (Pannunzio and Storey, 1998; Abele et al., 2002). These types of molecular responses have also been observed in previous studies (Madeira et al., 2012; Madeira et al., 2016b; Rosa et al., 2016).

Oxidative stress occurs from the inability of the antioxidant defense system to avoid damage caused by ROS and, although antioxidant enzymes (GST and CAT) acted to detoxify ROS under thermal stress levels, this significant increase was not enough to minimize the drastic increase in peroxidative damage in *A. AGASSIZII* and *H. MELAZONATUS*. The rise of lipid peroxidation levels means cell membrane disruption due to high cellular ROS, which could be related to malfunction of the mitochondrial complex enzymes, leading to a less effi ciency of the oxidative phosphorylation and reduced ATP generation. Accordingly, energetic depression is observed as lipid peroxidation rate increases (Abele et al., 2002; Rosa et al., 2016). In fact, these results are corroborated by the energy depletion in *A. AGASSIZII* and *H. MELAZONATUS*. Therefore, thermal and acidification stress might be related to

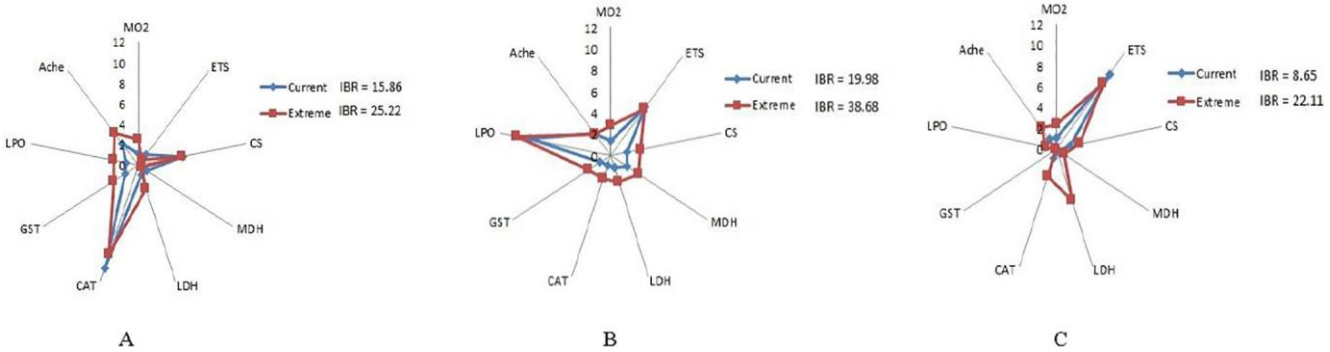


Fig. 3. Integrated biomarker response index (IBR) for muscle of (a) *APISTOGRAMMA AGASSIZII* (b) *PYRRHULLINA brevis* and (c) *Hyphessobrychon MELAZONATUS* acclimated to current and warming climate scenario (A2; IPCC, 2014).

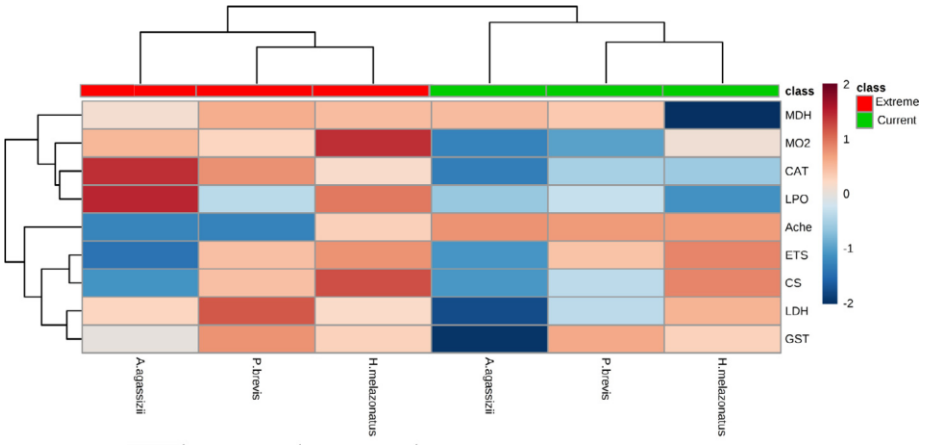


Fig. 4. Heatmap representation of the clustered data of three Amazon forest stream fishes (*APPISTOGRAMMA AGASSIZII*, *PYRRHULLINA brevis* and *Hyphessobrychon MELAZONATUS*) exposed current climate change scenario and extreme (A2, 4.5 °C and 900 ppm $CO_2$ higher from current scenario). Each cells denote the glog (generalized logarithm transformation) values of biomarker normalized concentration/activity. The colour scale ranges from red (higher than mean biomarker concentration/activity) to blue (lower than mean biomarker concentration/activity). Oxygen consumption ($MO_2$), Lactate dehydrogenase (LDH), Malate dehydrogenase (MDH, Citrate synthase (CS), Electron transport system (ETS), glutathione-S-transferase (GST, Catalase activity (CAT), lipoperoxidaton (LPO), and Acetilcolinestarase (Ache).

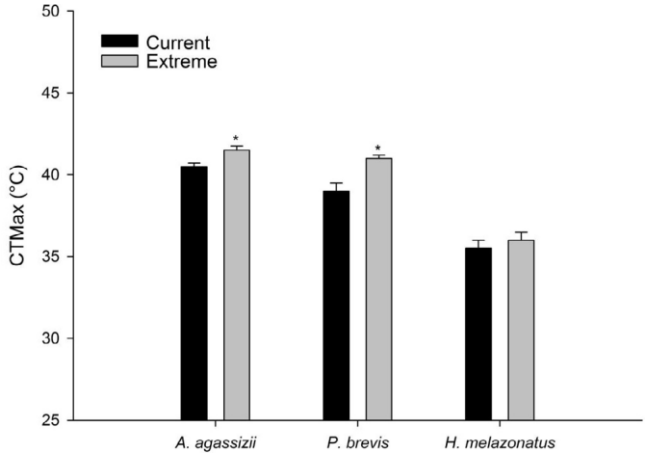


Fig. 5. Critical thermal maximum of three Amazon forest stream fishes, *APPISTOGRAMMA AGASSIZII* (current scenario 40 °C ± 0.2 and extreme 41.5 °C ± 0.2), *PYRRHULLINA brevis* (current 39 °C ± 0.4 and extreme 40 °C ± 0.2) and *Hyphessobrychon MELAZONATUS* (current 36 °C ± 0.5 and extreme 36 °C ± 0.5) acclimated for 30 days at climate change scenarios (IPCC, 2014). * indicates significant diff erent from current scenario using *t*-test ($p < 0.05$).

uncoupling mitochondrial function due to membrane cell disruption, with the potential occurrence of apoptosis and less ATP generation (Iftikar and Hickey, 2013). Our findings corroborate former oxidative stress studies with fish and crustaceos (Abele et al., 2002; Madeira et al., 2012; Sampaio et al., 2018).

Climate alterations and oxidative stresses are known to induce tissue damage with potential eff ects on muscle contraction ability and swimming performance (Rosa et al., 2016). In fact, all species presented AChE alterations in extreme climate scenarios. AChE is an essential enzyme in the cholinergic pathway that hydrolyses acetylcholine (ACh), regulating its levels in the synaptic cleft (Drever et al., 2011). *APISTOGRAMMA AGASSIZZI* and *P. brevis* increased Ache activities, the activation of AChE activity leads to a rapid degradation of ACh, an important neurotransmitter associated with locomotion (Jordan et al., 2014), suggesting that high temperature and $CO_2$ promotes synapse dysfunction, aff ecting the function of the cholinergic system. The rapid degradation of ACh can be associated with anxiety-like behaviour since the hyperactivation of the cholinergic system, in the brain is directly associated to increased anxiety-like behavior, as observed by Green and Jutfelt (2014) and Dixson et al. (2014). Instead, AChE inhibition in the species *H. MELAZONATUS* could lead to ACh accumulation in the nerve, resulting in the disruption of nervous activities by overstimulation (Sharbidre et al., 2011). The prolonged stimulation of the muscle fibers causes paralysis which ultimately results in death (Purves et al., 2008).

This result corroborated our findings that *H. MELAZONATUS* present electron transport chain impairment that leading to important energy impacts and compromising its swimming performance.

### 4.2. *The ACCLIMATION ABILITY sets THERMAL limits*

Our results show that *A. AGASSIZII* and *P. brevis* presented higher thermal tolerance compared to *H. MELAZONATUS*. In accordance, our previous findings (Campos et al., 2018) showed that *H. MELAZONATUS* present low thermal tolerance related to its active life style, are reflected in its lower thermal acclimation ability, compared to the other two species studied herein. Increasing maintenance costs reduce physiological tolerance, once high metabolic rates may increase critical oxygen tension and thermal limitation results from a decrease in systemic oxygen levels (hypoxemia), which consequently is determined by the animal ability to extract oxygen and to effi ciently deliver it to tissues (Pörtner and Knust, 2007).

Furthermore, critical thermal tolerances were significantly aff ected by climate scenario in *A. AGASSIZII* and *P. brevis*, but not in *H. MELAZONATUS*. *APISTOGRAMMA AGASSIZII* and *P. brevis* presented higher thermal limits at extreme climate change scenario indicating a degree of acclimation capacities, supporting IBR data (discussed below). Both species showed a significant CTMax acclimation ability: *A. AGASSIZII* increased its CTMax by 1.5 °C and *P. brevis* by 1.0 °C, in comparison to the current scenario. Our results corroborate previous studies with Amazon fish showing an acclimation capacity in their CTMax temperatures ranging from 1.0 °C to 1.8 °C (Campos et al., 2017). Acclimation capacity may actually be a mechanism that allows species to be resistant to climate changes; here we showed that some Amazon forest stream fishes have abilities to increase thermal tolerance, but *H. MELAZONATUS* did not change its thermal limits, suggesting that climate change will be specially a threat to this species.

### 4.3. *INTEGRATED BIOMARKER response AS A tool to identify ACCLIMATION CAPACITY*

Molecular mechanisms supporting thermal limits have been associated with functional acclimation capacity of energetic metabolism and antioxidant defense to maintain homeostasis (Pörtner and Knust 2007; Madeira et al., 2016a). Therefore, studying cellular and biochemical responses can be an important tool for defining the thermal window and thermal tolerance of a species (Deschaseaux et al., 2010). The use of a methodology that integrates the responses of diff erent biomarkers has been used to identify early thermal stress that could be related to acclimation abilities. Among these indices, Madeira et al. (2016b) have satisfactorily applied the Integrated Biomarker Response (IBR) in distinct tissues (including white muscle). In that work, the authors suggested that acclimation capacities in *Amphiprion OCELLARIS* are time-dependent and IBR values stabilized after 30 days of exposure;

therefore, no significant differences between the control and elevated temperature could be related to acclimation capacities.

Our findings, however, suggest that the three analyzed species presented physiological stress in the present study, since they all increased IBR values at extreme climate scenario compared to the current one. However, *H. MELAZONATUS*, the athletic species, had severe molecular alterations suggesting a narrow ability to tolerate further warming. The high increased IBR value results from oxidative stress, membrane damage, and loss of muscle neurotransmitter. Madeira et al. (2018) also observed an increase in IBR for distinct marine tropical species related to oxidative damage and induction of protective pathways against ROS damage. Therefore, the high mortalities and narrow thermal safety margin (the difference between thermal tolerance and thermal average) observed in the present study for *H. MELAZONATUS* results from low acclimation abilities and cellular stress. All these data are corroborated by cluster analyses and heatmaps that show a climate scenario cluster, suggesting a strong influence of temperature and $CO_2$ under biochemical and physiological parameters.

Our data suggest that all Amazonian species herein studied have low acclimation capacities at climate change scenario after 30 days exposure (Figs. 3 and 4). As far as we know, this is the first study that has examined thermal acclimation abilities in Amazon forest stream fish, which are adapted to a more thermally stable environment. Moreover, for most of the studied tropical species, including Amazonian fishes, studies have indicated that they have limited capacity for warming acclimation (Stillman, 2003; Campos et al., 2018). For instance, two congeneric species, *PARACHEIRODON AXELRODI* and *P. SIMULANS*, exposed for 28 days at 32 °C did not improve their aerobic capacity compared to natural temperature (Campos et al., 2017). Indeed, for these same species acclimated to A2 climate change scenario (4.5 °C and 900 ppm above current scenario), Fé-Gonçalves et al. (2018) found limited activation of glycolytic metabolism in *P. AXELRODI*, which was related to increased mortalities. In conformity, Madeira et al (2018), applying IBR methodology in four tropical species from distinct Phyla (Fishes, Mollusca and Crustacea), suggested low acclimation abilities for 3 °C warming, with all species presenting high IBR index values.

As for marine species, temperate streams species have high acclimation capacities compared to tropical ones. Rooke et al. (2017) showed that cold population of sunfish (*Lepomis gibbosus*) present high energetic and thermal plasticity compared to warm populations. Therefore, our results corroborate that tropical species are especially in threat under climate change scenario.

Our results suggest that Amazon forest streams fishes will be strongly affected by climate change scenario due to their low acclimation ability. Acclimation is the result of cellular and biochemical responses to maintain physiological homeostasis, which includes energy supply, and avoid cellular stress (Madeira et al., 2016b). In fact, the decrease in ROS formation, protein and lipid damage in fishes acclimated to warming has been related to their capacity to maintain thermal performance and improve their tolerance (Sampaio et al., 2018). Besides, our results confirm that IBR index is sensitive, provides precise information about species acclimation capacity, and should be used as a tool to evaluate the early effects of climate change on physiological stress as suggested by Madeira et al., 2018.

## 5. Conclusions

Overall, the Amazon species studied herein are susceptible to extreme climate change and stream warming caused by deforestation since them all present metabolic and oxidative alterations. The sedentary ones *A. AGASSIZII* and *P. brevis* seem to be less vulnerable to climate change because they present low levels of mortalities, reduced metabolic alterations and high thermal tolerance under a extreme warming scenario. In contrast, *H. MELAZONATUS*, the athletic species, presents the most severe damage and alterations, which is related to high mortality levels and narrow thermal safety margin.

These results corroborate our previous findings for fish acclimatized to stable thermal forest habitats, i.e., that athletic species are more vulnerable to warming (Campos et al., 2018), indicating that future climate scenario will have lower impact on low swimming performance species with potential effects on ecosystem metabolism.

Based in our findings, we suggest that *H. MELAZONATUS*, and possibly other athletic species, might be used for field monitoring studies applying the IBR methodology of aerobic metabolism and antioxidant defense enzymes to verify early effects of climate change. These studies provides important evidences for the impacts of climate change on population of ornamental fishes. In addition, future climate scenario and ornamental trade can both impact the genetic variability of Amazon species, which can be critical in species that are especially vulnerable as high activity species like *H. MELAZONATUS*.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2019.01.051.

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**Capitulo IV**

## Is the third universal response to warming mediated by aerobic metabolism or oxidative stress?

**In prep to Functional Ecology**

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Abstract

The body-size reduction at elevated temperature is a widely observed pattern, especially in aquatic organisms. Because of that, it has been suggested as the third universal response to global warming. Some studies have claimed that, at warming, the limitations on oxygen supply reduce the energy available to maintain growth rate. While other states that the as temperature increase the oxidative stress raises altering the cellular redox homeostasis driving energy supply to deal with ROS production. Therefore, empirical evidence has led to a debate about the proximate mechanisms that mediated the temperature size-rule. Thus, the aim of this work is investigate the body-size reduction at warming is mediated by limitation on oxygen supply or oxidative stress. For that question, we reared P. brevis under current and extreme climate change scenario (+4°C and 900 ppm above current scenario) for 180 days. We available the total weight, resting metabolic rate, maximum metabolic rate and aerobic scope at 7, 30, 60, 90, 120, 150 and 180 days of exposure. In addition, after 180 days exposure was available the mitochondrial respiration and ROS production and the activities of SOD, CAT, LPO, protein carbonyl and total protein in the muscle. Our results showed a 33% weight and protein reduction in individuals reared under extreme climate scenario. Besides, there were no differences in RMR, MMR, AE or mitochondrial respiration between scenarios. On the other hand, there was increase in SOD, LPO and protein carbonyl. In addition, LPO and protein carbonyl showed a negative relation with the protein amount. Our findings suggest that warming induce oxidative stress and alter redox homeostasis driven energy to deal with ROS production and produce a trade-off between self-maintenance and growth performance as suggested by the life-history trade-off theory.

Key-words: temperature size-rule, aerobic scope, oxygen supply, oxidative damage, redox homeostasis.

## 1. Introduction

Since the industrial revolution, global temperature has increased by ≈1°C due to the $CO_2$ emission. Global mean temperatures and the frequency of extreme temperature events are predicted to increase in the next century (Meehl & Tebaldi, 2004; IPCC, 2014). Global warming may have profound impacts on animal life since temperature is the main factor affecting the life-history of ectotherms; all aspect of animal life is virtually affected by temperature, since growth rate to reproduction. Atkinson (1994) showed that 80% of studies reported an inverse relationship between temperature and size, known as the temperature size-rule, demonstrating the generality of this response. This has led to the suggestion that body-size reduction is the third universal response to global warming (Gardner et al. 2011; Sheridan & Bickford 2011), besides changes in the phenology (Stenseth et al. 2002; Walther et al. 2002; Durant et al. 2007), and distributions (Parmesan & Yohe 2003; Root et al. 2003; Perry et al. 2005).

Even though that size reduction in warming is a well-known response, the physiological mechanism that mediates this process still in debate. Several authors suggest a possible role for oxygen creating the temperature size-rule (Hoefnagel and Verbeck, 2015). The central idea of this hypothesis is that oxygen become limiting under warm conditions since increase temperature raises the standard metabolism while oxygen supply fails to meet demand. Therefore, the aerobic scope (the difference between standard and maximum metabolic rate) decrease at warming conditions, which

could lead to oxygen supply restriction to maintain growth rate (Portner et al., 2010; Pörtner and Farrell, 2008).

The so-called oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis states that aerobic scope is reduced outside the thermal optimum and that limiting animal performance. For instance, Eliason et al., (2011) showed that migrations capacity of populations of Salmon (*Oncorhynchus nerka*) is related to their aerobic capacity. In addition, Pörtner and Knust (2007) showed that declines in abundance of eelpout, *Zoarces viviparus* at warming periods were related to aerobic scope reduction. Therefore, aerobic scope have been suggested to be a unifying physiological principle to explain constrains in growth rate, reproduction and locomotion (Farrell et al., 2009; Melzner et al., 2009; Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Rosa et al., 2017). However, the generality of OCLTT assumption have been contradicted and a series of studies provides data contrary to its main theoretical expectations (Clark et al., 2013; Audzijonyte et al., 2018; Jutfelt et al., 2018)

Increase in environmental temperature induces the production of reactive oxygen species, as a by-product of aerobic metabolism (Madeira et al., 2016). The concentration of ROS is controlled by antioxidants, which are consisted of non-enzymatic and enzymatic systems. Overproduction of ROS induces damage to lipids, proteins and deoxyribonucleic acid (DNA), leading to changes in cellular homeostasis (Braz-Mota et al., 2016; Prado-Lima and Val, 2016). Therefore, the oxidative stress is caused by the inability of cellular antioxidant systems deal with the reactive oxygen species. Because of that, it has been suggested that the increase in oxidative stress is one of the main reasons for reduced senescence, growth and reproduction (Costantini et al., 2010).

Recent findings suggest that oxidative stress provides a physiological mechanism in determining the consequence of life-history trade-offs between self-maintenance and activities, such as development and growth rate and, hence play a key role in animal fitness (Costantini 2008; Dowling and Simmons 2009; Metcalfe and Alonso-Alvarez 2010). As a life history strategy, fish may reduce investments in their own protection in favour of hatching success and survival chances (Fontagné et al., 2006). In fact, it has been suggested that oxidative stress constraint the growth performance (Monaghan et al. 2009; Costantini et al. 2010;Larcombe et al., 2015; Audzijonyte et al., 2018). However, experimental evidence for oxidative stress mediating growth rate under long-term warming acclimation still necessary.

Reduction in body size is one of the most concern for ecologists once it is determinant of intra- and interspecific interactions (Dell et al., 2011; Ohlberger & Fox, 2013), demographic processes (Barneche et al., 2016) and fisheries productivity (Baudron et al., 2014). Therefore, it is essential identifies the proximate mechanisms behind body size responses to temperature. The aim of the present study was to test if the size reduction at warming is mediated by aerobic metabolism or oxidative stress. Specifically, if the growth rate is limited by decrease in aerobic scope and oxygen supply to the tissues in accordance to the OCLTT hypothesis (Portner et al., 2010) or is limited by the increase in oxidative stress altering the cellular redox homeostasis in accordance to the life-history trade-off theory (Speakman et al., 2015). For that purpose, the effects on growth rate, the cascade of aerobic metabolism and oxidative stress were assessed in juvenile of *Pyrhullina brevis* reared for 180 days in the current and extreme climate change scenario (+4.5°C and 850 ppm above current scenario).

## 2. Material and Methods

### 2.1. Animal Collection and Maintenance

We collected juveniles of *P. brevis* (0.65 ± 0.1g) in small order streams at Reserva Florestal Adolpho Ducke (02°53'S, 59°58'W). The individuals were collected by hand nets (2-mm mesh) in the ponds that are used as reproductive area for this specie (Espirito-Santo et al., 2017). After capture, the juveniles were held in a 50-L aquarium with constant aeration and transferred to LEEM – Laboratory of Ecophysiology and Molecular Evolution at Brazilian National Institute for Research of the Amazon. At the laboratory, the fishes were held at 150-L tank (Fortlev®) outdoor (26°C ± 1) for two-weeks before experimental setup start. Half of the water was replaced every 24 hours. During this period, fish were fed *ad libittum* daily with TetraMin® Flakes. All housing and experimental sets are in accordance with CONCEA Brazilian Guide for Animal Use and Care and were authorized by INPA's Council for Ethics in Animal Use (CEUA - protocol number 027/2015).

### 2.2. Experimental Scenario exposure

The juveniles were reared in two distinct climate scenarios in climate rooms. These systems consist of climatic rooms with temperature and $CO_2$ computer controlled. Sensors measured temperature and $CO_2$ at Reserve Ducke every other minute and transmit the data to laboratory computers that control environmental rooms according to the climate scenarios. The climate scenarios provided by the Assessment Report of the IPCC (2014) for the year 2100 were simulated in the current scenario room (the natural temperature and $CO_2$ levels at Reserva Ducke) and extreme scenario room (4.5°C and 850 ppm $CO_2$ above current levels). Room temperature and $CO_2$ data are available in supplementary material (SM1).

The individuals were transferred to 10-L PVC tanks in three replicates containing five individuals per tank for each scenario with recirculating pump (N=30).

The fish were maintained in each climate room scenario for 180 days. To avoid ammonia accumulation, 30% of the water was replaced every day using environmentally stabilized room water. The pH, $O_2$, and temperature levels of the water were measured daily using a YSI probe, and $CO_2$ levels was measured by colorimeter method according to (Boyd and Tucker, 1992) (Table 1). The fish were fed *ad libitum* twice a day using commercial TetraMin®Flakes (40% protein) and fish were unfed 24 hours before experimental set-ups as described below.

2.3. Growth performance and Metabolic rate measurement

We measured the total mass of the all 15 individual of each climate room and the metabolic rates in 8 random individuals at 7, 30, 60, 90, 120, 150 and 180 days of exposure. The maximum and resting metabolic rate were measured in the intermittent-flow system. The individuals were placed in a 70-ml respirometer chamber to measure their metabolic rate ($n$ = 8). Intermittent flow respirometry was used to determine the metabolic rate of the fish (Norin and Clark, 2016). We used an automated apparatus DAQM (Loligo Systems, Tjele,Denmark), which consists of a recirculating circuit with 3 phases: flush, wait, and measurement. During the flush phase, peristaltic pumps were used to recirculate chambers with ambient tank water. The oxygen consumption was calculated following: $MO_2 = -\Delta O.Vresp.\ B^{-1}$, Where $\Delta O$ is the rate of change in oxygen concentration ($mgO_2\ h^{-1}$), V resp is the volume of the respirometry chamber, and B is the mass of the individual (kg). background respiration were also measured in a respirometer chamber without fish.

To determine maximum metabolic rate (MMR) individuals were manually chasing for 2 minutes (time required to exhaustion according previously test) (Norin and Clark, 2016). Then, they were placed in the static respirometer chamber and oxygen consumption was measured. The metabolic rate was measured at every 3 minutes with 1

min of water flux during 30 min and the highest value was considered the MMR. The individuals stayed in the chamber overnight (12h), during this time we collected 48 measurements of oxygen consumption with water flux of 3 min, wait of 2 min and 10 minutes of measurent. The resting metabolic rate (RMR) were calculated as the mean of the 10% lower values during the experimental test with $r^2$<0.9 (Norin and Clark, 2016). The aerobic scope (AS) was calculated as MMR – RMR. After measurement of the 180 days, the eight individuals were euthanized by a cerebral concussion and the muscle was harvested to mitochondrial respiration and oxidative stress analysis.

2.4. Mitochondrial respiration

After the fresh muscle were harvested, we immersed a small amount in ice-cold relaxing buffer (BIOPS, 2.8 mM $CaK_2EGTA$, 7.2 mM $K_2EGTA$, 5.8 mM $Na_2ATP$, 6.6 mM $MgCl_2 \cdot 6H_2O$, 20 mM taurine, 20 mM imidazole, 0.5 mM dithiothreitol, 50 mM K-MES, 15 mM Naphosphocreatine and 50 mM sucrose, pH 7.2). The muscle was teased into fiber blocks using a dissecting microscope and placed in 1 ml ice-cold BIOPSalong with 50 µg/ml. After 30 minutes, fibers were washed three times for 10min in 2ml of modified mitochondrial respiratory medium (MiRO5, 0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6H_2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM $KH_2PO4$, 20 mM HEPES, 160 mM sucrose and 1 g/l BSA, essentially free fatty acid, pH 7.2 at 25 °C; Gnaiger et al. (2000)). The fibers were blotted dry on filter paper and weighed into 5–10 mg bundles for respiration assays in 2mL of MIR05. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.4.1. *Mitochondrial respiration assay*

Mitochondrial respiration was measured in a high resolution respirometer, Oroboros Oxygraph-2k™ (Oroboros Instruments, Innsbruck, Austria) utilizing the

SUIT protocol (Braz-Mota et al., 2018) at mean acclimation scenario temperature (26°C for Current scenario and 30°C for extreme climate scenario). Oxygen was maintained above saturation to ensure saturationat respective assay temperatures tomaintain steady state oxygen flux, the SUIT protocol is following.

The Complex I (CI) substrates (2 mM malate, 10mM pyruvate and 10 mM glutamate) were added to measure state II respiration in the absence of ADP (denoted Leak). Excess ADP (2.0mM) to stimulate oxidative phosphorylation was added to saturate CI. Cytochrome c (10 μM) tested outermembrane integrity. Phosphorylating respiration with CI and CII substrates(OXP-I, II, state III respiration) was attained by addition of succinate(10mM). Followed by titrations of carbonyl cyanide p-(trifluoromethoxy)phenyl-hydrazone(CCCP, 0.5 μmol/l) to uncouple mitochondria (ETS). By the addition of rotenone (0.5 μM),and antimycin (1 μM),CI, and II were inhibited, respectively. The residual flux following theaddition of these inhibitors was attributed to background respiration.Cytochrome c-oxidase (CCO, CIV) was measured by the addition ofthe electron donor couple N,N,N′,N′-tetramethyl-p-phenylenediamine(TMPD, 0.5 mM) and ascorbate (2 mM). All the chemicals were purchase from Sigma Aldrich.

### 2.5. Oxidative stress biomarkers

For antioxidant enzymes and oxidative stress measurement, white muscle were homogeneized in 1:4 (wet tissue mass/buffer volume) in a PBS buffer (Madeira et al., 2016). The glutation-S-transferase (GST) activity in white muscle was determined using 1-chloro 2,4-dinitrobenzene (CDNB) as substrate, according to Keen et al. (1976). Changes in absorbance were recorded at 340 nm. The enzyme activity was calculated as nmol CDNB conjugate formed per min per mg protein. To determine the catalase (CAT) activity, the inhibition rate of $H_2O_2$ decomposition was monitored at 240 nm

(Beutler, 1975), and expressed as μM $H_2O_2$ $min^{-1}$ mg $protein^{-1}$. The lipid peroxidation (LPO) levels were quantified based on the oxidation of the $Fe^{+2}$ to $Fe^{+3}$ by hydroperoxides in acid medium in the presence of ferrous oxidation-xylenol orange, at 560 nm, according to the method described by Jiang et al. (1991). The protein carbonyl was quantifies according to Levine et al (1994) where 2,4-dinitrophenylhydrazine (DNPH) reacts with carbonylated proteins (aldehyde and ketone type only) to form dinitrophenyl hydrazine that can be detected at 358-370 nm. The protein amount was measured according to Bradfford (1976).

2.6. Statistical Analyses

The data are presented as mean ± SEM. All statistical analyses were performed in SigmaStat 3.1 using a significance level of 5% Parametric two-way ANOVA or non-parametric Kruskal-Wallis test (depending on whether the data met the assumptions of normality and homoscedasticity). The growth performance and metabolic rate (RMR, MMR and AE) were tested using a two-way Anova comparing climate scenario and time of exposure. *Post hoc* tests were carried out using Tukey's HSD. The results of mitochondrial respiration and oxidative stress biomarkers, as well protein in the muscle, were compared using Test t or Mann-Whitney U statistic (non-parametric) between climate change scenarios. In addition, Spearman correlations were analyzed between oxidative stress biomarkers (LPO and carbonyl protein) and protein with data log transformed.

3. Results

Regarding the abiotic factors measured between climate change scenarios the extreme climate scenario presented higher temperature and $CO_2$ and pH was lower

(mean of 30.5°C, 23ppm $CO_2$, and 5.9 pH) compared to the current scenario (mean of 26.5°C, 8ppm $CO_2$,and 6.3 pH) (Table 1).

The two-way analyses for weight showed significant differences between scenario (F= 25.73, p> 0.001) and among time of exposure (F= 24.94, p>0.001). Also, the interaction factor showed differences between climate change scenario and time exposure (F=3.38, p>0.004) (figure 1). The differences between climate scenarios started at 90 days and at the end of 180 days exposure the individuals acclimated at extreme climate scenario showed a weight reduction of 33.41%. In addition, there was a significant decrease in protein amount of fishes acclimated for 180 days in the extreme climate change scenario (table 2).

The routine metabolic rate did not differ between a climate scenario and among time of exposure (fig 2A). Also, maximum metabolic rate (MMR) did not differ between climate scenario (F=0.128, p=0.722), however, there were differences among time of exposure (F= 16.18, p>0.001). A decrease of MMR was observed at 90, 150 and 180 days compared to the others time. The interaction showed significant differences (F=3.29, p>0.005). In accordance with MMR, aerobic scope showed no differences between climate scenario (F=1.175, p=0.130), but there were differences among time of exposure (F=7.73, p>0.001). A decrease of aerobic scope was observed at 90, 150 and 180 days compared to the others time (fig 2A). In addition, there were significant differences in the interaction between climate scenario and time of exposure (F=4.46, p>0.028) (fig. 2B).

There were no significant differences in mitochondrial physiology at any respiration complex between climate change scenarios exposed for 180 days (fig 3). Regarding oxidative stress status, we observed an increase in the maximum activities of

Superoxide dismutase (t= 2.321, p>0.05), lipid peroxidation (U=0.00, p>0.05) and carbonyl proteins (U= 8, p>0.05). The maximum activities of Catalase and Glutatione-S-transferase did not present significant differences between climate scenario (table 2). In addition, the Spearman analysis between lipid peroxidation by protein ($R^2$= -0.68, p>0.00001) and carbonyl proteins by proteins ($R^2$=-0.56, p>0.0001) showed negative correlation (figure 4).

## 4. Discussion

In the present work, we observed that *Pyrrhulina brevis* reared under high temperature and $CO_2$ levels (±4.0 °C and 900 ppm above current levels) decreased body weight. However, metabolic rates, aerobic scope and mitochondrial respiration are not compromised under long-term acclimation predicted for the near future. Therefore, our results indicate a mismatch between aerobic metabolism and growth performance in opposite to the OCLTT hypothesis. On the other hand, after 180 days exposure, we observed an increase in the SOD activity, LPO and carbonyl protein indicating a redox disturbance that oxidative stress in accordance with the life-history trade-off hypothesis.

The reduction in body-size is being reported from a number of species (Daufresne, et al., 2009) and have been pointed as the third ecological universal response to warming (Gardner et al., 2011) and the cause of the decrease in the fish biomass production (van Dorst et al., 2018). In the Amazon, Ilha et al (2018) studying deforested streams observed a 6°C increased in temperature compared to the forested stream; and fish population that lives in the warm environment presented a 36% reduction in body weight. In addition, the authors reported a 27% of reduction in weight of *Pyrhullina australis* a congeneric species of the present study. In fact, our results are in accordance

with Ilha et al (2018) since we observed that after 180 days exposure to warm and acidic environment *P. brevis* showed a 33% reduction in weight.

In addition, our results showed a decrease in muscle protein of individuals exposed to extreme climate scenario. In a previous study, we have observed increases in ammonia excretion of adults of *P. brevis* acclimated for 15 days to warming scenario (personal observation). The ammonia is a by-product of the oxidation processes of proteins or amino acids. The increase in ammonia excretion indicates an increase in the oxidation of proteins in order to supply the metabolic demand. In fact, acclimation to warming has been related to an increase in the protein metabolism in O. mykiss (Lauff & Wood, 1996; Kieffer et al., 1998). Therefore, our findings suggest that under warming and acidic conditions *P. brevis* increases protein catabolism to meet energy supply, which could limit growth capacity.

In addition, our findings suggest a mismatch between growth rate and oxygen supply capacity in dissimilarity to OCLTT hypothesis. The OCLTT states that at pejus temperature (where the aerobic scope starts to drop), the specie performance decreases because of a decrease in aerobic scope related to a cardiorespiratory impairment, which reduce tissue oxygen levels leading to mitochondrial dysfunction and tissue hypoxia (Pörtner and Farrell, 2008). According to that, limitations on tissue oxygen suplly can be used to explain tolerance, performance, and growth (Pörtner and Farrell, 2008; Pörtner and Knust, 2007). Therefore, reduction in size should be related to a decrease in aerobic scope and mitochondrial respiration capacity under extreme climate change exposure. However, The *P. brevis* exposed to the extreme climate scenario showed a size reduction, nevertheless, it maintains the aerobic scope and mitochondrial respiration capacity as in the current climate scenario. The fact that aerobic scope did not change at the warm and acidic condition while growth declines, have already been

reported. For instance, Gräns et al. (2014) observed that at the warmest and acidic (high $pCO_2$) acclimation condition the Atlantic halibut (*Hippoglossus hippoglossus*) increased aerobic scope, however, growth rate decreased. In addition, Healy and Schulte, 2012 have shown that growth rate was negative in killifish (*Fundulus heteroclitus*) where aerobic scope was increased (25–30°C). Therefore, our recent findings corroborates that growth performance and oxygen transport capacity are governed by distinct physiological process.

Although aerobic metabolism did not change during the experiment, we observed that *P. brevis* increased SOD activity, LPO and carbonyl protein reared at extreme climate change scenario indicating an increase in ROS production and cellular damage. Although the antioxidant activity increased (SOD), the increase in LPO and protein carbonyl indicates that the antioxidant mechanism were not sufficient to prevent against oxidative damage. The LPO and carbonyl protein are the two major types of cellular oxidative damage and their increase at elevated temperature is well documented in the literature and seems a general response to warming as such reduction in body-size (Vinagre et al., 2012, Rosa et al., 2016). The overproduction of ROS damage proteins, and lipids, and initiate a cascade of events, which impaired cellular function and can promote apoptosis events constrain survival, reproduction and growth performance (Iftikar et al., 2013). For instance, Rosa et al., (2017) showed that the epaulet shark (*Hemiscyllium ocellatum*) reared under elevated temperature (+4°C) and pCO2 (900ppm) increased antioxidant systems (CAT) and oxidative damage (LPO) and decreased developmental time and size.

The life-history theory postulate that trade-offs can mold the patterns of investment by organisms among reproduction, growth, and survival, and those are mediated by free radical production and oxidative stress (Speakman, 2005). According to this hypothesis,

elevated cellular damage limits the investment resource to allocate in animal performance (Speakman and Selman, 2011), since allocation of limited resources to one trait has negative consequences for other traits requiring the same resource. Therefore, our results suggest that size-reduction is associated with increase in oxidative stress. In fact, it has been suggested that oxidative stress might play a key role as a constraint on, and cost of, growth (Monaghan et al. 2009; Costantini et al. 2010; Larcomber et al., 2015). In a recent meta-analyses review, Smith and colleagues (2016) proposed that oxidative stress could act as a constraint on growth, supporting the theoretical links between oxidative stress and animal life histories and provide evidence for a growth–self-maintenance trade-off. In addition, Kacienė et al., (2015) showed a reduction in the size of the plant (*Hordeum vulgare* L) related to the increase in oxidative stress when exposed to different stress factors. Therefore, our results suggest that there are costs in terms of oxidative damage for individuals living in a warming scenario and, consequently, a trade-off between growth and redox maintenance.

Hence, in accordance with the life-history theory, we propose that oxidative stress mediates the size reduction at warming in *P. brevis*. In fact, the Spearman analysis showed a negative correlation between the levels of lipid peroxidation and carbonyl protein with proteins amount, supporting, at the tissue level, that oxidative stress imposes physiological impacts that can constrain the growth performance. The mechanism by which ROS is linked to the growth is not well understood, however, Morales and Munné-Bosch (2016) proposed for plants that NADPH oxidases are activated during growth, thus generating transient ROS increases in the apoplast. Through this mechanism, ROS gradients act down regulating growth gene in leaves and roots. However, the physiological cascade of ROS signaling growth genes must be tested to identify this mechanism in fishes.

Summarizing, our findings showed that reared under extreme climate change scenario, *P. brevis* decreased body-size in accordance to the third universal response to warming. The decrease in body-size has important ecological, including increasing predation risk, earlier time to sexual maturity, and decreased lifetime reproductive success. Besides, it has been suggested that elevated temperature is already affecting the fish biomass production by increase small fish abundance (van Dorts et al., 2018). Therefore, studies investigating the global warming effects on the biomass of economic species is necessary to predict the impacts on food security of the traditional population and in the local economy.

In addition, our results showed no effects of climate scenario on aerobic metabolism and mitochondrial respiration indicating a mismatch between oxygen supply and growth in opposite to the OCLTT hypothesis. However, we found increased ROS production and oxidative stress and, furthermore, protein content and oxidative stress were correlated. The strong correlation between oxidative stress biomarkers and protein suggests that cellular stress markedly determines growth performance in *P. brevis*. Therefore, for the first time, we propose a link between oxidative stress and growth capacity at warming in accordance with the Life-history trade-off theory. Increase in oxidative stress at warm is widely reported as well as size-reduction; therefore, we suggest that the third universal response to warming is mediated by the oxidative stress.

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Table 1. Temperature, $O_2$, $CO_2$, and pH means in the water and in the air of experimental climate rooms; The rooms are real-time computer controlled simulating current levels and extreme climate scenarios (A2, plus 4.5°C and 900ppm $CO_2$) proposed by IPCC 2014.

| | Water | | | | Air | |
|---|---|---|---|---|---|---|
| Scenario | Temperature °C | $O_2$ (mg.$L^{-1}$) | $CO_2$ (ppm) | pH | Temperature °C | $CO_2$ (ppm) |
| Current | 26.5 ± 1.0 | 6.5 ± 0.5 | 8±2 | 6.3 ± 0.3 | 27.5 ±1.7 | 456 ± 15 |
| Extreme | 30.5 ± 1.0 | 6.3 ± 0.4 | 23 ±2 | 5.9 ± 0.5 | 32.0 ± 1.8 | 1254± 16 |

Table 2. The antioxidant enzyme activity of Superoxide dismutase (SOD), catalase (CAT), glutathione –S – transferase (GST); the oxidative damage stress indicator lipid peroxidase (LPO) and protein carbonyl (CARB); and the protein concentrarion in white muscle of of *P. brevis* acclimated to current and extreme climate change scenario (4.5°C and 900 ppm plus current scenario). Asterisks indicate significant differences between climate change scenario by test t analyses.

| Scenario | SOD | CAT | GST | LPO | CARB | Protein |
|---|---|---|---|---|---|---|
| Current | 0.078±0.008 | 0.016±0.004 | 0.010±0.005 | 27.6 ± 5.39 | 0.004±0.0001 | 8.54±0.78 |
| Extreme | 0.105±0.008* | 0.020±0.005 | 0.013±0.004 | 153.7±43.92* | 0.008±0.0003* | 5.49±0.4* |

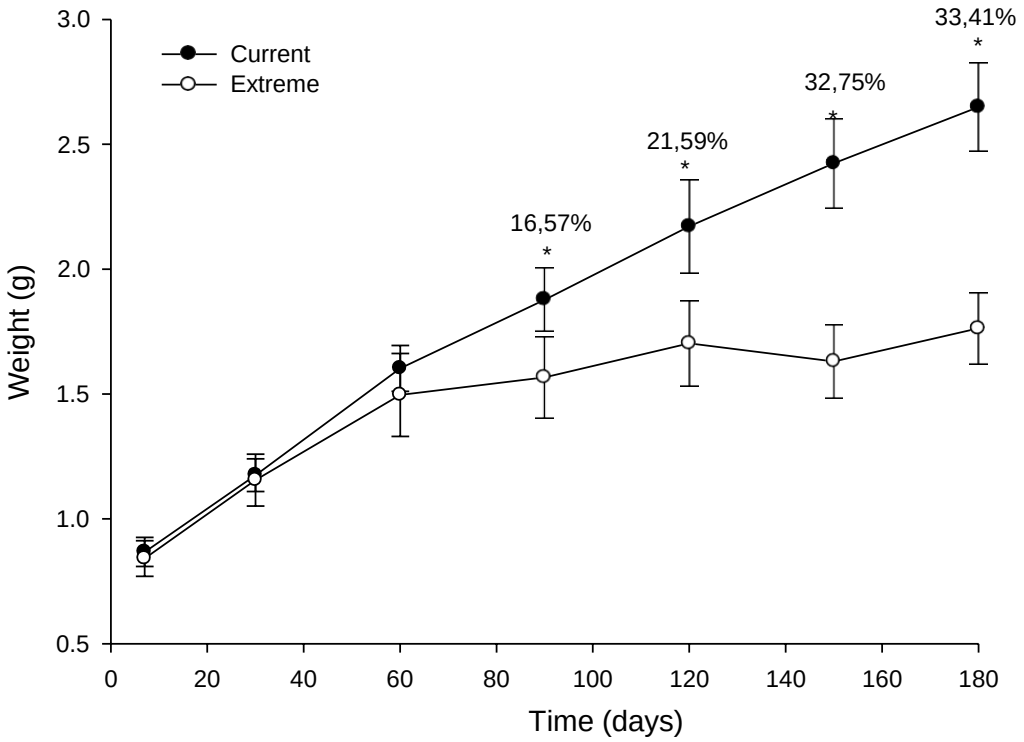
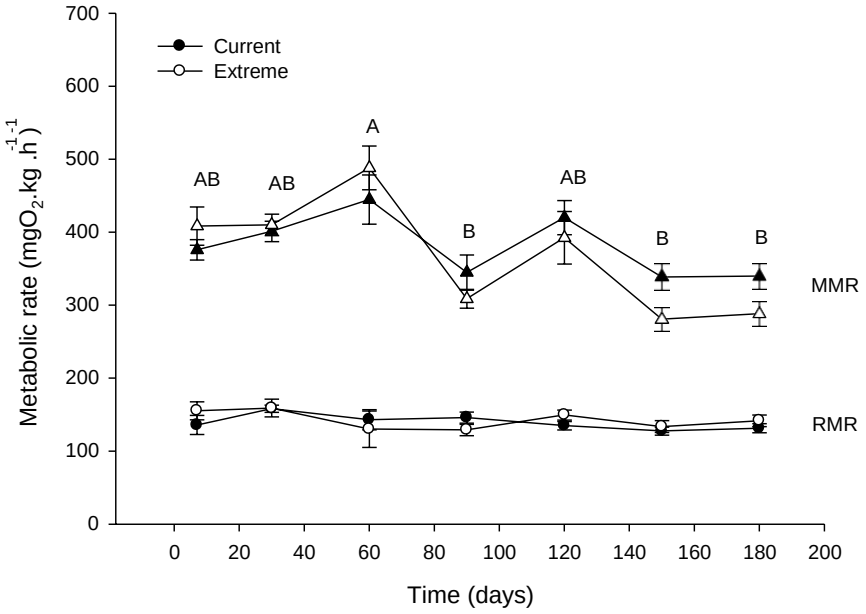
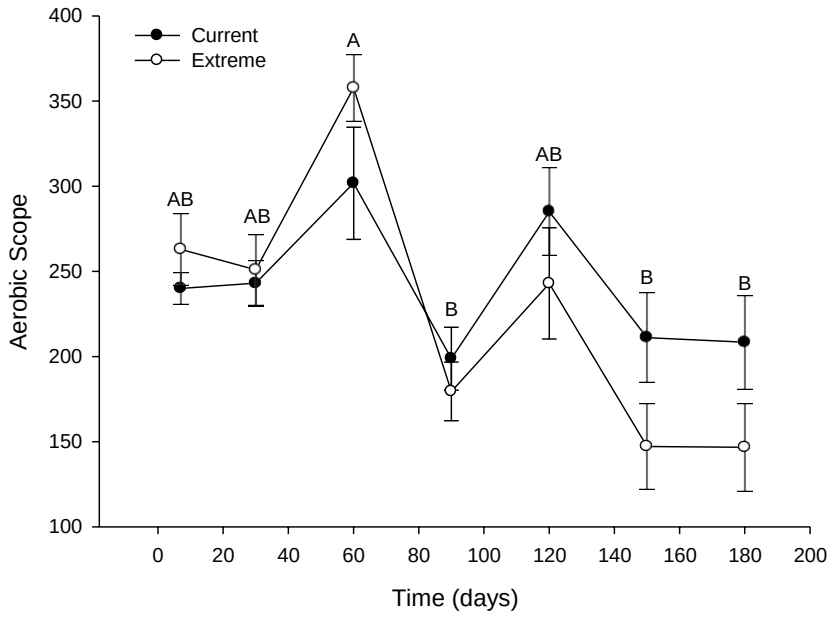



Figure 1. The weight variation along the 180 days exposure of *P. brevis* to current and extreme climate change scenario (4.5°C and 900 ppm plus current scenario). Different letters indicate significant differences by Anova two-way analyses and post-hoc of Tukey.




A




B

Figure 2. A) The resting metabolic rate (RMR) and maximum metabolic rate (MMR) along the 180 days exposure of *P. brevis* to current and extreme climate change scenario (4.5°C and 900 ppm plus current scenario). B) The aerobic scope (AE) of *P. brevis* to current and extreme climate change scenario (4.5°C and 900 ppm plus current scenario). Different letters indicate significant differences by Anova two-way analyses and post-hoc of Tukey.

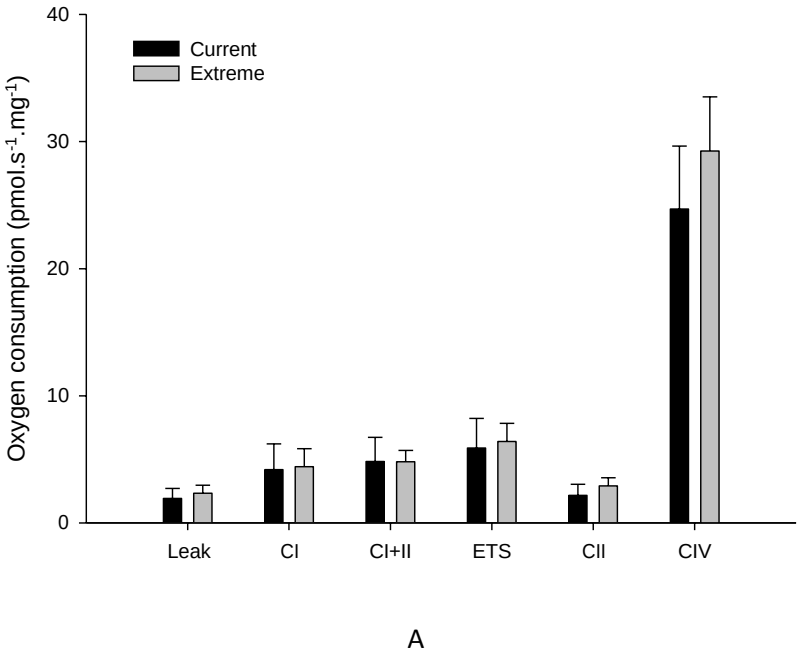
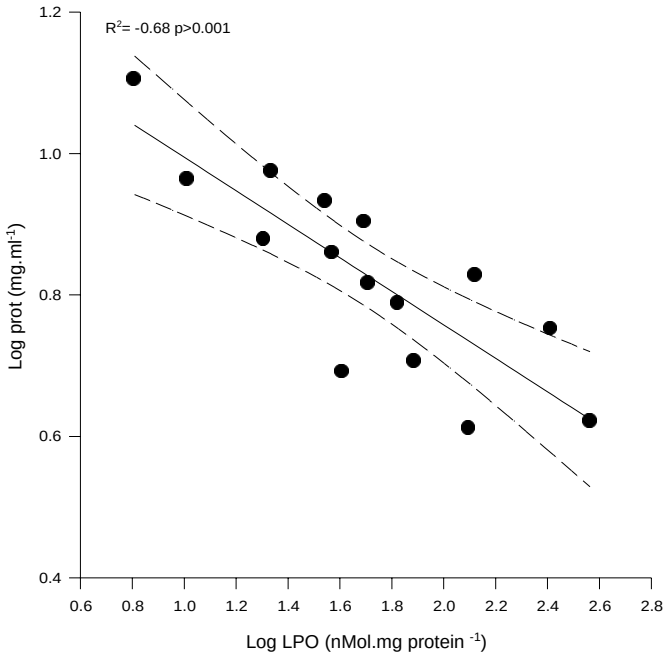
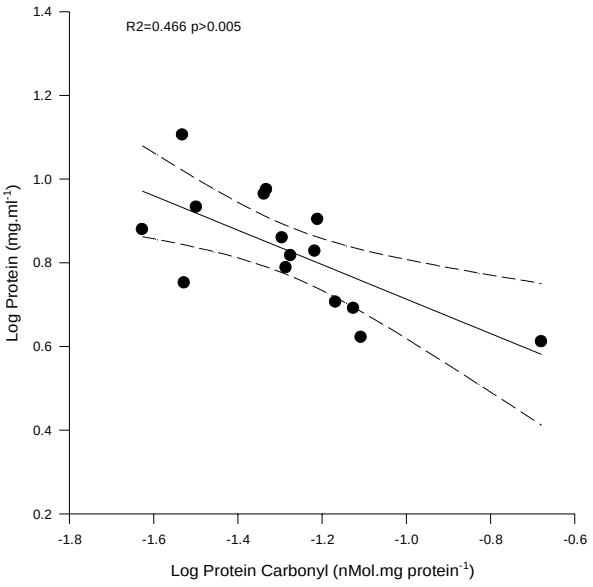



Figure 3. The A) mitochondrial respiration in Leak, Complex I (CI), complex I+II (CI+II), electron transport system (ETS), Complex II (CII) and cytocrome c oxidase (CIV) of *P. brevis* acclimated to current and extreme climate change scenario (4.5°C and 900 ppm plus current scenario).

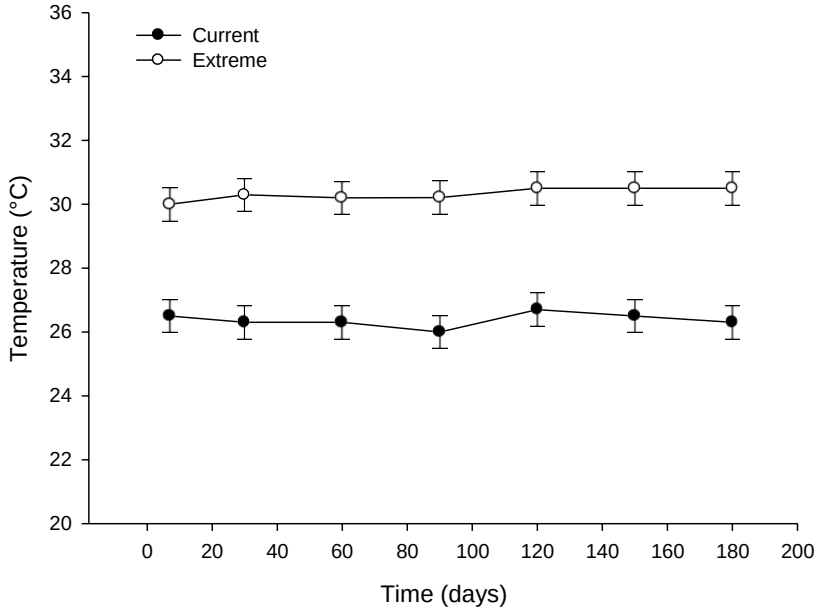



A

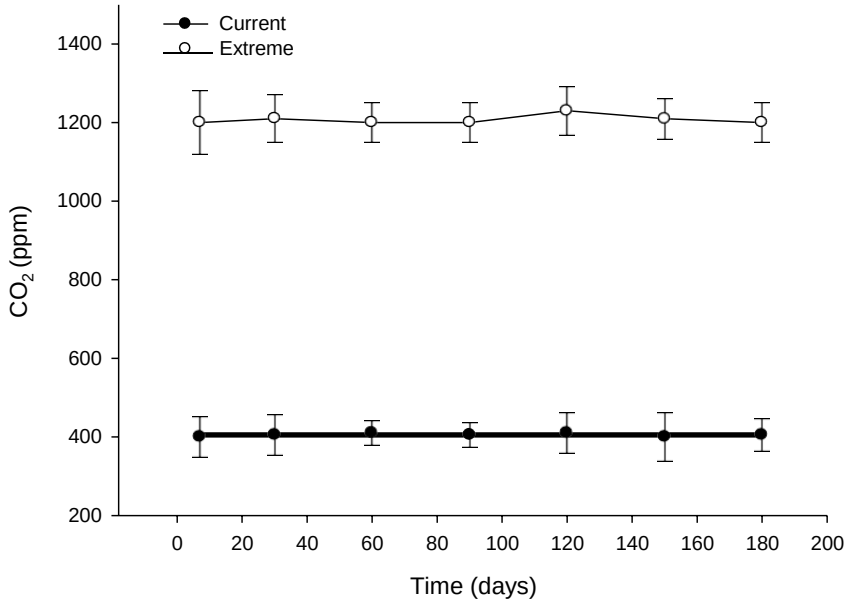



B

Figure 4. The spearman correlations analyses between A) LPO and protein and B) Carbonyl protein and protein. The data were log transformed.




A




B

SM. The A) temperature and B) $CO_2$ variation along the 180 days exposure of *P. brevis* to current and extreme climate change scenario (4.5°C and 900 ppm plus current scenario).

## 6. Conclusão Geral e Perspectivas

A presente tese contribui para o conhecimento sobre a vulnerabilidade e a capacidade de aclimatação para enfrentar as mudanças climáticas previstas para o ano de 2100 em peixes de Igarapé da Amazônia. A aclimatação é um dos mecanismos mais importantes que permitirão aos organismos enfrentar as mudanças ambientais e, portanto, é essencial analisar a plasticidade fenotípica em resposta ao aumento de temperatura e $CO_2$ a fim de obtermos poder preditivo. A capacidade de espécies tropicais sobreviverem às mudanças climáticas tem se mostrado restrita, portanto, é de suma importância compreender os mecanismos pelos quais espécies amazônicas com diferentes estilos de vida conseguem se adaptar às novas condições relacionadas ao clima.

Na presente tese nós estudamos uma variedade de respostas fisiológicas em espécies com distintos estilos de vida. Nossos resultados contribuem para o conhecimento sobre a suscetibilidade das espécies amazônicas frente ao aumento de temperatura e $CO_2$ e fornece ferramentas que ajudam nas previsões futuras sobre as mudanças na composição e estrutura das populações naturais.

Os resultados obtidos mostram que as espécies de Igarapé da Amazônia apresentam baixa capacidade de enfrentar os desafios climáticos previstos para o ano de 2100 e o grau de vulnerabilidade está diretamente relacionado com o estilo de vida das mesmas. No capitulo I foi mostrado que a demanda energética e a tolerância térmica dos peixes de Igarapé da Amazônia são relacionados com o estilo de vida destas espécies. Espécies ativas, comumente encontradas no canal do Igarapé nadando contra a corrente, apresentam altas demandas enérgicas de rotina, devido ao alto custo de manutenção de um estilo de vida mais ativo, contudo, sua capacidade de incremento do escopo aeróbico (escopo aeróbico fatorial) é limitada, reduzindo a tolerância destas espécies a situações estressoras como aumento de temperatura. Estes resultados sugerem que uma extensão

dos períodos de seca com ondas de calor, como os previstos para a região amazônica, irão impactar primeiramente as espécies ativas levando a uma mudança na composição e estrutura das assembleias locais.

No segundo capítulo, nós investigamos o efeito do aumento da temperatura e $CO_2$ sobre o compromisso osmorespiratório em três espécies com estilos de vida diferentes. Nossos resultados mostraram que as respostas iônicas e metabólicas são espécie-especificas. No geral, quando expostas ao cenário extremo as espécies aumentam a demanda energética e fazem isso às custas do aumento na perda de ions. Nossos resultados sugerem que a modulação branquial é um importante mecanismo de aclimatação aos cenários climáticos extremos uma vez que aumenta a tomada de oxigênio. Ainda, *H. melazonatus*, espécie ativa, apresentou grandes distúrbios osmorregulatórios, que podem ter importantes impactos na capacidade de sobrevivência a longo prazo.

Em concordância com os resultados obtidos nos capítulos anteriores, no capitulo 3 nossos resultados mostram ajustes metabólicos nas três espécies aclimatadas aos cenários climáticos extremos. Nossos resultados mostram que as três espécies estudadas têm baixa capacidade de aclimatação aumentando a demanda energética, ativando mecanismo de defesa antioxidante e apresentando danos celulares. Mais uma vez, *H. melazonatus* mostrou menor habilidade para enfrentar os desafios climáticos extremos. Nossos resultados mostram danos celulares, elevada taxa de mortalidade e diminuição da janela térmica no cenário climático extremo. Portanto, nós podemos esperar uma diminuição populacional em *H. melazonatus* que acarretará numa menor variabilidade genética em suas populações.

No último capitulo, capítulo 4, nossos resultados mostraram uma diminuição no tamanho de *P. brevis* aclimatadas por 180 dias no cenário climático extremo. Estes dados

corroboram com a hipótese de que a diminuição no tamanho das espécies é uma resposta universal ao aumento de temperatura. Nossos resultados ainda mostram que a redução no tamanho não está ligada a capacidade de fornecimento de energia celular, mas sim a um aumento nos danos celulares, provocado por mecanismos oxidantes. Estas respostas têm impactos profundos na estrutura populacional, podendo levar a uma diminuição na capacidade reprodutiva e de dispersão das espécies. Ainda, vale à pena lembrar que a região amazônica é o principal consumidor, e um dos principais produtores, de peixes do Brasil, portanto, uma diminuição no tamanho de peixes com interesses comerciais irá impactar profundamente a alimentação e a economia da região.

O ambiente aquático amazônico é composto por uma variedade de habitats com características físicas e químicas distintas e uma alta diversidade de espécies de peixes, portanto, avaliar a capacidade de aclimatação em espécies que ocorrem em diferentes habitats, em diferentes estágios de vida e com diferentes estilos de vida fornecerá importantes informações para verificar resistências das espécies e determinar quais ambientes são prioritários para programas de conservação. Além de medir a capacidade de aclimatação de alguns traços fisiológicos, é imprescindível também avaliar o potencial de adaptação através de alterações genéticas. Uma vez que alguns genótipos podem ter vantagens em um ambientes de alta temperatura e altos níveis de $CO_2$, os mesmos podem promover a resiliência frente às mudanças climáticas. Portanto, nós sugerimos estudos transgeracionais para verificar o potencial de adaptação das espécies amazônicas frente aos cenários de mudanças climáticas.

Nossos resultados ressaltam a importância de políticas públicas voltadas para a diminuição dos agentes causadores das mudanças climáticas e para a preservação das áreas de floresta que têm papel fundamental na manutenção da temperatura dos igarapés.

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