PROGRAMA INTERINSTITUCIONAL DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS – UFSCar/UNESP

EFEITOS DA ACLIMATAÇÃO TÉRMICA NO METABOLISMO AERÓBICO, PARÂMETROS CARDIOVASCULARES E TOLERÂNCIA TÉRMICA EM GIRINOS DE *BOKERMANNOHYLA IBITIGUARA*

"EFFECTS OF THERMAL ACCLIMATION ON AEROBIC METABOLISM, CARDIOVASCULAR PARAMETERS AND THERMAL TOLERANCE IN TADPOLES OF *BOKERMANNOHYLA IBITIGUARA*"

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Novembro
2020

UNIVERSIDADE FEDERAL DE SÃO CARLOS
UNIVERSIDADE ESTADUAL PAULISTA
PROGRAMA INTERINSTITUCIONAL DE PÓS-GRADUAÇÃO EM CIÊNCIAS
FISIOLÓGICAS - PIPGCF UFSCar/UNESP

Efeitos da aclimatação térmica no metabolismo aeróbico, parâmetros cardiovasculares e tolerância térmica em girinos de *Bokermannohyla ibitiguara*

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Dissertação apresentada ao Programa Interinstitucional de Pós-graduação em Ciências Fisiológicas – UFSCar/UNESP como parte dos requisitos para obtenção do título de Mestre em Ciências. Área de concentração: Fisiologia.

Jaboticabal
2020

Folha de Aprovação

Defesa de Dissertação de Mestrado do candidato Leonardo Sambini Longhini, realizada em 20/11/2020.

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O Relatório de Defesa assinado pelos membros da Comissão Julgadora encontra-se arquivado junto ao Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas.

O luto é o preço que pagamos pelo amor.

A todes que suportaram a dor nesse ano de 2020.

AGRADECIMENTOS

Primeiramente preciso agradecer à minha amada mãe, Silvia Sambini, um exemplo de mulher guerreira e perseverante. Obrigado por sempre apoiar meus sonhos e minhas lutas.

Outro exemplo de mulher e professora extremamente forte é minha orientadora, Luciane Gargaglioni, que possibilitou toda essa jornada acadêmica desde a iniciação científica. Muito obrigado por todo aprendizado e oportunidades, Lu.

Agradeço imensamente ao meu co-orientador e amigo, Lucas Zena, que esteve presente em cada etapa desse projeto mesmo distante fisicamente em alguns momentos. Sou grato por toda sua paciência e saiba que o tenho como exemplo de pesquisador dedicado e curioso.

À Prof.ª Cynthia Prado, Prof. Renato Nali e Natalia Salles por contribuírem imensamente com as coletas e identificação dos girinos.

Um agradecimento especial à toda equipe do Laboratório de Fisiologia: Prof.ª Kênia, Prof. Glauber, Carol Scarpellini, Carol Silva, Lays, Elisa, Aline, Carlos, Luis Gustavo, Danuzia, Kassia, Lara, Tiago, Gabriela, Daniel, Welex, Isabela, Mariane, Victor, Camila, Vivian, Lívia, Ane, Gabriel, Damares, Euclides, Dona Ângela e Rodrigo. Sou eternamente grato por toda ajuda, aprendizado e experiências que compartilhamos ao longo desses anos.

Obrigado aos meus queridos e estimados amigos que sempre me apoiaram, mesmo nos momentos de loucura. Talita, Vitor, Natalia, Guilherme, Aline, Marina, Silvia, Jaime e Enrico, sem vocês não teria chegado até aqui.

Obrigado à minha avó, Helena e meu primo Marcos, por todo o apoio nos estudos.

Agradeço à Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pelo apoio financeiro e bolsa concedida (processo n° 2018/01899-0) que possibilitou a conclusão desse projeto com sucesso.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo apoio financeiro.

Ao Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas e todos os funcionários da FCAV, UFSCar e FOAr.

Não posso deixar de agradecer às professoras e professores que contribuíram para a minha formação desde a educação básica até a pós-graduação. Espero que um dia nossa profissão seja realmente valorizada no Brasil.

Por fim, um NÃO obrigado ao atual governo, pois apesar de todo desmonte na educação, ciência e saúde, esse projeto foi realizado e é um símbolo de resistência.

"A coisa mais bela que podemos experienciar é o misterioso. É a fonte de toda verdadeira arte e ciência." – Desventuras em série

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Resumo

É proposto que a aclimatação térmica oferece uma vantagem seletiva em resposta às mudanças ambientais, conferindo resiliência em um cenário de mudanças climáticas. Uma vez que os anfíbios são um dos grupos mais vulneráveis às mudanças ambientais, é fundamental identificar a capacidade de aclimatação das espécies em climas mais quentes. O objetivo do trabalho foi investigar os efeitos da aclimatação térmica em girinos da perereca-arbórea de riacho do Cerrado (*Bokermannohyla ibitiguara*), e a relação entre as condições térmicas experienciadas em seu micro-habitat. Nós avaliamos o seu escopo aeróbio mensurando a taxa metabólica de rotina e máxima em diferentes temperaturas testes (15, 20, 25, 30 e 34°) em dois grupos aclimatados cronicamente (18 e 25°). Medimos também os parâmetros morfométricos dos dois grupos, a frequência cardíaca (*f*c) e o tônus autonômico em repouso e após bloqueio autonômico seletivo. Além disso, determinamos a temperatura crítica máxima (CT_{max}) e a tolerância ao aquecimento (CT_{max}-T_{max}), os quais não foram afetados pela aclimatação. As taxas metabólicas e *f*c aumentaram com a exposição aguda a temperaturas elevadas, porém a aclimatação não teve efeito significativo na frequência cardíaca basal e após bloqueio autonômico, bem como no tônus colinérgico e adrenérgico. Somado a essa ausência de compensação térmica da *f*c observamos uma diminuição no escopo aeróbio no grupo aclimatado a 25°C, acompanhado de uma redução de 53,8% da massa corporal nos girinos desse grupo. Dessa forma, concluímos que os girinos de *B. ibitiguara* são termo-especialistas, uma vez que habitam um micro-habitat com pouca variação de temperatura e possuem limitada capacidade de aclimatação em altas temperaturas. Assim, pequenos aumentos de temperatura acima das encontradas no seu micro-ambiente torna a espécie mais vulnerável às mudanças climáticas e/ou degradação do seu habitat.

1 – Introdução geral

1.1 – Um Cenário de Mudanças Climáticas

Muitos estudos têm evidenciado a influência do ambiente sobre a ecologia e a fisiologia de organismos e como as variações ambientais podem influenciar nas repostas comportamentais e fisiológicas de um indivíduo (Adolph; Porter, 1992; Navas, 1996; Mesquita et al., 2007). Além disso, os animais são capazes de modificar seus hábitats, ou seja, animais e ambiente são interdependentes. Uma vez que selecionam por meio do comportamento seu ambiente real a partir de uma variedade que poderiam viver, e sua presença em determinado local com frequência pode alterar as características físicas e químicas do local. A alteração ambiental causada pelo animal pode, então, mudar seu comportamento ou sua própria fisiologia (Hill et al., 2012).

O aquecimento global representa um exemplo de dimensão mundial desse fenômeno. O histórico de uso de combustíveis fósseis pela população humana tem modificado a atmosfera terrestre em direção a uma composição que aumenta a retenção de calor planetário, aumentando as temperaturas nas quais os seres vivos irão experimentar no futuro (Cramer et al., 2014). As temperaturas médias globais da superfície aumentaram $0,87 \pm 0,10°C$ no período de 2006-2015 em relação ao período pré-industrial (1850-1900), com um recente aquecimento de $0,2°C$ por década. E a previsão é que o aumento de um $1,5°C$ ou mais pode impactar gravemente os sistemas naturais e humanos (IPCC, 2018).

O clima exerce um papel dominante na distribuição geográfica das espécies, uma vez que a temperatura ambiental (Ta) é um dos fatores que mais afeta os processos biológicos. As espécies, em resposta a um longo tempo evolutivo, apresentam características e adaptações que as permitem estabelecer, desenvolver e reproduzir sobre determinadas condições climáticas. As variações diárias ou anuais das condições climáticas também interferem no funcionamento (fisiologia), comportamento e ciclo de vida dos organismos (Bellard et al., 2012).

Desse modo, qualquer mudança em grande escala no clima da Terra afeta drasticamente a biosfera. Na realidade, já estamos presenciando mudanças na distribuição de diversos grupos de organismos em decorrência desse cenário de alterações climáticas (Reece et al., 2015). Estudos têm demonstrado que as espécies estão tendendo a se deslocar em direção aos polos, por exemplo, de 36 espécies de peixes estudados no Mar

do Norte, 15 mudaram seus centros de distribuição latitudinal nos últimos 25 anos, das quais 87% migraram para latitudes mais altas do hemisfério norte (Perry et al., 2005). Em outro estudo, de quase 900 espécies entre animais e plantas, constatou-se que 434 ampliaram seus territórios em um período médio de 66 anos, sendo que 80% mudaram sua distribuição como esperado em resposta ao aquecimento global. (Pörtner; Knust, 2007; Sunday et al., 2011).

Contudo, o deslocamento geográfico não é a única resposta possível frente as mudanças climáticas. Independente dos mecanismos envolvidos, em teoria as espécies podem mudar, e como proposto por Bellard e colaboradores (2012) essas mudanças podem se dar em três eixos distintos, mas não exclusivos. O primeiro eixo seria o espacial, o qual implica na capacidade de dispersão como também na mudança de hábitat a níveis locais ou de micro-habitat. Essa é a resposta mais bem documentada, desde registros paleontológicos até os atuais, sendo que mudanças na amplitude latitudinal e altitudinal já foram observadas em mais de 1000 espécies (Parmesan, 2006).

O segundo eixo é o de ajuste temporal, em que os indivíduos mudam seus traços de história de vida no tempo, isto é, ajustam eventos do ciclo de vida como migração, florescimento e amadurecimento, em escalas diárias ou sazonais para tentar acompanhar as mudanças dos fatores abióticos, como a temperatura em um período diário ou anual. O que temos observado é uma mudança nos eventos fenológicos em resposta às mudanças climáticas, porém mudanças temporais também podem ocorrer em uma pequena escala temporal, por exemplo, com comportamentos ou padrões de atividade ajustados nos ritmos de atividade diária para compensar os custos energéticos de uma condição climática diferente (Bellard et al., 2012).

O terceiro eixo, por sua vez, corresponde às mudanças na própria história de vida para tentar lidar com as novas condições em sua distribuição local, ao invés de recorrer às mudanças ideais no espaço e tempo. As espécies podem lançar mão desse terceiro eixo por meio de alterações fisiológicas que permitam, por exemplo, tolerância ao aquecimento ou ambientes secos, ou então através de mudanças comportamentais de sua dieta, atividade ou desprendimento energético.

Apesar de serem menos evidente algumas respostas fisiológicas já foram relatadas durante as mudanças climáticas do último século, especialmente de vários ectotérmicos, os quais tem sua locomoção, crescimento, reprodução e determinação sexual sensíveis à temperatura (Tewksbury et al. 2008; Johansen; Jones 2011). Entretanto, para muitos parâmetros, as respostas fenotípicas plásticas devem atingir um limite fisiológico e saturar

em ambientes extremos. Por exemplo, o tamanho do corpo ou a taxa metabólica não podem aumentar ou diminuir indefinidamente sob mudanças ambientais sustentadas (Chevin et al. 2010). Nesse caso, uma seleção forte é necessária para lidar com as mudanças climáticas (Bellard et al., 2012).

Assim, a grande questão sobre os efeitos das mudanças climáticas é se as espécies serão capazes de se adaptar rápido o suficiente para acompanhar o ritmo acelerado das mudanças no clima global. Qualquer que seja o tipo de respostas adaptativas, os mecanismos envolvidos são devidos à microevolução, ou seja, as espécies podem se adaptar geneticamente a novas condições por meio de mutações ou seleção de genótipos existentes (Salamin et al. 2010). Ou usar a plasticidade fenotípica (produto da seleção natural) que oferece uma gama de respostas de curto prazo dentro uma mesma geração de indivíduos para promover a sobrevivência ou tolerância às mudanças ambientais (Charmantier et al. 2008).

Inclusive algumas evidências sugerem que a contribuição da plasticidade é comumente mais importante do que a genética (Hoffman; Sgro 2011). Portanto, a plasticidade fenotípica é muito relevante, pois se configura como a habilidade de um único genótipo de expressar formas alternativas de morfologia, estado fisiológico e/ou comportamento frente diferentes condições ambientais, podendo ser reversíveis ou irreversíveis. (Westeberhard, 1989; Botkin et al. 2007; Chevin et al. 2010).
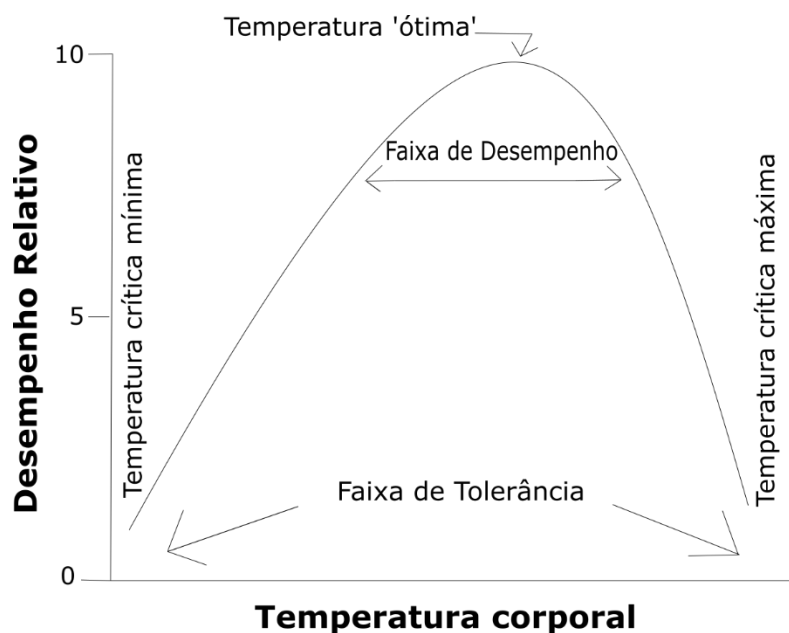
1.2. Os ectotérmicos frente às mudanças climáticas

A temperatura corporal (Tc) é talvez a mais importante variável ecofisiológica que afeta o desempenho de organismos ectotérmicos (Angilletta, et al., 2002), uma vez que dependem primariamente de fontes externas de calor e de ajustes comportamentais para a regulação da Tc. Isso implica em taxas metabólicas consideravelmente menores, comparada à de animais endotérmicos, e um sistema cardiorrespiratório com menor capacidade para transporte de grandes quantidades dos gases respiratórios, necessários para manter um metabolismo elevado (Gatten et al., 1992; Pough et al., 2001).

Dessa forma, o modo de termorregulação dos ectotérmicos pode restringir sua capacidade de dispersão espacial e/ou temporal, limitando-os a nichos térmicos adequados (Bovo et al., 2020). Além desses aspectos levantados, os organismos ectotérmicos que habitam regiões tropicais (menor latitude) são atualmente descritos como mais vulneráveis aos impactos das mudanças climáticas, uma vez que vivem em ambientes mais homogêneos e estariam sujeitos a menores flutuações da temperatura

anual, o que os situariam mais próximos de seus limites térmicos (Tewksbury et al. 2008; Simon et al., 2015; Madeira et al., 2016). Além disso, as espécies de zonas tropicais não só seriam mais sensíveis aos aumentos de temperatura, como também apresentariam limitada plasticidade fenotípica de tolerância térmica (aclimatização térmica) (van Berkum, 1988; Addo-Bediako et al., 2000; Hoffmann et al., 2003; Gilman et al., 2006; Ghalambor et al., 2006).

Em ectotérmicos, grande parte dos processos fisiológicos varia rapidamente de acordo com a Tc, definindo curvas de desempenho térmico (Huey; Stevenson, 1979). Esta curva de sensibilidade térmica apresenta uma faixa de temperatura ótima na qual o desempenho do organismo é máximo, e reduz à medida que a temperatura cai até um mínimo crítico, ou então aumenta até atingir a temperatura ótima e declina rapidamente até um máximo crítico. Assim, os limites térmicos críticos estabelecem a faixa de tolerância térmica (Fig.1) de um organismo (Katzenberger et al., 2012).

Figura 1. O esquema representa uma curva de desempenho hipotética de um ectotérmico em função da temperatura corporal. Os limites térmicos entre as temperaturas críticas mínima e máxima estipula a faixa de tolerância, fora desse intervalo o desempenho do indivíduo se torna comprometido. Também está evidenciada a temperatura ótima, na qual o desempenho (e.g. taxa metabólica) é máximo. Adaptado de Huey e Stevenson, 1979.

No entanto, as curvas de desempenho das taxas fisiológicas não são fixas e podem mudar em resposta às mudanças nas condições ambientais, independentemente

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das mudanças genotípicas (Fry, 1958; Bonduriansk; Day, 2009). Assim, determinar como as curvas de desempenho térmico podem variar nos indivíduos frente aos sinais ambientais é fundamental para compreender o efeito da variabilidade climática nas populações (Chevin; Lande, 2009; Seebacher; Grigaltchik, 2014).

Nesse sentido, a hipótese de tolerância térmica limitada pela capacidade de oxigênio (OCLTT, sigla do inglês para Oxygen and Capacity-Limited Thermal Tolerance) sugere que as capacidades bioquímicas e fisiológicas de ectotérmicos aquáticos evoluíram de tal modo que o escopo aeróbio (a diferença entre a taxa metabólica basal e máxima) é potencializado dentro de uma determinada faixa de temperatura (Temperatura ótima) a fim de otimizar o desempenho relacionado à aptidão, como crescimento, reprodução e locomoção (Pörtner, 2001, Claireaux; Lefrançois, 2007; Pörtner; Farrell, 2008). Enquanto o desempenho, por sua vez, diminuiria à medida que o escopo aeróbio diminui em temperaturas mais altas e mais baixas (Clarck et al., 2013).

Assim, uma elevação da temperatura para além do ótimo fisiológico pode comprometer a integridade de vários processos biológicos, como metabolismo, crescimento, alimentação, mecanismos bioquímicos e até a integridade de estruturas moleculares que ativam resposta de choques térmicos (Pörtner et al., 2006; Pörtner, 2010). Por exemplo, Pörtner e Knust (2007) mostraram que a redução na capacidade cardíaca em elevadas temperaturas levava a uma redução do escopo aeróbio e um aumento na mortalidade de peixes da espécie *Zoarces viviparus*. Os autores atribuíram a diminuição do escopo aeróbio com a redução da abundância desta espécie em anos de altas temperaturas.

Entretanto, a generalidade dessa teoria vem sendo questionada (Clark et al., 2013, Norin; Clark, 2016), e dados sugerem que essa Temperatura ótima diz pouco sobre a temperatura de preferência ou desempenho dos ectotérmicos aquáticos, uma vez que o escopo aeróbio pode continuar a subir até próximo de temperaturas letais, diminuindo rapidamente conforme a morte ocorre (Clark et al., 2011; Healy; Schulte, 2012; Clark et al., 2013). Assim, não é regra que a Temperatura ótima irá coincidir com o escopo aeróbio máximo, podendo estar muitas vezes abaixo desse ponto na curva de desempenho térmico (Clark et al., 2013).

Dentre os vertebrados ectotérmicos, o grupo dos anfíbios desperta um enorme interesse para estudos de biologia térmica, devido às suas estratégias complexas de termorregulação que não podem ser dissociadas de um balanço adequado de água. Além disso, observamos uma elevada diversidade de espécies e modos de vidas nesse grupo,

que vem sendo descrito como um dos mais vulneráveis frente aos cenários extremos previstos em decorrência das mudanças climáticas, devido a sua limitada capacidade de dispersão e dependência da água para completa conclusão de seu ciclo de vida (Lawler et al., 2010).

Pouco se sabe sobre a fisiologia de espécies nativas de anfíbios, também desconhecemos quais os efeitos da temperatura a curto e longo prazo sobre os mecanismos fisiológicos da fase larval dessas espécies, especialmente em relação às respostas cardiorrespiratórias. Os sistemas respiratório e cardiovascular funcionam sincronicamente na manutenção do transporte de oxigênio (O_2) que se dá pela combinação de meios convectivos e difusivos, e que podem ser alterados de maneira a atender a demanda aeróbica metabólica de cada organismo (Storz et al., 2010). Assim, em organismos ectotérmicos, a taxa metabólica é consideravelmente afetada pela temperatura (Bícego et al., 2007), e em anfíbios, efeitos agudos da temperatura resultam em aumento exponencial da taxa metabólica e outros parâmetros fisiológicos como a frequência cardíaca (f_C) e respiratória (Rocha; Branco, 1998; Bícego-Nahas; Branco, 1999; Zena et al., 2016; Longhini et al., 2017).

Em anfíbios anuros, a taxa metabólica é dependente da temperatura, geralmente dobrando ou triplicando com o aquecimento, ou seja, seguindo um Q_{10} (coeficiente térmico) igual a 2,0-3,0 (Overgaard et al., 2012; Rocha; Branco, 1998). Durante aumentos agudos de temperatura, a f_C em anfíbios aumenta cerca de 2 vezes a cada 10°C de aumento da temperatura, juntamente com a taxa metabólica (Q_{10} = 2,0) (Hedrick et al., 1999; Overgaard et al., 2012; Seebacher; Franklin, 2011; Zena et al., 2015; Zena et al., 2016a). Entretanto, a longo prazo (e.g. aclimatação), as espécies apresentam considerável capacidade de aclimatação fisiológica (e.g. plasticidade fisiológica reversível), permitindo a estas compensar os efeitos térmicos diretos sobre a taxa metabólica e, consequentemente, a f_C. Isto é, uma determinada taxa fisiológica pode permanecer relativamente constante, apesar da variação de temperatura. Quanto mais próximo de 1 for o Q_{10} para um parâmetro fisiológico, maior a resiliência deste organismo às mudanças de temperatura (Seebacher et al., 2015).

Em condições de repouso, o coração dos anfíbios opera sob um balanço da atividade simpática (tônus adrenérgico), atividade parassimpática (tônus vagal inibitório) e atividade intrínseca determinada pela despolarização das células marca-passo, além da ação das catecolaminas circulantes (Andersen et al., 2001; Zena et al., 2015; Zena et al.,

2016). Neste contexto, o balanço autonômico simpático e parassimpático em anfíbios, como consequência da aclimatação térmica (crônica), ainda permanece inexplorado.

Um tipo de controle cardiovascular foi verificado em duas espécies de peixes de águas temperadas: a perca (*Perca fluviatilis*) e a truta arco-íris (*Oncorhynchus mykiss*) (Ekström et al., 2016; Sandblom et al., 2016). Devido à influência da poluição térmica advinda de efluentes despejados por uma usina nuclear no Mar Báltico, a f_C intrínseca de uma população de percas cronicamente aclimatadas à temperatura de 22°C é reduzida e o tônus autonômico vagal é elevado quando comparados a populações de percas aclimatadas à temperatura de 16°C, e que vivem em uma área de referências sem a influência da poluição térmica advinda da usina nuclear (Sandblom et al., 2016). Um elevado tônus vagal também foi observado na truta arco-íris, uma vez que o efeito da temperatura elevada (16°C; aclimatação) após 39 dias foi capaz de induzir significativa plasticidade do controle autonômico sobre o coração, reduzindo a f_C para valores próximos ao de repouso a 9°C ($Q_{10} = 1,2$), principalmente devido a uma aumentada inibição colinérgica (Ekström et al., 2016).

1.3. Anfíbios do Cerrado

O Cerrado cobre 22% do território brasileiro e é considerado a maior região de savana tropical da América do Sul, e um dos 35 *hotspots* de biodiversidade do planeta (Mittermeier et al., 2004). Esse título é em decorrência do bioma ser extremamente rico em número de espécies, com aproximadamente 12.000 espécies de plantas, cerca de um terço delas endêmicas, e 2.373 espécies de vertebrados registradas, sendo quase um quinto delas endêmicas (CEPF, 2017).

Em relação aos anfíbios são conhecidas 204 espécies, dessas 72 são endêmicas deste bioma (CEPF, 2017). A fauna de anuros no Cerrado, por sua vez, está interligada com a heterogeneidade ambiental, formada por uma variedade de hábitats horizontalmente contrastantes no espaço, como áreas florestais, abertas, com diferentes tipos de vegetação, úmidas e secas que ofertam elevado número de recursos para as espécies. Isto favorece a ocorrência tanto de espécies especialistas como generalistas, mantendo uma alta diversidade (Colli et al., 2002; Bridgewater et al., 2004; Gambale et al., 2014).

Entretanto, nas últimas décadas mais da metade de sua área foi degradada em função da criação de pastagens, plantações de grãos e outros tipos de uso (Klink; Machado, 2005). Como consequência, as taxas de desmatamento anuais no Cerrado são

mais elevadas do que na Amazônia, esses índices tornam-se mais alarmantes se consideramos que apenas 8% de sua área está legalmente protegida (CEPF, 2017).

Dessa forma, pesquisas no cerrado são extremamente necessárias, não só para conhecermos a diversidade de espécies endêmicas, mas para traçar medidas de conservação, pois a intensa ação antrópica, ocasionada principalmente por expansões agrícolas, tem modificado hábitats naturais e ecossistemas, levando muitas espécies a serem extintas (Klink; Machado, 2005; Pombal; Gordo, 2004; DinizFilho et al., 2009; Gambale et al., 2014).

Além disso, de acordo com o Painel Brasileiro de Mudanças Climáticas, as principais tendências identificadas para o Cerrado indicam: (a) aumento de 1°C na temperatura do ar, com uma diminuição de 10% a 20% na precipitação ao longo das próximas três décadas (até 2040); (b) aumento entre 3 e 3,5ºC na temperatura do ar e redução entre 20% e 35% da precipitação, até meados do século (2041-2070); e (c) aumento da temperatura entre 5 e 5.5ºC e desaceleração mais crítica na precipitação, com reduções entre 35% e 45% para o final do século (2071-2100).

Assim, se o período seco se tornar mais longo, a menor cobertura de nuvens elevaria ainda mais as temperaturas no verão, que é o período chuvoso (CEPF, 2017). Tais modificações de habitat são uma ameaça direta à vida dos organismos, especialmente aos anfíbios anuros, pois muitas espécies são extremamente dependentes de determinadas condições climáticas e disponibilidade de ambientes aquáticos para a reprodução (Haddad; Prado, 2005).

A maioria dos anuros se reproduzem através de fertilização externa e apresenta um desenvolvimento indireto com um estágio larval aquático e um adulto terrestre (Duellman; Trueb, 1994). A metamorfose dos girinos, além de ser um processo de reorganização sistêmica, pode ser influenciada por uma série de fatores como nível de água (Crump, 1989; Székely, Cogălniceanu; Tudor, 2010), presença de predadores (Laurila; Kujasalo, 1999; Richardson, 2001), disponibilidade de alimento (Kupferberg, 1997, Álvarez; Nicieza, 2002) e temperatura (Newman, 1992; Angilletta et al., 2004).

Os girinos são excelentes candidatos aos estudos de tolerância térmica por diversos motivos, uma vez que sua temperatura corporal é igual à do ambiente e por viverem em ambiente aquático não correm risco de desidratação. Contudo, podem enfrentar outros problemas com o aumento da temperatura, como redução do oxigênio dissolvido na água e estresse osmótico, fatores que podem interferir na tolerância térmica (Spotila et al., 1992; Gomez-Mestre; Tejedo, 2004; Re et al., 2006; Pörtner; Knust, 2007; Katzenberger

et al., 2012). Além disso, a fase larval é um período no qual o gasto energético é voltado para o crescimento e desenvolvimento e todas as características analisadas são independentes da condição reprodutiva (Katzenberger et al., 2012).

Desse modo, o nosso modelo de estudo foram os girinos de um anuro pertencente à família Hylidae, a *Bokermannohyla ibitiguara* (Cardoso, 1983). A espécie é endêmica do bioma Cerrado, ocorrendo no Parque Nacional Serra da Canastra e em fragmentos florestais ao redor do estado de Minas Gerais, sendo considerada totalmente dependente de áreas florestadas, uma vez que é fortemente associada a matas de galeria (Fig.2) durante todas as fases de sua vida (Cardoso 1983, Haddad et al. 1988, Nali; Prado 2012). Sua história natural e biologia pouco conhecida conferiram à espécie o status de Dados Deficientes na lista de espécies ameaçadas da IUCN (sigla em inglês para União Internacional para a Conservação da Natureza; Caramaschi; Eterovick, 2004).

Figura 2. Riacho associado a mata de galeria na zona rural de Sacramento (MG), onde os girinos de *B. ibitiguara* foram coletados.

2. Objetivos

Em vista das evidências expostas acima, o objetivo geral do trabalho foi avaliar se girinos de *B. ibitiguara* apresentam compensação térmica quando aclimatados em temperatura elevada (18 *vs* 25°C), bem como investigar os mecanismos fisiológicos

envolvidos na tolerância térmica. Especificamente os principais objetivos da dissertação são:

- Investigar as variações de temperatura dos micro-habitats larvais de *Bokermannohyla ibitiguara;*

- Determinar a Temperatura Máxima Crítica (CTmax) nos diferentes grupos de aclimatação (18 e 25°C), por meio de rampa de aquecimento (0,1°C.min^{-1});

- Avaliar o efeito da aclimatação térmica (mínimo de 3 semanas) (18 e 25°C) sobre os parâmetros cardiovasculares como a frequência cardíaca (f_C) basal e o tônus autonômico cardíaco em girinos pré-metamórficos de *B. ibitiguara;*

- Avaliar os efeitos de diferentes temperaturas (resposta aguda: 15, 20, 25, 30 e 34°C) na taxa metabólica de rotina e máxima nos diferentes grupos de aclimatação (18 e 25°C) de girinos pré-metamórficos de *B. ibitiguara.*

O artigo a seguir apresenta mais detalhes sobre a pesquisa desenvolvida, as hipóteses, e os métodos e análises utilizados. O manuscrito foi redigido nas normas da revista *Journal of Experimental Biology* (https://jeb.biologists.org/), e se encontra em processo de revisão pelos autores para submissão.

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Effects of thermal acclimation on aerobic metabolism, cardiovascular parameters and thermal tolerance in tadpoles of *Bokermannohyla ibitiguara*

In prep to: Journal of Experimental Biology

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Abstract

Thermal acclimation is suggested to provide a reversible physiological adjustment in response to environmental changes, conferring resilience in a climate change scenario. Since amphibians may be more vulnerable to habitat modification because of their life cycle dependence on both land and water, it is critical to identify species' ability to acclimate in warming climates. Here, we investigate the effects of temperature acclimation on tadpoles of a stream-breeding savanna tree frog (*Bokermannohyla ibitiguara*) and the relationship between the thermal conditions experienced in their microhabitat. We quantified variation in thermal physiology by measuring routine and maximum metabolic rate (RMR and MMR, respectively) at different test (15, 20, 25, 30 e 34°C) and acclimation temperatures (18 and 25°C). In addition, we measured body characteristics of the two groups and the heart rate (f_H) before and after autonomic blockade with atropine and sotalol. Further, we determined the critical thermal maximum (CT_{max}) and warming tolerance ($WT=CT_{max}-T_{max}$), which were not affected by acclimation. The routine and maximum metabolic rate and f_H increased with acute exposure to high test temperatures, although the acclimation did not affect significantly the maximum metabolic rate, f_H or the autonomic tones. In addition to this absence of physiological compensation, we observed a decrease in aerobic scope in the group acclimated to 25 °C, followed by a 53.8% reduction in body mass in the tadpoles of this group. In conclusion, the tadpoles of *B. ibitiguara* inhabit in a thermally stable environment and are not able to thermally acclimate to high temperatures like 5°C above those found in their micro-habitat, making the species more vulnerable to climate changes or/and with degradation of their habitat.

Keywords: Oxygen consumption; CT$_{max}$; heart rate; autonomic tonus, climate change.

1. Introduction

It is undeniable that climate change is happening. Predictions of extreme events and elevated temperature peaks are already taking place (Schär et al., 2004; Diffenbaugh and Ashfaq, 2010; Clusella-Trullas and Chown, 2013). The past decade presented an astonishing run of record-breaking storms, forest fires, droughts, heat waves, and floods around the world with global average temperature warmed just 1.0°C beyond preindustrial levels (IPCC, 2018). Global warming also has a remarkable effect on the behavior, distribution and physiology of many animal species (Parmesan and Yohe, 2003; Parmesan, 2006; Chen et al., 2009; Charmantier et al., 2008; Foden et al., 2013; Settele et al., 2014, Pacifici et al., 2017; Seebacher et al., 2015; Sandblom et al., 2016).

Since ectotherms depend primarily on external heat sources, temperature imposes significant constraints on their behavior and physiology (Bícego et al., 2007). Notably, many ectotherms can remodel their physiology to minimize the extent to which physiological rates change in response to variations in ambient temperature (whether chronic, recurrent or extemporaneous). This flexibility can occur by thermal compensation through the process of thermal acclimation (response to a single environmental variable under experimental conditions) or by acclimatization (in response to several environmental variables under field conditions) (Pough et al., 1992; Rome et al., 1992; Seebacher et al., 2015). Thermal acclimation provides a reversible physiological adjustment in response to environmental conditions and offers an energetic advantage over individuals that experience a time lag in physiological response (Angilletta, 2009). Allowing an ectotherm to acclimate in different thermal conditions may generate more realistic interpretations of presumable climate warming effects on physiological parameters. One of the possible responses is thermal compensation in which a physiological rate remains relatively constant despite variations in ambient temperature (Seebacher et al., 2015).

Also ectotherms inhabiting tropical regions are currently described as more vulnerable to the impacts of rises of average temperature, as these species experience smaller annual temperature fluctuations, which would bring them closer to the thermal tolerances (e.g., difference between critical thermal minimum [CT_{min}] and maximum [CT_{max}] temperatures) (Somero and DeVries, 1967; Ghalambor et al., 2006; Deutsch et al. 2008; Nilsson et al., 2009; Huey et al., 2012; Simon et al., 2015). Nevertheless, there is a lack of a comprehensive analysis of the capacity for physiological plasticity across taxonomic groups and geographic regions, which precludes generalizations regarding

thermal plasticity based on species' geographical distributions. Therefore, to make predictions of the impacts of climate changes on ectotherms species is important to study the interactions between physiology and temperature.

The respiratory and cardiovascular systems are tightly coupled to maintain adequate oxygen delivery to metabolically active tissues, and cardio-respiratory adjustments are always required whenever metabolic demands change, such as during environmental temperature changes. Thermal acclimation of metabolic rate and cardiorespiratory functions seems to be crucial for many ectotherms favoring those plastic phenotypes by conferring resilience against predictable (e.g. seasons) and unpredictable changes in ambient temperature (Seebacher et al., 2015; Sandblom et al., 2016a).

Current forecasts have shown amphibians vulnerability regarding future climate change events, mainly regarding their limited dispersibility and water dependence for completing their life cycle (Lawler et al., 2010). In most anuran amphibians, the metabolic rate is temperature-dependent, rising exponentially when acutely increased from 10 to 35°C, with a thermal coefficient (Q_{10}) of 2.0 (Rocha and Branco, 1998; Overgaard et al., 2012). During acute temperature increases, heart rate (f_H) also increase about 2 times with each 10°C increase in temperature, thereby following the same trend as metabolic rate (Hedrick et al., 1999; Bícego-Nahas and Branco, 1999; Overgaard et al., 2012; Seebacher and Franklin, 2011; Zena et al., 2015; Zena et al., 2016). However, in a long term, species may exhibit considerable physiological acclimation capacity (e.g. reversible physiological plasticity), allowing them to compensate for the direct thermal effects on the cardiovascular system and consequently in aerobic metabolism (Seebacher et al., 2015).

Considering that Brazil holds the most diverse amphibian fauna of the world (Segalla et. al., 2014), with the Brazilian Cerrado recognized as one of the world hotspots of biodiversity, with a high endemism of amphibian species (CEPF, 2017), we aimed to study the tadpoles of an endemic anuran amphibian from the savanna-like Cerrado, the *Bokermannohyla ibitiguara* (Cardoso, 1983). This species inhabits preserved areas of the Serra da Canastra National Park (20°15'29''S, 46°24'55''W and 20°109'S, 46°309'W, 900–1,496 m), in addition to some modified forest fragments in the state of Minas Gerais. This tree frog is strongly associated with gallery forests during all phases of its life cycle (Cardoso 1983, Haddad et al. 1988, Nali and Prado 2012; Nali et al., 2020).

The aquatic larval amphibians are an ideal model to analyze thermal influences on physiological adaptations, since their relatively small size, together with the high heat capacity and thermal diffusivity of water, makes them isothermal with the environment (Lutterschmidt and Hutchison, 1997). Besides that, *B. ibitiguara* is included by the International Union for Conservation of Nature (IUCN) as data deficient and nothing is known about its vulnerability to environmental changes such as temperature and their relationship with the microhabitat. Thus, our aim was to investigate the interacting effects of thermal acclimation (as a form of phenotypic plasticity) and rate of temperature change on thermal tolerance and physiological mechanisms on tadpoles of *B. ibitiguara* acclimated in different temperatures *(*18 vs 25°C*)*. For this we determined the maximum critical temperature (CT_{max}) during acute gradual temperature increases and calculated the warming tolerance (WT, the difference between CT_{max} and maximum temperature of micro-habitat). We also evaluated the aerobic scope by measuring routine and maximum metabolic rate at different test temperature. Additionally, the body characteristics of tadpoles of both acclimation groups were valued, and f_H was measured before (e.g., routine f_H) and after autonomic blockade performed with a muscarinic antagonist (atropine) and a β-adrenergic antagonist (sotalol), in order to quantified the relative cardiac autonomic tone at both acclimation temperatures. We hypothesized that the existence of thermal compensation in tadpoles of *B. ibitiguara* would result in higher thermal tolerance for tadpoles acclimated to elevated (25°C) relative to lower temperature (18°C). Considering that acclimation results in compensation of resting f_H in fish (Ekström et al., 2016; Sandblom et al., 2016b), we also hypothesized that chronic exposure of tadpoles to high temperatures would result in cholinergic inhibition and/or reductions of the intrinsic heart rate.

2. Material and methods

2.1. Animal collection and maintenance

Premetamorphic tadpoles (between 26-30 stage according to Gosner, 1960; see table 1 for biometrics) of *B. ibitiguara* ((Hylidae, Cardoso, 1983) were collected in one permanent stream (Fig. 1) located at the rural area of Sacramento (20º16'21,9"S; 47º04'24,5"W; 677m elevation), Minas Gerais State, Southeastern Brazil. By using an aquarium fishing net, we collected tadpoles during both day and nighttime on February, April and December of 2019. Animals were transported in plastic bags to the our laboratory at the Department of Animal Morphology and Physiology, UNESP,

Jaboticabal, Brazil (approximately 21°14′S and 48°17′W) where they were maintained in glass aquariums (90 L) under natural photoperiod.

After two days of habituation to the new environment, tadpoles were divided between two acclimation groups, 18 and 25°C (hereafter G18 and G25, respectively). Both acclimation temperatures were chosen based on previous water temperature records reported for the same stream environment (Renato Nali, personal communication). For acclimating tadpoles to 18 ± 0.01°C, a stainless-steel coil was positioned inside the aquarium and connected by plastic tubes to an external circulation bath (PolyScience 9112A11B Programmable, Model 9112 Refrigerated Circulator). For acclimating tadpoles to 25 ± 0.02°C, we used a heater controlled by a thermostat (Roxin Ht-1300, 100w) maintained inside the aquarium. Each acclimation temperature was achieved by increasing or decreasing water temperatures by 2°C per day until it reached the desired temperature. All individuals were acclimated at their final treatment temperatures for at least 3 weeks (which is considered the typical acclimation time for small aquatic organism (Barrioneuvo and Fernandes, 1998). Animals were fed daily with an herbivore fish food (Maramar, maxi green, 75% vegetable origin). To ensure good water quality, an external filtration system (mechanical, chemical and biological filtration (model HF-0400, Atman, Santo André, São Paulo, Brazil) was used in each aquarium along an external air pump to maintain water oxygen saturation. Furthermore, twice a week 20-30% of the aquarium water was removed with animal waste (via a siphon) and replaced with clean water from an artesian well. Animal collection was approved by the Brazilian environmental agency (SISBIO-ICMBio, #621361), and all experimental protocols were approved by the Animal Care and Use Committee of São Paulo State University (CEUA-FCAV; #02205/18).

2.2. Microhabitat temperature

The stream temperature where tadpoles were collected was recorded for every field trip (four in total: February, April and December of 2019 and July of 2020) at three different site points along the stream extension. For this, we used a portable dissolved oxygen and temperature polarographic meter (YSI, Model 550A). Additionally, a temperature logger (iButton; Maxim Integrated, San Jose, CA, USA), previously coated in a biologically inert wax mixture (20% Elvax®; DuPont, NC, USA; 80% histological paraffin wax), was positioned in the water close to the bottom of the stream where the

tadpoles were found in order to record water temperature fluctuations every hour during a whole year.

2.3. Body characteristics

After chronically acclimated for at least 3 weeks, the tadpoles of each acclimation group (G18 and G.25) were individually weighed and measured (metric pachymeter) to obtain the average body mass, total body length (TL, beginning of the head to the end of the tail), partial length (PL, beginning of the head to the insertion of the tail) and body width (BW).

2.4. Upper thermal limits

Critical thermal maximum (CT_{max}), defined as the thermal point at which activity becomes disorganized and the animal loses its ability to escape from conditions that lead to death (Cowles and Bogert, 1944), was determined using the dynamic method previously performed in tadpoles (Lutterschmidt and Hutchinson, 1997; Duarte et al., 2012; Kern et al., 2015; Agudelo-Cantero and Navas, 2019). The experiment started at the acclimation temperature of each group, then animals were exposed to a constant heating rate of 0.1°C min^{-1} inside a waterbath, until we observed immobility after five consecutive taps on tale by using a glass stick (Badr et al., 2016; Simon et al., 2015; Moyano et al., 2017; Agudelo-Cantero and Navas, 2019). The ramp increases in temperature experienced by the tadpoles were continuously measured by using a temperature sensor (MLT415/M Thermistor temperature sensor, ADInstruments®, Sydney, Australia). Once an individual reached its CT_{max}, we quickly transferred it into a plastic container with water at ~25°C to allow recovery. Only animals that survived after 24 hours were included in the analysis, of the 16 animals tested, only 1 died within 24 hours.

We also estimated the warming tolerance (WT), which provides a measure of the relative amount of warming that each species can withstand before reaching critical performance levels (Deutsch et al., 2008). This metric was calculated as the difference between the organism's critical thermal maximum and the microhabitat maximum environmental temperatures (CT_{max} – Tmax) (Duarte et al., 2012). We considered Tmax as the average of maximum temperatures recorded at the stream during the months we collected the tadpoles.

2.5. Measuring oxygen consuption in tadpoles

Oxygen consumption (metabolic rate = $\dot{M}O_2$) was measured in resting tadpoles and after forced activity at five test temperatures (15, 20, 25, 30 and 34°C) by using intermittent respirometry (Steffensen, 1989; Clarck et al., 2013; Svendsen et al., 2016; Rosewarne et al., 2016). Each animal was placed in a cylindrical acrylic respirometer (total volume of 43 mL) submerged in an experimental tank filled with aerated water. Through a hole in the upper part of the respirometer, we placed an oxygen sensor (PSt3, PreSens, Regensburg, Germany), and signal was continuously monitored by a software (FIBOX3, PreSens, Germany). Inside the experimental box were also an aerator to ensure adequate oxygenation; one submerged circulation aquarium mini-pump (mini pump A, Sarlobetter, Brazil) that flushed water in the respirometer chamber, and one circulation pump (ECEEN, 43GPH) to ensure proper water mixing when the respirometer was closed for measurements of oxygen consumption by the animal. Adjustments and maintenance of each test temperature were made through a serpentine connected to an external circulation bath (PolyScience 9112A11B Programmable, Model 9112 Refrigerated Circulator). Oxygen probe was calibrated daily at the test temperatures using 100% (aerated distilled water) and 0% (100 ml distilled water with 1 g dissolved Na_2SO_3) air saturated water.

Tadpoles were placed into the respirometer for habituation at the first test temperature (15°C) for at least one hour, which is sufficiently long for recovery from handling stress (Kern et al., 2014; Seebacher and Grigaltchik, 2014; Longhini et al., 2017). After one hour, the respirometer was sealed and oxygen concentration determined twice at each test temperature (15, 20, 25, 30 e 34°C), always ensuring that oxygen levels were not falling below 80% air saturation.

At the end of the experimental protocol for resting measurements of $\dot{M}O_2$, animals were removed from the respirometer and transferred to plastic containers with cool water at ~25°C. Tadpoles were gently dried and weighed with a precision scale (0.01g, Model LW 303i, Bel Engineering, Italy). Since it was not possible to keep the tadpoles immobile during respirometry trials, $\dot{M}O_2$ measurements represent routine metabolic rates (RMR) of oxygen consumption, indicating the rate of oxygen consumed during low levels of voluntary activity (Seebacher and Grigaltchik, 2014).

For measuring maximum metabolic rate (MMR), we used the manual chasing method immediately before tadpoles being introduced into the respirometer (Clarck et al.,

2013). This method was chosen because *B. ibitiguara* tadpoles are bottom dwellers, found mostly resting on rocky or silty substrates (Leite and Eterovick, 2010), under or above submerged leaves in the stream. This method makes it possible to achieve maximum $\dot{M}O_2$ levels due to excessive post-exercise oxygen consumption (Reidy et al., 1995). Thus, the animals were placed in a 500 mL beaker placed in the same experimental box used for measurements of RMR, and with a glass stick, we chased the individual for 5 minutes continuously or until exhaustion (no response after 5 consecutive taps on the tail). After chasing protocol, tadpoles were immediately placed into the respirometer that was sealed for measurement of maximum $\dot{M}O_2$. Tadpoles were exposed to the same test temperature (14, 20, 25, 30 and 34°C) randomly for both acclimation groups. The respirometry system (acrylic chamber, tubes and pumps) was cleaned daily at the end of each experimental protocol by using chlorine to avoid any microbial/algae growth. The background oxygen consumption was low and never exceeded 5% of tadpole's consumption.

The $\dot{M}O_2$ (µmol.g.h^{-1}) during each measurement phase was derived from the slope (K) of the linear regression of oxygen content (kPa) over time (h) according to the equation:

$$y = V_{RE} W_o^{-1} \frac{dCO_2}{d\tau}$$

where *y* is $\dot{M}O_2$, V$_{RE}$ is the effective respirometer volume of water in the respirometer, calculated as the total respirometer volume minus the organism volume, W$_o$ is the organism mass (we assumed a density of 1 kg.L^{-1}) and $dCO_2/d\tau$ is the slope of the linear decrease in oxygen content during the time the chamber was sealed (Svendsen et al., 2016). For final $\dot{M}O_2$ calculations we only considered slopes with r^2 ≥0.95.

2.6. Drugs

For autonomic blockade we utilized drugs and doses previously used in the larval stage and adults of anuran amphibians (Zena et al., 2016; Longhini et al, 2017). Atropine (cholinergic muscarinic antagonist; 3.0 mg kg^{-1}) and sotalol (β-adrenergic antagonist; 3.0 mg kg^{-1}) were purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in

amphibian Ringer solution (composition in mmol l^{-1}: 46.9 NaCl; 21.0 KCl; 2.40 CaCl; 1.29 MgCl; 3.14 NaHCO$_3$; according to Zena et al., 2016; Longhini et al, 2017).

2.7. Autonomic control of heart rate (f_H) in tadpoles

Heart rate (f_H) was measured by a non-invasive methodology as previously described (Longhini et al., 2017). Briefly, we coupled two parallel electrodes, made out of hypodermical needles (40x1.20mm, 18G), to a 20 mL plastic syringe that remained positioned into the experimental tank connected to a circulation pump to ensure adequate water oxygenation between the outside and the inside syringe. The electrodes were wired and connected to a signal amplifier (A-M Systems, model 1700, Sequim, WA, USA), allowing the collection of electrical signals from the tadpole's heart. Biological signals were recorded at a sampling rate of 1 kHz by an acquisition system (PowerLab System, ADINSTRUMENTS®, Sydney, Australia) and further analyzed offline (Chart Software, version 7.3, ADINSTRUMENTS®, Sydney, Australia) using the software's built-in filters (low-pass: 50 Hz) over the raw signals. The online signals were amplified (10.000x gain) and filtered (bandpass: 0.1-5 KHz). In addition, the water system was grounded to attenuate the noise using a ground wire connected to the amplifier.

The experimental protocol for the blockade of sympathetic and parasympathetic modulation on the heart was initiated after one hour of the tadpoles' habituation to the experimental apparatus, which was followed by recordings of baseline f_H measurements for an additional one hour. After baseline recordings, tadpoles were gently removed from the experimental apparatus (hereafter "syringe") and handled to receive intraperitoneal injection of atropine. An additional one hour was used for recording f_H after the muscarinic blockade. Subsequently, sotalol hydrochloride injection was performed in order to achieve a full autonomic blockade, and f_H was recorded for an additional hour. Intraperitoneal injections were performed using a dental needle (Mizzy, 200 μm outside diameter) connected by a polyethylene tube (PE-10, Clay Adams, Parsippany, NJ, USA) to a Hamilton syringe (5 μL). Injections were standardized so that the volume injected into the peritoneal cavity was 0.46 μL g^{-1}. The movement of an air bubble inside the PE-10 polyethylene tube that attached the micro-syringe to the dental needle confirmed the flow of the drugs.

Autonomic blockade protocol was performed twice with the same tadpoles, at first the experiment was conducted with each group (G18 e G25) in their respectively acclimation temperatures (18 and 25°C). After a total of 7 days, the groups were again

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subjected to autonomic blockade now for an acute exposure in the opposite temperature of acclimation. Thus, G18 was exposed for 1 hour to 25°C and selective autonomic blockade performed as previously described. Conversely, G25 was exposed for 1 hour to 18°C and selective autonomic blockade performed. At the end of experiments, tadpoles were euthanized by placing in a solution of benzocaine hydrochloride (250 mg L^{-1}) buffered to pH 7.7 with sodium bicarbonate (Longhini et al., 2017).

2.8. Statistical analysis

The f_H averages were obtained using the final 5 minutes of a stable record and calculated by using the LabChart software's signal detection tools (version 7.3, Sydney, Australia) as previously described (Longhini, et al., 2017). In addition, we calculated the cholinergic and adrenergic tones of the heart, based on the animals' cardiac intervals using the equations proposed by Altimiras et al. (1997), as previously described in Longhini et al. (2017). For comparing the thermal tolerances parameters (CT$_{max}$ and WT) and body characteristics of tadpoles between the two acclimation groups we used an unpaired t test.

To verify the effect of acclimation (18 vs. 25°C), test temperatures (18 vs.25°C), treatment effects (selective autonomic blockade), and their interaction on f_H (response variable), we fitted linear mixed models by using the package nlme (Pinheiro et al., 2018). Percentage change in autonomic tone (response variable) was also analyzed using linear mixed model with acclimation (18 vs. 25C), test temperatures (18 vs. 25C), autonomic tone (cholinergic vs. adrenergic) and their interaction included as fixed factors. We also fitted linear mixed models for studying the effect of acclimation (18 vs. 25°C), test temperatures (15, 20, 25, 30 and 34°C) and their interaction on RMR and MMR. In all cases, individual identification was included as random effects (intercept) to account for the repeatability of the data throughout the study. All statistical analysis were performed using R software (R Core Team, 2020). For all analyses, statistical significance was accepted when $P \leq 0.05$. Normality of the residuals were visually inspected by using histograms and boxplots. Homogeneity of variance for each model was visually inspected and tested by using a Levene's test. When necessary, appropriate data transformations were performed (log and square root transformation). Data are presented as mean ± s.e.m.

3. Results

3.1. Microhabitat predictors

Figure 1 presents data logger's recordings for seasonal temperature changes in the stream where tadpoles of *B. ibitiguara* inhabits (from April 24 of 2019 to July 25 of 2020). During the dry seasonal we observed that the flow of the stream ceases completely, leaving only a few permanent ponds with no connection, which explain highly daily temperature variation between August and November (see Figure 1), in which we confirmed during one field trip that the data logger was completely out of the water in one of the stream point that has drown out. Considering only the months when the stream was certainly full (December to April) according to field observations, Tmax (maximum temperature) was 23.6 ± 0.6; Tmin (minimum temperature) was 21.2 ± 0.7 and Tmean (mean temperature) was 22.4 ± 0.7.

For each field trip we also measured stream water temperature manually at the points where we collected tadpoles, either during daylight or nighttime: February 2019: 24.2°C (15h50); 23°C (16h00) and 22.6°C (8h30); April 2019: 22.5°C (19h30); 22.3°C (20h07) and 22.2°C (10h10); December 2019: 23°C (16h33); 22.7°C (19h48); and July 2020: 21.1°C (12h05); resulting in a Tmean of 22.1 ± 0.6. We also measured the dissolved oxygen in the same points of collection: 5.6 ± 1.7 mg/L (mean values for field trip on February 2019); 7.7 ± 0.1 mg/L (mean values for April 2019); 5.7 ± 0.6 mg/L (mean values for December 2019) and 4.6 ± 0.3 mg/L (mean values for July 2020).

3.2. Body characteristics of acclimation groups

The acclimation treatment significant affected (see Table 1) all morphological traits: body mass (G18: 1.3 ± 0.1 *vs*. G25: 0.7 ± 0.07 g; $t_{(17)=}4.76$; $P<0.001$); total length (G18: 54.7 ± 1.5 *vs*. G25: 46.7 ± 1.3 mm; $t_{(17)=}3.96$; $P<0.001$); partial length (G18: 17.9 ± 0.6 *vs*. G25: 14.8 ± 0.3 mm; $t_{(17)=}4.13$; $P<0.001$) and body width (G18: 9.9 ± 0.5 *vs*. G25: 7.6 ± 0.3 mm; $t_{(17)=}3.66$; $P<0.001$).

3.3. Thermal Tolerance

Tadpoles of *B. ibitiguara* of both acclimation groups exhibited similar CT_{max} (G18: 36.8 ± 0.2°C *vs*. G25: 36.7 ± 0.09°C; P=0.84; Fig. 2). The heating rate did not differ between the two groups (Slope G18: 0.0014 *vs* Slope G25: 0.0015; $F_{(1,107)} = 0.61$, $P=0.43$; see supplementary material, Fig. S1). Since Warm Tolerance is dependent on CT_{max}, the

WT was the same for both acclimated groups (18°C: $12.4 \pm 0.5°C$ *vs*. 25°C: $12.4 \pm 0.2°C$; P=0.83).

3.4. Effects of temperature on aerobic metabolism on tadpoles

Routine metabolic rate increased exponentially with temperature (from 15 to 34°C) in both acclimation groups (18 and 25°C), particularly at higher test temperatures (Test temperature effect: $F_{1,62}$= 302.5, $P < 0.0001$; Fig. 3A). Acclimation also affected significantly RMR ($F_{1,14}$= 68.2, $P < 0.0001$), and tadpoles acclimated to 25°C showed a higher RMR, which represents the interaction between acclimation and test temperature ($F_{1,62}$= 4.58, $P = 0.03$), therefore no thermal compensation occurs.

Maximum metabolic rate also increased with temperature and reached a maximum of 26.4 μmol g^{-1} h^{-1} at 33.7°C on G18, although acclimation was not significant ($F_{1,15}$= 0.044, P=0.83). But test temperature ($F_{1,54}$= 216.9, $P < 0.0001$) and the interaction between acclimation and test temperature ($F_{1,54}$= 7.1, $P < 0.0001$) significantly affected the MMR (Fig. 3B). Aerobic scope is presented as the absolute difference between mean values of RMR and MMR in the figure 4A and in factorial terms as the ratio of MMR to RMR (Fig. 4B). The absolute aerobic scope shows that G25 has a smaller amplitude with the peak moved to a lower temperature compared to G18. The factorial scope also demonstrates a reduced aerobic scope of the group acclimated to 25°C (Fig. 4B). We can visually note a reduced aerobic scope for G25 in relation to G18, in both absolute and factorial aerobic scope.

3.5. Temperature and selective autonomic blockade effects on f_H

Heart rate (f_H) at rest increased significantly with experimental temperature ranging from 51.8 ± 5.0 to 79.4 ± 6.7 beats.min^{-1} (Q_{10}=1.9) between 18 and 25°C, respectively for G18 (Fig. 5A). The same pattern was observed for G25, varying from 52.3 ± 3.1 to 81.3 ± 3.8 beats.min^{-1} (Q_{10}=1.9) between 18 and 25°C, respectively; (test temperature effect: ($F_{(1,62)}$=635.997; P<0.001; Fig. 5B).

Acclimation temperatures did not affect f_H responses to acute changes in temperature (Acclimation effect: $F_{(1,12)}$=0.014; P=0.90). Pharmacological treatment with atropine and sotalol significantly affected f_H. Atropine increased f_H, while the subsequent treatment with sotalol caused a slightly reduction in f_H (Treatment effect: $F_{(2,62)}$=33.372; P<0.001). While atropine and sotalol had similar effects on f_H in both test temperatures,

the amplitude of their effects were more pronounced at 25 compared to 18°C (interaction effect: ($F_{(2,62)}$=5.707; P=0.005).

As expected regarding the effect of atropine and double autonomic blockade on f_H, acclimation did not influence autonomic tone (acclimation effect: $F_{(1,11)}$=0.012; P=0.91). Although pharmacologic treatment with atropine resulted in a large amplitude change in f_H independent of acclimation at the higher temperature (25°C), most of this effect was due to increases in the intrinsic f_H, since autonomic tone did not differ between temperatures (Test temperature effect: $F_{(1,3)}$= 2.442; P=0.12). However, cholinergic tonus tends to be higher in the group acclimated to 18°C at 25°C (22.2±3.8%) in comparison with test temperature of 18°C (11.9 ± 4.5%). The other hand, the adrenergic tone remained relatively constant between the test temperatures of 18°C (G18: 9.8 ± 2.6% and G25: 9.2 ± 1.5%) and 25°C (G18: 11.5 ± 4.5% and G25: 13.1 ± 4.0%) in both acclimated groups.

4. Discussion

4.1. Thermal Tolerance of larval stage

The native *B. ibitiguara* tadpole from Cerrado did not have upper thermal tolerance modified by chronic acclimation at high temperature (18°C: 36.8 ± 0.2°C *vs*. 25°C: 36.7 ± 0.09°C). Although a general pattern has been suggested regarding CT_{max} increasing at high acclimation temperatures, some anuran amphibians show limited or absence of acclimation capacity (Rome et al., 1992; Bovo et al., 2020), which seems to be case for tadpoles of *B. ibitiguara*. Neotropical Brazilian anuran tadpoles found in contrasting biomes, such as *Rhinella ornata* in the Atlantic Rainforest (CT_{max}: 42.5°C) and the *Rhinella granulosa* in the Caatinga (CT_{max}: 44.4°C) exhibit high CT_{max} compared to *B. ibitiguara* (Simon et al., 2015). Species with restricted geographical distributions and a narrower range of thermal extremes may have less opportunity to evolve thermal acclimation (Huey and Kingsolver, 1993; Brattstrom, 1968; Bernardo and Spotila, 2006; Gifford and Kozak, 2012). Thus, low CT_{max} values for tadpoles of *B. ibitiguara* seems to be a result of adaptation and reflect selection for safety margins that allows survival in their specific micro-habitat. Indeed, *B. ibitiguara* range is known only from the Serra da Canastra Mountain in the Brazilian Cerrado, inhabiting streams surrounded by gallery forests in a topographically complex landscape in altitudes up to 1.500 m (Nali, et al., 2020). In the present study, we have sampled tadpoles in a 670 m altitude stream, in which tadpoles were not exposed to large daily or seasonal temperature fluctuations in their

micro-habitat, which may be related to the presence of a gallery forests alongside streams where tadpoles inhabit (Fig. 2). Conversely, tadpoles that develop in tropical temporary ponds may exhibit CT_{max} above 40°C because they experience large daily temperature fluctuations (Abe and Neto, 1991).

In order to evaluate the risk that tadpoles from *B. ibitiguara* are prone to suffer from heat shock, that is, how fast tadpole's performance would decline as it approach the upper thermal limit, we estimated their warming tolerance (WT= CT_{max} – Tmax) (Duarte et al., 2012). Since CT_{max} was virtually the same between both acclimation groups (18 and 25°C), values estimated for WT were similar and considerably high (G18: 12.44±0.5 and G25:12.42±0.2). This suggests that tadpoles of *B. ibitiguara* have a safety margin regarding the direct impact of peak environmental temperatures, meaning these tadpoles are in some way resistant to thermal stress episodes (Duarte et al., 2012; Gutiérrez-Pesquera et al., 2016). Such elevated WT values are in between data from tadpoles living in cool ponds and streams of the subtropical Atlantic Forest in northern Argentina (WT = 13.2°C) reported by Duarte et al. (2012), and from tadpoles in the Atlantic Forest in southeastern Brazil (WT = 9.0°C) reported by Simon, et al. (2015). Even though canopy-protected cool species have larger warming tolerance and thus should be less vulnerable to warming temperatures, it does not mean they are not at risk of extinction. As for the case of *B. ibitiguara*, which is a habitat specialist, adults are only found reproducing in cool streams protected by gallery forests, they are likely at risk of succumbing from anthropogenic land-use changes such as deforestation, in which expose streams to much high daily and seasonal changes temperature.

4.2. Effect of temperature on Aerobic Metabolism

Routine and maximum metabolic rate are two fundamental physiological variables providing the floor and ceiling in aerobic energy metabolism. The total amount of potential energy available between these two parameters constitutes the aerobic metabolic scope (AMS). We verified that both routine and maximum metabolic rate are considerably influenced by increases in temperature (from 15 to 34°C), with values for RMR relatively elevated for tadpoles acclimated at 25°C, showing the limited capacity for acclimation in *B. ibitiguara* tadpoles. Although previous studies have shown that small aquatic ectotherms may be able to acclimate in a relatively short time (Brown et al. 2004; Rohr et al., 2018), it is clear that small increases in the average stream temperature would impact considerably *B. ibitiguara* tadpoles' survival. Since tadpoles acclimated at

25°C were unable to compensate for the direct effect of temperature on their RMR, aerobic scope was significantly impaired as judged by the fact that MMR seems to be slightly reduced above test temperatures of 20°C, when compared to G18. In fact, we opted for not performing MMR at the test temperature of 34°C as tadpoles from the acclimation group at 25°C did not stand the chase protocol and started to die during the O_2 measurements. Although CT_{max} encountered for those tadpoles was relatively higher (36.7 ± 0.09°C) than the temperature in which tadpoles died, we must consider that in order to reach each temperature before the chase protocol, tadpoles were slowly warmed (or cooled) relative to the acclimation temperature (e.g., 25°C) thereby remaining at each test temperature for up to one hour. Hence, we must recognize that the chosen warming protocol for obtaining the CT_{max} was relatively fast (0.1°C min^{-1}), which is suggested to overestimate CT_{max} values, in which a more slow heating rate returns lower CT_{max} values (Chown et al., 2009; Rezende, et al., 2011; Ribeiro et al., 2012; Simon et al., 2015). Yet, we opted for letting the tadpole to habituate to the new environment (i.e., respirometer) before chasing the tadpole for obtaining MMR.

In addition to the acclimation temperature at 25°C, we also tested a higher temperature (28°C) in which tadpoles were maintained for up to 3 weeks, but animals exhibited signs of reduced food intake in addition to show poor body condition (visual observation). Other studies have also observed such deleterious effects of high acclimation temperatures in several species, such as arthropods, urchin, zooplankton and salmon (Rall et al., 2010; Lemoine and Burkepile, 2012; Alcaraz et al., 2014; Hvas et al., 2017). For instance, Healy and Schulte (2012) studying the fish *Fundulus heteroclitus,* found that at temperatures where both RMR and MMR are still increasing exponentially with temperature, and aerobic scope is maximal, the fish have difficulty to maintain body mass during long-term acclimation. This suggests there are limitations on the ability to take up, process or assimilate enough nutrients to support the high metabolic rates at high acclimation temperatures (Schulte, 2015). This hypothesis also agrees with the fact that acclimated tadpoles at 25°C were also struggling to maintain body condition because of an already elevated RMR. (Fig. 3B). Indeed, we observed a clear difference in body measurements between tadpoles from different acclimation groups. While tadpoles from G18 exhibited a more robust health condition, like larger total body length (TL: 54.7±1.5 mm) and body width (BW: 9.9±0.5 mm), tadpoles acclimated at 25°C showed a more skinny body shape (TL: 46.7±1.3 mm and BW: 7.6±0.3 mm). This could imply that food

transit is accelerated thereby impairing food assimilation (Edwards, 1971; Marian and Pandian, 1985), despite both groups being kept at the same feeding regime.

After the completion of the effects of acclimation and test temperature on RMR and MMR, tadpoles were returned to their respective aquariums at their acclimation temperature, thus we could follow tadpoles through their metamorphosis. It is interesting to note that tadpoles acclimated to 18°C took a while to start the transition from an aquatic larval phase. Normally, environmental stressors such as temperature, prolonged droughts and hypoxic environments would accelerate metamorphosis by increasing the hypothalamus-pituitary-interrenal axis activity (Owerkowicz et al., 2009; Heinrich et al., 2011; Rollins-Smith, 2017). However, during our field trip in the middle of the dry season (July 2020), *B. ibitiguara* tadpoles could still be found in what seems to be permanent water ponds, despite the stream flow has ceased. We confirmed that these ponds exhibited a temperature of 21.1°C (time of the day 12h05, similar to other months) and oxygen solubility not different from values when the stream had a running flow. Therefore, it seems that *B. ibitiguara* tadpoles can survive through the dry season in suitable ponds by delaying metamorphosis until the following rainy season.

In contrast, the tadpoles acclimated at elevate temperature (25°C) did not arrive at climax to undergo metamorphosis like tadpoles of G18, since low temperatures promote larger size at metamorphosis but extend the larval duration; while high temperatures accelerate larval development but at the cost of size (Marian and Pandian, 1985). Therefore, the reduced growth and development and thus the delay in metamorphosis at G25 may indicate changes in energy allocation with most of it being diverted to maintain a high routine metabolic rate. Besides that, environmental stressors (e.g. desiccation/ low pH/ hypoxia or even high temperature) could influence the hormonal control of metamorphosis (Kikuyama et al., 1993). Both thyroid and glucocorticoid hormones are known to trigger metamorphosis in amphibians and elevate temperature may activate the hypothalamus-pituitary-interrenal axis and accelerate metamorphosis (Duellman and Trueb, 1994; Crespi and Denver, 2004; Ruthsatz et al., 2018). Thus, the release of hormones for metamorphose can demand a high metabolic cost for this transition, which was impossible for *B. ibitiguara* tadpoles at high temperature due to the high routine metabolic demand, leading to a trade-off between maintaining the body condition or metamorphose.

Likewise, Seebacher and Grigaltchik (2014) also observed an exponential increase in RMR of frog tadpoles, *Limnodynastes peroni*, especially in animals

acclimated to the cold (15°C), which showed significantly higher O_2 consumption at higher experimental temperatures (20 and 25°C) compared to group acclimated at higher temperature (25°C). In addition, the authors reported that tadpoles acclimated to 15°C were more active than animals acclimated to 25°C, which may be an indication that more oxygen was used by tadpoles acclimated to 15°C for a given level of activity. An alternative explanation is that low temperature activity requires more ATP per unit of muscle power than high temperature. In our case, both RMR and MMR increased exponentially in parallel, up to temperatures close to 25°C, before the critical maximum temperature that can be tolerated by the tadpoles. In more stable environments such as the stream where the tadpoles inhabit, physiological processes should be thermally sensitive with maximal performance falling within the limited range of environmental temperatures (Gabriel, 2005; Gabriel et al., 2005), thus our data corroborate with this prediction. In addition, as water temperatures rise animals will face a double problem of progressively reduced oxygen solubility in the water and enhanced costs reflected in increased metabolic rates (Pörtner et al., 2006).

$B.$ $ibitiguara$ tadpoles are not athletic species, but they also need an active $\dot{M}O_2$ for feeding, growth, reproduction and locomotion (used for foraging as well as escape from predators). However, in terms of temperature dependence of active oxygen consumption the MMR failed to continue increasing with temperature (Fig. 3), while RMR continued its exponential increase in G18, may until temperature approached a lethal level, while RMR in G25 reaches a plateau at 30°C. This same response was observed by Fry (Fry, 1947; Fry and Hart, 1948) when exercising goldfish (*Carassius auratus*), predicting that the optimal temperature for aerobic scope is created by the failure of MMR to continue increasing with temperature (Farrell, 2009).

Thus, in a scenario with an increase of just 2 °C in the micro-habitat of tadpoles, it would be enough to increase their aerobic metabolism to the maximum in the face of metabolic demand, and with that their levels of intake and body mass decreased, consequently the metamorphosis will also be compromised, since the animal needs to accumulate energy for this process.

4.3. Effect of temperature and autonomic blockade on routine heart rate

In this study, the effect of a long-term acclimation (weeks) did not cause any compensatory response in the autonomic control. The treatment with atropine increased tadpole's heart rate in both test temperatures, although the magnitude of the response was

temperature dependent, with a more pronounced tachycardia at the higher experimental temperature. In addition, sotalol treatment following atropine reduced f_H to near baseline values, suggesting routine f_H and intrinsic f_H are very similar.

Regarding routine values for f_H tadpoles acclimated at 18°C exhibited increases in f_H at the test temperature at 25°C with a Q_{10} coefficient of 1.9. Likewise, tadpoles acclimated to 25°C when acutely cooled to 18°C exhibited a reduction in heart rate with the same Q_{10} coefficient of 1.9. Interestingly, as previously discussed (see 4.2), routine metabolic rate was always higher in the acclimation group at 25°C relative to 18°C across all test temperatures. In a previous study with larval stages of *Xenopus leavis*, the authors show that tadpoles are able to adjust cardiac output by increasing f_H and stroke volume when exposed to acute hypoxia (Francis Pan and Burggren, 2013). Therefore, since f_H in *B. ibitiguara* during acute warming from 18 to 25°C or during acute cooling from 25 to 18°C exhibited the same magnitude response in both acclimation group (see supplementary material, Fig. S2), elevated oxygen demand observed for G25 may be explained by either increases in cardiac output due to adjustments in stroke volume, in addition to increases in arteriovenous extraction.

Also, the thermal acclimation did not affect the autonomic tones on the heart of tadpoles of *B. ibitiguara*, since cholinergic tone is very similar to adrenergic tone, as atropine increases f_H and sotalol reduces with the same magnitude. There was a trend toward an increase in cholinergic tone at G18, however the individual variation was too large for the increase to be statistically significant. In contrast, in larvae of *Xenopus laevis* no cholinergic tone was found in all stages studied by Jacobsson and Fritsche (1999), but there is an active adrenergic tone on the heart early in the development. While in premetamorphic tadpoles of *Lithobates catesbeianus* the cholinergic tone is more predominant for the thermal interval between 15 and 25°C; otherwise at higher temperatures (30°C) the cholinergic tone decreases and the adrenergic tone increases (Longhini et al. 2017). Therefore, the autonomic tone can change according to the stage of development between species and the temperature experienced (Burggren, 1995).

5. Perspectives

Since the individuals of *B. ibitiguara*, inhabiting in a protected area by vegetation with permanent ponds throughout the year, they can find favorable conditions to have a prolonged larval phase and possibly adjust the metamorphosis time for the beginning of the next rainy season. Further, they do not experience large temperature variations in the

water, which contributes to a high O_2 solubility. However, in a scenario with increased water temperatures due to gallery forest degradation, stressors such as temperature, prolonged droughts and nutritional deficiency would accelerate metamorphosis by increasing the activity of the hypothalamo-pituitary-interrenal axis, responsible for mediates the response of the animal to its environment (Rollins-Smith, 2017).

6. Conclusions

Our study demonstrated that tadpoles of *B. ibitiguara* has a limited phenotypic plasticity in response to acclimation at high temperatures, since the thermal tolerance traits, such as CT_{max} and WT, were not different between acclimation groups (18 and 25°C). In addition, our results also show that *B. ibitiguara* tadpoles' exhibit limited plasticity regarding metabolic and cardiac parameters, once aerobic metabolic scope was reduced in animals acclimated to higher temperature (25°C). These findings may be related to the fact that *B. ibitiguara* tadpoles develop in a stable micro-habitat in which daily and seasonal changes in water temperature are narrow and possibly reflect the characteristics of the gallery forests alongside streams where tadpoles inhabit. In addition, our hypothesis that there would be f_C compensation due to high cholinergic tone or reductions of intrinsic f_C was not confirmed, since cholinergic and adrenergic tone did not differ between both groups. This lack of plasticity during the larval phase of *B. ibitiguara* has important conservation implications, because the adult phase of this anuran amphibian is a habitat specialist, always associated to a topographically complex landscape that has so far endured anthropogenic modification (Nali et al. 2020). Therefore, in a scenario with prolonged droughts, gradual increases in ambient temperatures and degradation of what remains from gallery forest habitats in non-protected areas of the Brazilian savannah-like Cerrado will likely affect the species survival.

Supplementary material

Supplementary material is available at…

Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contribution

LSL, LAZ and LHG designed the research. LSL, LAZ, ACGR and GSL performed the experiments and LSL, LAZ and ETP analyzed the data. All authors interpreted the data and provided critical and intellectual input during the preparation of the manuscript and approved the final version.

Funding

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Table 1. Comparisons of body characteristics of tadpoles in different laboratory acclimation groups (G18 and G25°C). Values shown are means ± s.e.m. for total body length (TL), partial length (PL), body width (BW) in millimeter (mm) and body mass in grams (g). We tested differences with unpaired t test at a 0.05 significance level. T values and the corresponding degrees of freedom are also shown, all parameters are significant different between acclimation groups.

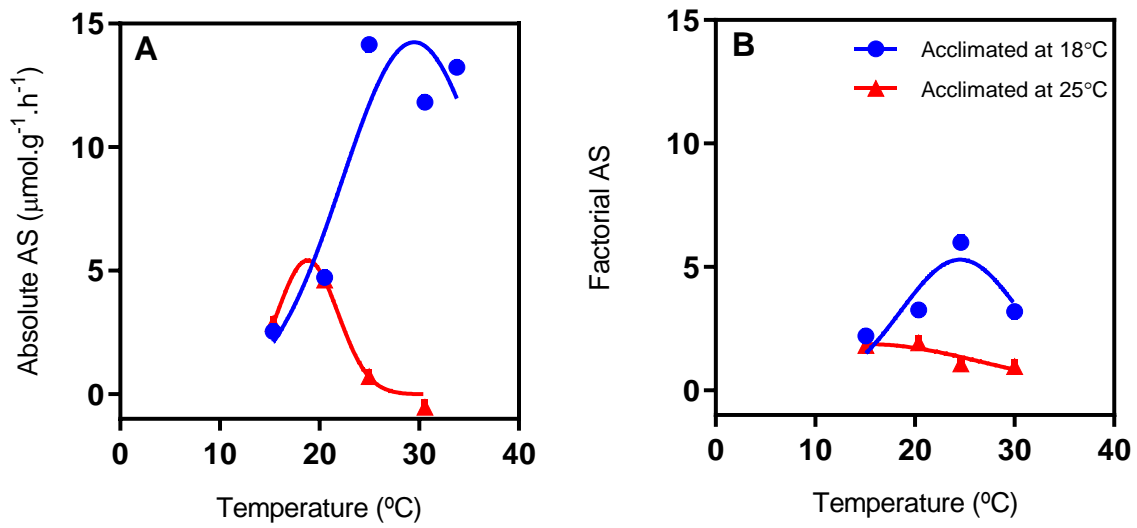| Groups	TL (mm)	PL (mm)	BW (mm)	Body mass (g)
G18 (N=9)	54.7 ± 1.5	17.9 ± 0.6	9.9 ± 0.5	1.3 ± 0.1
G25 (N=10)	46.7 ± 1.3	14.8 ± 0.3	7.6 ± 0.3	0.7 ± 0.07
G18 *vs.* G25	$t_{(17)}=3.96$; $P<0.001$	$t_{(17)}=4.13$; $P<0.001$	$t_{(17)}=3.66$; $P<0.001$	$t_{(17)}=4.76$; $P<0.001$

Figures

Figure 1. Annual records of stream water temperature where tadpoles of *B. ibitiguara* were collected. Data logger was programmed to collect and store temperature values every hour, starting April 24 of 2019 to July 25 of 2020. The sensor was replaced on December 14 of 2019 in a new field trip. On July 25 of 2020, the location where the sensor was dried up completely due to the dry season, thus, we infer that the area with the greatest temperature variation represents air temperature.

Figure 2. Critical thermal maxima (CT$_{max}$) for tadpoles of *B. ibitiguara* and maximum temperature (Tmax) of their micro-habitat. Acclimation treatment (18 and 25°C) did not affect CT$_{max}$ of premetamorphic tadpoles (*P*=0.84). Data are presented as mean ± s.e.m. (18°C, N= 7; 25°C, N=8).
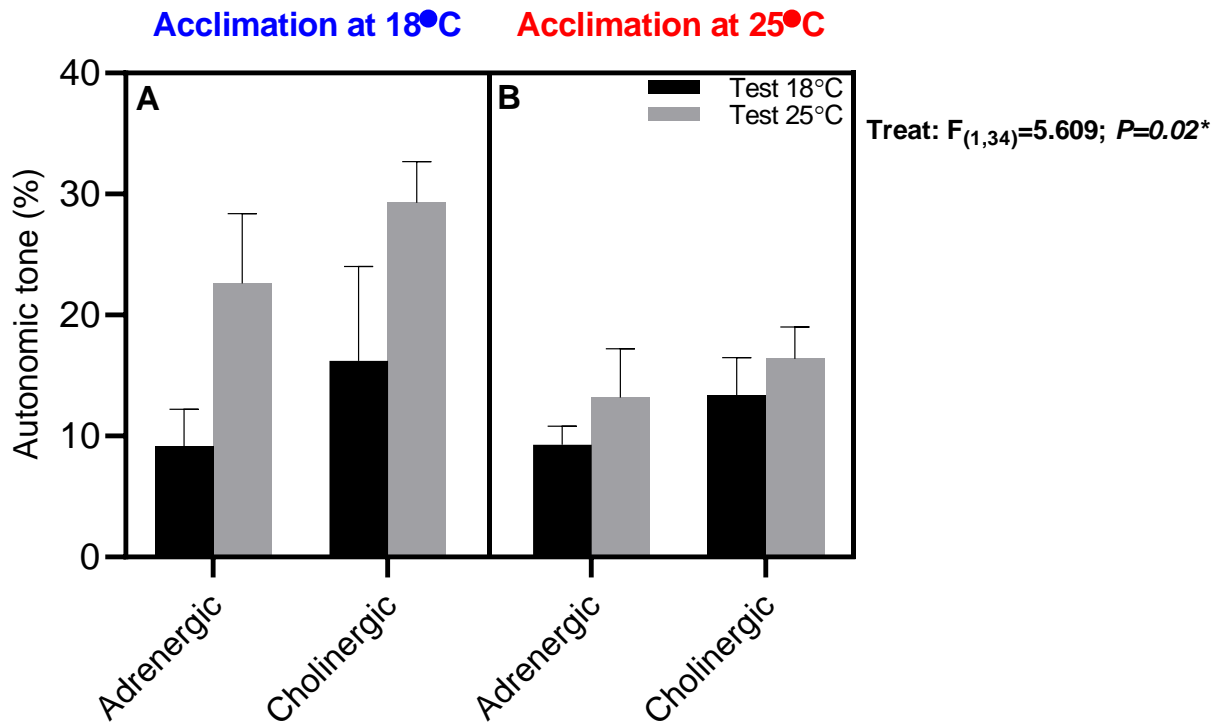
Figure 3. Temperature effects on aerobic metabolism parameters in tadpoles of *B. ibitiguara*. Routine metabolic rate (A; RMR) and maximum metabolic rate (B; MMR) of tadpoles acclimated to 18°C (N=8) and 25°C (N=8), and exposed to different test temperatures (15, 20, 25, 30 e 34°C). The results from the linear model for the variables that showed statistical significance are presented in the panel. Data are presented as mean ± s.e.m.

Figure 4. The estimate of absolute aerobic scope (AAS) and factorial aerobic scope (FAS) of tadpoles of *B. ibitiguara*. The absolute aerobic scope (A; aerobic scope) is calculated from the difference between maximum metabolic rate (MMR) and routine metabolic rate (RMR). Factorial aerobic scope (B) is calculated from the ratio between MMR and RMR.

Figure 5. Effect of pharmacological blockade on heart rate in tadpoles of *B. ibitiguara*. The effects of pharmacological blockade (atropine alone and double blockade = atropine + sotalol) on heart rate (f_H) in tadpoles acclimated at 18°C (N = 8) and tadpoles acclimated at 25°C (N=7) at different experimental test temperatures (18 and 25°C). Regardless of the treatment used, the f_H was significantly altered by temperature of 25°C (*P*<0.001). The results from the linear model for the variables that showed statistical significance are presented in the panel. Dissimilar letter means significant difference between treatments. Data are shown as means ± S.E.M
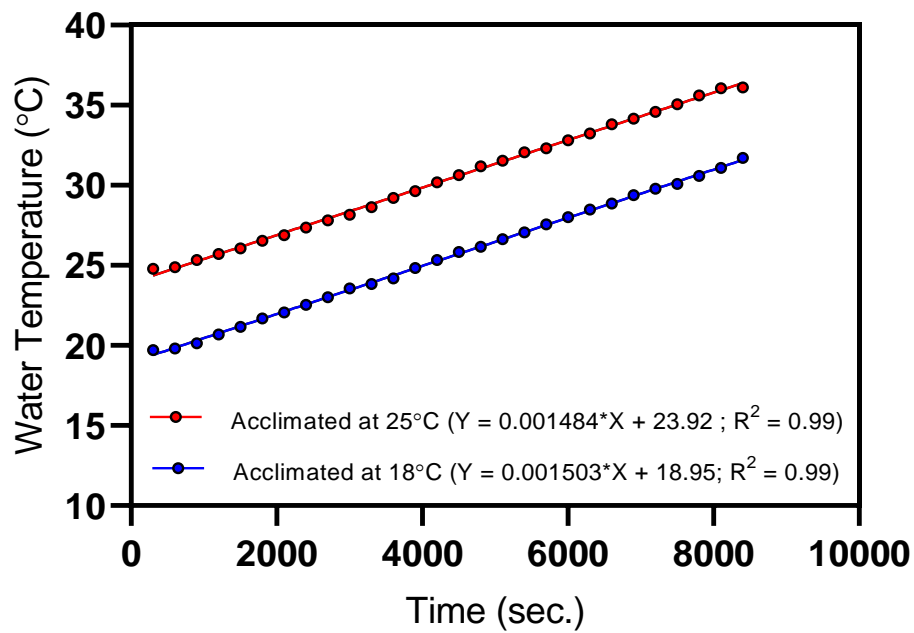
Figure 6. Calculated autonomic tones on the heart of resting tadpoles of *B. ibitiguara* acclimated at 18°C and 25°C at different test temperatures (18 and 25°C). The results from the linear model for the variables that showed statistical significance are presented in the panel. Data are represented as means ± S.E.M., N = 7.
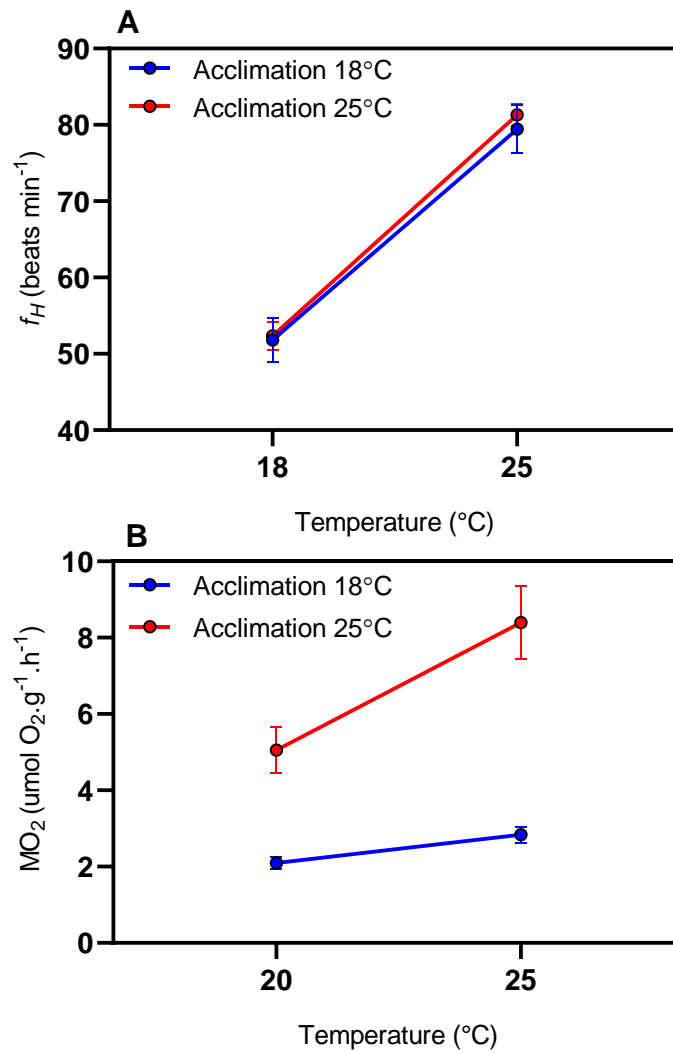
Supplementary information

Figure S1. The linear regression of slopes indicates the corresponding heating rates (ΔT) in the experiments for upper thermal limits (CT_{max}) of acclimated group to 18°C (blue line) and 25°C (red line). There are no statistical differences between slopes (P=0.436).

Figure S2. Thermal responses of heart rate (f_H) and routine metabolic rate (RMR) on tadpoles of *B. ibitiguara* chronically acclimated at 18° and 25°C and exposed to acute test temperatures. Blue and red line: acute thermal effect (test temperature) on f_H (A) and RMR (B) in tadpoles acclimated to 20 (blue) and 30 °C (red).

Figure S3. Tadpoles of *B. ibitiguara* **from group acclimated at 18°C (A) and acclimation group at 25°C.**