Eduardo Hermógenes Moretti

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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciência, na Área de Fisiologia Geral.

Orientador(a): Prof. Dr. Fernando Ribeiro Gomes

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_____Dedicatória

Dedicatória

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_____Epígrafe

You running and you running
And you running away
You running and you running
But you can't run away from yourself
Can't run away from yourself
Can't run away from yourself
Can't run away from yourself

Bob Marley, “*Running away*” song

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Apresentação

Esta tese está composta por um resumo inicial, seguido de uma contextualização teórica do assunto e suas referências. Em seguida, encontra-se um artigo científico em inglês com os resultados da tese. O artigo está composto por: Introduction, Materials and Methods, Results, Discussion, References and Tables and Figures. Para encerrar estão descritas as conclusões gerais.

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Índice

Resumo/Abstract

Resumo: Anfíbios tem a habilidade de manifestar febre comportamental em ambientes heterotermais durante infecção a um custo metabólico associado à elevação da temperatura corpórea e à ativação do sistema imune. Apesar do custo metabólico, a temperatura corpórea febril otimiza a resposta imune no combate à infecção e aumenta as chances de sobrevivência do indivíduo. Contudo, devido à limitada capacidade de termorregular, os anfíbios enfrentam variações diárias e sazonais na temperatura corpórea e na resposta metabólica de reação à infecção. O nosso objetivo foi medir a variação da resposta metabólica à infecção dentro da variação de temperaturas ecológicas relevantes do sapo Cururu. Testamos a hipótese de que a infecção aumenta as taxas metabólicas do sapo Cururu, mas o custo energético da resposta imune deve ser menor na temperatura febril dos sapos infectados. Para testarmos as hipóteses, nós medimos a temperatura operacional dos sapos no campo, a preferencia termal dos sapos hígidos e a temperatura preferencial dos sapos infectados. Depois, medimos a taxa metabólica e a resposta metabólica dos sapos antes e depois da infecção por LPS nessas temperaturas. Nossos resultados mostraram que as temperaturas ecológicas relevantes dos sapos variaram entre 17°C e 26°C. A temperatura influenciou a taxa metabólica dos sapos, mas só na temperatura preferencial dos sapos hígidos houve custo metabólico associado à infecção. Contudo, na temperatura corpórea dos sapos infectados a resposta metabólica de reação à infecção foi menor, indicando que o controle regulado no ponto de ajustes “set-point” da temperatura corpórea durante a infecção coevoluiu com um custo energético otimizado da resposta imune.

Palavras chaves: anfíbios; termorregulação, taxa metabólica; infecção

Abstract: Anphibians have the ability of manifested behavioral fever in heterothermal environments during infection with a metabolic cost associated to elevated body temperature set-point and due to activation of immune system. Despite the metabolic cost, fever body temperature optimizes immune response to combat infection and increase the survival of the host. However, because of the limited capacity for thermoregulation, amphibians can confront daily and seasonal variation in body temperature and in the metabolic response of reaction to combat infection. So, we measured the variation in metabolic response of reaction to infection at ecology relevant body temperature range in Cururu toads. We hypothesized that infection increases metabolic rates of the Cururu toads due to the activation of the immune system at different temperatures, but the energetic cost of immune response is lower at preferred body temperature of infected toads (behavioral fever). To test these hypotheses we measured the operative body temperature in the field, the preferred body temperature of higid toads, and the preferred body temperature of infected toads. After, we measured metabolic rate and metabolic response of the toads before and after injection of LPS at these temperatures. Our results showed that the ecology relevant temperature range of Cururu toads (*R. icterica*) varies between 17°C and 26°C, respectively, at operative temperature and at preferred body temperature in infected toads when exposed to heterothermal environment. The temperature had the major impact on metabolic rate of the toads during infection. But, at fever body temperature toads decrease the metabolic response of reaction to infection, indicating that the regulated control of body temperature set-point during infection coevolved with an optimized energetic cost of immune response.

Key words: amphibians; thermoregulation; metabolic rate; infection

Introdução Geral

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Aspectos gerais da termorregulação em anfíbios

A temperatura corpórea dos anfíbios, animais ectotérmicos, é uma complexa integração entre comportamento, fisiologia, morfologia e características físicas do ambiente que ocupam ao longo do espaço e tempo (Brattstrom, 1963; Carey, 1978; Hutchison & Dupré, 1992). Sua variação afeta a cinética de processos bioquímicos e fisiológicos, inclusive comportamentos relacionados, e também a taxa na qual energia e materiais são extraídos do ambiente e usados nos diversos processos metabólicos do animal, como por exemplo, manutenção, crescimento e reprodução (Carey, 1978; Hutchison & Dupré, 1992). Devido a essas características, que afetam o metabolismo de uma forma geral e valor adaptativo (Darwinian fitness), ajustes regulatórios são empregados por muitas espécies para lidar com esses desafios térmicos espaciais e temporais (Crawshaw et al., 1985; Angilletta et al., 2002).

Anfíbios, por exemplo, tem o potencial de controlar a temperatura corpórea através da interação entre ajustes comportamentais (e.g.: permanência em abrigos, seleção de temperaturas, exposição ao sol), fisiológicos (e.g.: aclimatação, resfriamento por evaporação) e a taxa de transferências termais (de calor) com o meio ambiente (e.g: irradiação, condução, convecção e evaporação pela pele) (Brattstrom, 1963; Hutchison & Dupré, 1992). Contudo, a limitação de controle fisiológico sugere que a regulação comportamental deve ser dominante na regulação da temperatura corpórea em anfíbios (Hutchison & Dupré, 1992). O tempo de permanência ou evasão em um abrigo, toca ou corpos d’água; o microclima do abrigo; quantidade de tempo ao sol; nível de hidratação do animal; orientação do animal para o sol ou vento; postura; velocidade do vento; hora do dia ou estação do ano; entre outros, são exemplos dessa interação entre

comportamento, fisiologia e condições microclimáticas que afetam a temperatura corpórea dos anfíbios (Brattstrom, 1963; Hutchison & Dupré, 1992).

Para uma melhor compreensão do grau de dependência de mecanismos regulatórios da temperatura corpórea dos animais ectotérmicos para a manutenção do seu metabolismo e desenvolvimento de suas atividades no ambiente que ocupam, devem ser integradas as relações entre: 1) a variação da temperatura ambiental que o animal ocupa; 2) variação da temperatura de um modelo nulo nos microhabitats ocupados (temperatura operacional); 3) o intervalo de temperatura corpórea do animal na natureza (temperatura de atividade); 4) além do comportamento termorregualatório em um gradiente térmico (intervalo "*set-point*" da temperatura preferencial do animal em condições laboratoriais) (Hertz et al., 1993; Hutchison & Dupré, 1992; Navas, 1996; McNab, 2002).

De acordo com a análise dessas variáveis citadas acima, os animais ectotérmicos podem ser definidos como termorreguladores, ou seja, capazes de manter a temperatura corpórea em um estreito intervalo em relação a uma grande variação da temperatura ambiental, ou termoconformadores, que implica pouca ou ausência de termorregulação, ou seja, a temperatura corpórea acompanha as variações na temperatura ambiental (Hutchison & Dupré, 1992; Hertz et al., 1993). Vale salientar, contudo, que ectotérmicos termoconformadores em ambientes termais constantes apresentarão poucas variações na temperatura corpórea (Hutchison & Dupré, 1992; Hertz et al., 1993). Ademais, muitas espécies podem ser termorreguladores durante determinado período, mas termoconformadores em outros. Por exemplo, ectotérmicos que mantém atividades de forrageamento diurnas devem ser termorreguladores comportamentais efetivos durante o dia, mas na inatividade noturna devem ser típicos termoconformadores

(Hutchison & Dupré, 1992). Ou seja, os termos termorregulador e termoconformador são extremidades opostas de um contínuo (Hutchison & Dupré, 1992).

Devido à pele altamente permeável, os fluxos de água, energia, osmólitos e gases respiratórios nos anfíbios são comparativamente altos entre os vertebrados. Sendo assim, os anfíbios dependem de um equilíbrio entre os requerimentos para respiração e hidrorregulação no controle da temperatura corpórea. Esses fatores também são influenciados pela relação corpórea entre área de superfície:volume com fatores ambientais que o animal ocupa (Tracy, 1976; Feder, 1982). Por exemplo, o tamanho corpóreo limita a dependência dos sapos recém-metamorfoseados (*Rhinella marina*) à proximidade de corpos d'água para evitar dissecação (Freeland & Kerin, 1991; Cohen & Alford; 1993). Juntamente com o fato de que a dispersão noturna é evitada, os sapos recém-metamorfoseados só são capazes de dispersarem durante o período diurno, quando atingem determinado estágio de desenvolvimento na qual a perda de água por evaporação reduz em relação ao tamanho corpóreo (Freeland & Kerin, 1991; Cohen & Alford; 1993; Pizzatto et al., 2008).

Devido a esses requerimentos para respiração e hidrorregulação em conflito com a termorregulação, sugere-se que os anfíbios são maus termorreguladores, sendo caracterizados por uma alta variabilidade diária e sazonal de temperatura corpórea (Rome et al., 1992). Dessa maneira, anfíbios devem ter desempenhos de atividades em temperaturas sub-ótimas e raramente experimentar temperaturas corpóreas que permitem altos níveis de desempenho (Rome et al., 1992). Entretanto, ajustes comportamentais que integram demandas associadas ao balanço hídrico e à termorregulação, em conjunto com baixa variabilidade da temperatura ambiental, podem amenizar essa alta variabilidade na temperatura corpórea prolongando o período de atividade dos anfíbios (Carey, 1978; Feder, 1982; Hutchison & Dupré, 1992; Navas

et al., 2008). Por exemplo, salamandras neotropicais ocupam microhabitats que apresentam baixa variação temporal e espacial de temperatura. Nesses microhabitats, as salamandras são incapazes de termorregular alternando entre locais frios e quentes, contudo permitem pouca variação da temperatura corpórea (Feder, 1982). Por outro lado, através de ajustes comportamentais e fisiológicos, pererecas (*Hyperolius marmoratus*) conseguem reter calor do ambiente, e assim manter a temperatura corpórea maior que a temperatura ambiente e altas taxas de vocalização durante o período noturno (Passmore & Malherbe, 1985). Essa retenção de calor deve ser proporcionada pela exposição das pererecas ao sol durante o dia e diminuição nas taxas de perda de água por evaporação durante a noite (Withers et al., 1982; Passmore & Malherbe, 1985). O comportamento de exposição ao sol é comum em anfíbios, mas depende da disponibilidade de recursos hídricos no ambiente, e também, de ajustes comportamentais e fisiológicos para o anfíbio estabilizar a temperatura corpórea (Lillywhite, 1970; Lillywhite, 1971; Hutchison & Dupré, 1982).

Aclimatização e diferenciação genética podem maximizar a habilidade de resposta aos fatores ambientais. A capacidade da aclimatação termal do metabolismo basal e do metabolismo pós-atividade locomotora permite salamandras de clima temperado reduzir os requerimentos energéticos e as taxas de depleção das reservas energéticas em temperaturas mais elevadas (Feder, 1978). Em contrapartida, a incapacidade de aclimatação metabólica em salamandras neotropicais deve restringir sua distribuição geográfica a estreitas zonas climáticas em comparação à distribuição de salamandras de zona temperada (Feder, 1978). Anuros tropicais de alta altitude, por exemplo, não apresentam respostas de aclimatação da taxa metabólica durante a atividade locomotora (Navas, 1996). Contudo, são caracterizados por menor sensibilidade do desempenho locomotor à variação de temperatura e maior taxa

metabólica durante a atividade locomotora do que espécies congêneres de baixa altitude (Navas, 1996). Estes resultados sugerem que a habilidade de anuros de alta altitude de se moverem a baixas temperaturas envolve diferenciação genética entre espécies relacionadas ao longo de gradiente altitudinal (Navas, 1996). Esses ajustes, em conjunto, tendem a melhorar a capacidade de atividade dos animais nas condições termais que prevalecem no ambiente em que vivem, permitindo aos anfíbios apresentar tanto amplas faixas de tolerância termal, sem uma temperatura preferencial ou ótima, quanto apresentar uma estreita faixa preferencial ou ótima para atividades (Brattstrom, 1963; Carey, 1978; Hutchison & Dupré, 1992; Navas, 1996; McNab, 2002; Navas et al., 2008). Dessa forma, animais que são ativos em um grande intervalo de temperatura devem experimentar seleções para desempenho independente da temperatura, em contrapartida aos animais que são ativos em um estreito intervalo de temperaturas (Rome et al., 1992).

Sendo assim, as temperaturas ótimas de desempenho de atividades podem tanto coincidir quanto não coincidir com as temperaturas preferenciais dos animais, ou até mesmo com o intervalo de temperatura normal de atividade. Navas (1996), por exemplo, mostrou que as temperaturas de atividade dos anuros nas altitudes que ocupam tendem a coincidir com as temperaturas para melhor desempenho locomotor. Adicionalmente, espécies de alta altitude apresentam maior flexibilidade de amplitude termal para atingirem 80% do pico de desempenho ao salto em comparação aos anuros que ocupam altitude de menor variabilidade termal (Navas, 1996). Por outro lado, a distância alcançada durante o salto horizontal no anuro *Limnodynastes tasmaniensis* é maior nas temperaturas entre 30°C e 33°C, temperaturas estas que são raramente frequentadas no gradiente térmico e maiores que a preferência termal de 24°C (Whitehead et al., 1989; Hutchison & Dupré, 1992). Dessa forma, uma imensa variação

entre temperatura preferencial e temperaturas ótimas para atividades pode ser encontrada para anfíbios, refletindo os diversos ajustes regulatórios da temperatura corpórea para lidar com os desafios térmicos espaciais e temporais do ambiente que ocupam (Carey, 1978; Crawshaw et al., 1985; Hutchison & Dupré, 1992; Angilletta et al., 2002).

Regulação dos pontos de ajustes "set-point" da temperatura corpórea

Estudos em vertebrados indicam que o controle de respostas efetoras para um conjunto de ajustes termorregulatórios (e.g.: comportamentais, fisiológicos ou aclimatórias) são integrados por neurônios termo e não termosensíveis (Crawshaw et al., 1985; Bicego et al., 2007). Esses neurônios, localizados na região pré-óptica do cérebro, são responsáveis por receber e integrar as informações dos termosensores periféricos e centrais (Crawshaw et al., 1985; Bicego et al., 2007). Esse conjunto de reguladores é responsável em determinar e estabilizar o ponto de ajuste "*set-point*" da temperatura corpórea do indivíduo, que é influenciado pelos processos de aclimação/aclimatização e adaptação (Kluger, 1991; Hutchison & Dupré, 1992; IUPS Thermal Commission, 2001). Entretanto, quando a temperatura do corpo desvia significativamente do ponto de ajuste "*set-point*" o indivíduo pode se encontrar hipotérmico (desvio inferior) ou hipertérmico (desvio superior), principalmente por causa da capacidade efetora insuficiente e que gera consequente falta de estabilização (Kluger, 1991; IUPS Thermal Commission, 2001).

Por outro lado, a hipotermia e a hipertermia podem ser uma resposta controlada pelos reguladores centrais e não ser considerada um desvio do ponto de ajuste "*set-point*" (Kluger, 1991). A hipotermia controlada (anapirexia) permite aos indivíduos, explorar e ocupar ambientes geralmente desfavoráveis (Hutchison & Dupré, 1992;

Boutilier, 2001; Bicego et al., 2007). O sapo comum Europeu (*Rana temporaria*), por exemplo, submerge em lagos hipóxicos durante o inverno até as condições climáticas e disponibilidade de alimentos voltarem ao normal. Durante esse período, os animais diminuem de forma controlada a temperatura corpórea e entram em um estado hipometabólico, que aumenta o tempo de sobrevivência por diminuir o impacto das demandas de ATP sobre os substratos energéticos endógenos (Boutilier, 2001). Por outro lado, a hipertermia controlada (febre) faz parte da resposta do organismo no combate à infecção. A presença de aumento da temperatura corpórea em resposta à infecção nos diversos grupos de animais, desde invertebrados até vertebrados ectotermos e endotermos, sugere que a febre tem uma história filogenética antiga e importante valor adaptativo (Kluger, 1979; Hasday et al., 2000; Bicego et al., 2007).

A febre é parte de uma resposta inflamatória sistêmica aguda em resposta ao desequilíbrio homeostático causado, principalmente, por microparasitas (vírus, bactéria) (Ashley et al., 2012). Essa resposta inflamatória consiste de uma cascata finamente regulada de processos imunológicos, fisiológicos e comportamentais que é orquestrada por citocinas proinflamatórias (IL-1, IL-6 e TNF-α) (Hasday et al., 2000; Ashley et al., 2012). Estas citocinas ativam no cérebro a febre e o comportamento doente, além de induzir a secreção de proteínas de fase aguda do fígado (Ashley et al., 2012). Em anfíbios e animais ectotermos de uma forma geral, estudos indicam que pirógenos exógenos ou endotoxinas (e.g.: bactérias gram-positivas, vírus, fungos e lipopolissacarídeo de bactéria gram-negativa - LPS) ativam a liberação de citocinas proinflamatórias, que estimulam mediadores piréticos (prostaglandinas) a agirem no sistema nervoso central, especificamente na região pré-óptica, e estimula uma resposta efetora comportamental nos anfíbios de busca por ambientes mais quentes (Myhre et al., 1977; Hutchison & Erskine, 1981; Bicego et al., 2007). Vale salientar, contudo, que

ajustes fisiológicos como fluxo sanguíneo cutâneo, refletância da pele, secreção de hormônios (e.g.: hormônios da tireoide, melatonina e catecolaminas) e neurotransmissores (e.g.: acetilcolina, histamina) também influenciam a preferência termal nos anfíbios (Hutchison & Dupré, 1992). Porém, não se sabe se esses ajustes participam da resposta efetora em resposta à febre nos anfíbios.

Estudos, principalmente com vertebrados, mostram que esse aumento controlado da temperatura corpórea é um importante mecanismo que aumenta as chances de sobrevivência do indivíduo à infecção através de dois possíveis mecanismos não excludentes (Kluger et al., 1975; Hasday et al., 2000; Blatteis, 2003): 1) inibição do crescimento, desnaturação de proteínas e redução da atividade infecciosa dos microrganismos invasores que possuem intervalo de temperatura ótima de crescimento abaixo da temperatura corpórea do hospedeiro durante a febre (Kluger et al., 1975; Hasday et al., 2000; Blatteis, 2003); 2) melhora da resposta imunológica do hospedeiro, principalmente por criar um ambiente termal ótimo para expressão e apropriada ação coordenada de citocinas. Entre outros exemplos, aumenta a emigração de granulócitos da circulação para o local da inflamação e modula passos críticos no recrutamento de linfócitos T, potencializando a adesão destas células ao endotélio dos vasos sanguíneos de órgãos linfoides secundários e do tecido inflamado (para revisão: Hasday et al., 2000; Blatteis, 2003).

Apesar dos benefícios no controle, eliminação da infecção e aumento nas chances de sobrevivência do indivíduo, a resposta inflamatória que resulta em febre gera custo energético ao indivíduo (Muchlinski, 1985; Sherman & Stephens, 1998; Lochmiller & Deeremberg, 2000). O aumento no ponto de ajuste "*set-point*" acarreta em um aumento na taxa metabólica do animal, como consequência da relação direta entre taxa metabólica e temperatura corpórea (McNab, 2002). Em mamíferos, espera-se

que o aumento de 1°C na temperatura corpórea aumente em 10% a taxa metabólica do indivíduo, além de um aumento na taxa de oxidação de glicose (Roe & Kinney, 1965; Ashley et al., 2012). Estudos com anfíbios mostraram que injeção de bactérias inativadas (*Aeromonas hydrophila*) ou LPS pode aumentar de 24% a 100% a taxa metabólica dos anuros, e indicam que o aumento no custo energético pode refletir um efeito do aumento da temperatura como também um custo adicional da estimulação imune (Muchlinski, 1985; Sherman & Stephens, 1998; Llewellyn et al., 2011). Além do custo energético, a resposta de combate à infecção pode causar danos teciduais induzidos pela liberação de espécies reativas de oxigênio e nitrogênio por neutrófilos no local da inflamação (Ashley et al., 2012). Adicionalmente, as citocinas proinflamatórias inibem comportamentos associados à reprodução em favor do comportamento doente, que envolve diminuição na atividade de forrageamento, anorexia e redução de ingestão de água (Adelman & Martin, 2009; Ashley et al., 2012). Essa mudança comportamental tem sido interpretada como uma adaptação associada à conservação de energia, redução da ingestão de micronutrientes importantes para o crescimento do patógeno e minimização do risco de predação (Aubert, 1999; Adelman & Martin, 2009). Sapos (*R. marina*), por exemplo, apresentaram redução de ingestão de grilos e da atividade locomotora quando injetados com LPS (Llewellyn et al., 2012).

Apesar dos diversos custos, estudos com camundongos e peixes mostram que a elevação controlada da temperatura corpórea é essencial para uma efetiva resposta imune no combate à infecção (Jiang et al., 2000; Boltaña et al., 2013). Contudo, nem sempre animais ectotérmicos tem oportunidade de termorregular para obter temperaturas ótimas no desempenho de suas atividades (Hutchison & Dupré, 1992; McNab, 2002). Esses efeitos devem ser proeminentes em anfíbios, que dependem de mecanismos comportamentais, fisiológicos, além de ambientes com alta diversidade

termal e disponibilidade hídrica para ajustar sua temperatura corpórea (Rome et al., 1992; Hutchison & Dupré, 1992). Esses fatores serão essenciais para determinar a capacidade efetora do indivíduo durante o combate à infecção, no qual um novo ponto de ajuste "*set-point*" é regulado (Bicego & Branco, 2002; Bicego et al., 2002). Uma possível falta de estabilização da temperatura corpórea pode gerar uma resposta inadequada com alto custo metabólico e possíveis consequências no valor adaptativo do indivíduo (Jiang et al., 2000; Boltaña et al., 2013).

Isso torna os anfíbios um excelente modelo para entender o custo energético da infecção em intervalos de temperaturas ecológicas nas quais o indivíduo tem ou não a oportunidade de termorregular, simulando situações que o animal pode confrontar na natureza, como por exemplo: A) temperatura operacional de atividade no campo (animal termoconformador); B) preferencia termal independente de restrições e custos ecológicos (animal termorregulador hígido); e C) na preferência termal regulada durante infecção (animal termorregulador durante infecção). A partir dessas relações é possível determinar o efeito da temperatura e da estimulação imune na taxa metabólica, e também, a resposta metabólica em resposta à infecção em temperaturas que o animal termoconforma ou termorregula. A partir dessas considerações testamos a hipótese de que a infecção aumenta as taxas metabólicas do sapo Cururu devido à ativação do sistema imune em diferentes temperaturas, mas o custo energético da resposta imune deve ser menor na temperatura preferencial dos sapos infectados (febre comportamental). Sendo assim, os resultados previstos foram:

1) Os sapos manifestam febre comportamental no gradiente térmico em resposta à injeção com lipopolissacarídeo de bactéria gram-negativa (LPS);

2) A temperatura preferencial dos sapos hígidos (antes dos tratamentos) será maior que a temperatura operacional do campo;

3) A temperatura preferencial dos sapos infectados (LPS) será maior que dos sapos controle (Salina) e da temperatura preferencial dos sapos hígidos;

4) As taxas metabólicas dos sapos antes (padrão) e depois dos tratamentos serão positivamente associadas à temperatura;

5) A simulação de infecção por LPS aumentará as taxas metabólicas dos sapos nas diferentes temperaturas;

6) A resposta metabólica à infecção será menor na temperatura preferencial dos sapos infectados (febre comportamental).

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_____Artigo

Behavioral fever decreases metabolic response of reaction to infection in Cururu toads (*Rhinella icterica*)

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Running title: Fever decrease metabolic response in toads

Abstract: Anphibians have ability of manifested behavioral fever in heterothermal environments during infection with a metabolic cost associated to elevated body temperature and to activation of immune system. Despite the metabolic cost, fever optimizes immune response to combat infection and increase survival of the host. However, the limited capacity for thermoregulation, amphibians can confront daily and seasonal variation in body temperature and in the metabolic response of reaction to combat infection. So, we measured the variation in metabolic response of reaction to infection at ecology relevant body temperature range in Cururu toads. We hypothesize that thermal ecology of temperature range varies when toads thermoconform and thermoregulate, and that metabolic rate of the toads increase during infection at different temperatures, but the energetic cost of immune response is lower at fever body temperature. We measured the operative body temperature in the field, the preferred body temperature of higid toads, and the fever body temperature of infected toads. After, we measured metabolic rate and metabolic response of the toads before and after injection of LPS at these temperatures. Our results showed that the mean ecology relevant temperature range of Cururu toads (*R. icterica*) varies between 17°C at operative temperature, and 26°C at fever body temperature in toads injected with LPS. The temperature had the major impact on metabolic rate of the toads during infection. But, at fever body temperature, toads decrease the metabolic response of reaction to infection, indicating that energetic cost of immune response coevolved to optimize at fever body temperature.

Key words: Toads, infection, behavioral fever and metabolic response

Introduction

In ectothermic animals, preferred body temperatures likely reflects an integrated physiological response at which several physiological processes approach maximal efficiency (Hutchson & Dupré, 1992) .However, amphibians may experience a high daily and seasonal variability of body temperature due the conflicts of the requirements for respiration and hydroregulation with those for thermoregulation (Rome et al., 1992). Thus, optimal performance temperatures activities can both match as does not match the preferred temperature or even the normal temperature range of activity of the animal (Huey et al., 1989; Hutchison & Dupré, 1992; Navas, 1996).

During infection, amphibians develop behavioral fever when exposed to a heterothermal gradient in laboratory condition or outdoor enclosures (Kluger, 1977; Myhre et al., 1977; Sherman et al., 1991; Bicego-Nahas, 2000; Llewellyn et al., 2012). There is also some evidence that, at least for one anuran species, infected individuals show higher body temperatures than uninfected ones in natural environments with high thermal quality (Richards-Zawacki, 2010). Studies conducted with different vertebrates indicated that fever shortens the duration of infection and enhances survival rates of the host, however at the expense of energetic metabolic costs to generate and maintain febrile response (Kluger, 1991; Sherman & Stephens, 1998; Lochmiller & Deeremberg, 2000; Jiang et al., 2000; Hasday et al., 2000; Blatteis, 2003; Ashley et al., 2012). Furthermore, fever can indirectly cause collateral tissue damage, as a consequence of the enhanced microbial killing mechanisms (Hasday et al., 2000; Blatteis, 2003; Ashley et al., 2012). Possibly, immunological processes that are usually active in the presence of fever have evolved to function optimally at febrile rather than higid body temperatures (Hasday et al., 2000; Jiang et al., 2000; Blatteis, 2003; Boltaña et al., 2013). Fever, for example, suppressed plasma TNF-α expression, delayed gamma

interferon expression, improved survival, and reduced the bacterial load in mice infected with *Klebsiella pneumoniae* peritonitis (Jiang et al., 2000). Jiang and collaborators (2000) suggested that the increase in core temperature that occurs during bacterial infections is essential for optimal antimicrobial host defense. Additionally, behavior fever induces a major, coordinated upregulation of anti-viral genes in zebrafish (*Danio rerio*) infected with carp virus. Fish that cannot express behavioral fever show decreased survival under viral challenge (Boltaña et al., 2013). Boltaña and collaborators (2013) suggested that behavioral fever act as integrative signal that orchestrates biological output by promoting specific protein production in responding cell populations. This, in turn, leads to increased efficacy of defence traits and provides a positive adaptive value to the host (Boltaña et al., 2013).

Different from endothermic animals, that generate high-metabolic derived heat production and heat-seeking behavior, ectothermic animals depend only on behavioral mechanisms to raise body temperature during infection response (Vaughn et al., 1974; Kluger, 1977; Sherman et al., 1991; Hasday et al., 2000). Additionally, metabolic rates during infection might be much more variable in ectotherms than endotherms (McNab, 2002). Given the greater dependence on the environment thermal quality to regulate body temperature, ectothermic vertebrates might be active in the field at temperatures that do not achieve even the higid preferred temperatures, the more thermal optima for immunological processes that characterize a fever response (Hutchison & Dupré, 1992; Hertz et al., 1993; McNab, 2002). This effect should be more prominent in amphibians, that depends of environments with high thermal diversity and water availability to elevated and stabilize its body temperature, and because they are active mainly during the night, when environment generally has low thermal quality (Tracy, 1976; Hutchison & Dupré, 1992; Navas et al., 2008; Noronha-de-Souza et al, 2015).

Additionally, given that body temperature varies directly with environmental temperature, it is possible to disassociate the contribution of elevated temperatures and upregulated immune function to the metabolic energy cost of behavioral fever in amphibians. For example, toads (*Rhinella marina*) injected with lipopolysaccharide of gram-negative bacteria (LPS) showed higher metabolic rates than control at fever body temperature, suggesting that immune stimulation generated additional metabolic cost (Sherman & Stephens, 1998).In this way, amphibians are an excellent model to test hypotheses about the energetic cost of infection in a range of ecologically relevant temperatures, simulating situations that the animals can confront in nature and during which the individuals have the opportunity or not to thermoregulate, including: A) operative temperature in the field (thermoconformer animal); B) preferred temperature independent of ecological costs and constraints (higid thermoregulator animal); and (C) preferred body temperature during regulated elevation of set-point body temperature (thermoregulator animal during infection). Behavioral fever in amphibians is commonly studied by exposing an individual to pyrogenic substance lipopolysaccharide (LPS) (Sherman et al., 1991; Bicego-Nahas et al., 2000; Llewellyn et al., 2012). LPS is a cell-wall component of gram-negative bacteria that is recognized by pathogen-associated molecular patterns (PAMPs) cell membrane, triggering host immune response (Beutler, 2004; Ashley et al., 2012). The advantage of purified-LPS injection is that simulates bacterial infection independent of other pathogen-imposed costs of disease (Adelman & Martin, 2009).

From these considerations, we hypothesized that infection increases metabolic rates of the toads (*Rhinella icterica*) due to the activation of the immune system at different temperatures, but the energetic cost of immune response is lower at fever body temperature. To test this hypothesis we first collected data of operational temperature

using agar models connected to temperature data loggers placed in the microhabitats used by the toads during reproductive activity. Second, in the laboratory, we described the set-point range of the preferred temperature before and after infection simulation through LPS injection. Third, we measured standard metabolic rates of toads at mean operative and preferred body temperatures before and after infection simulation. Lastly, we calculated the metabolic response of the toads to infection simulation in each of these mean temperatures.

In this way, the predicted results were:

1) Toads develop fever behaviorally in the thermal gradient after to be injected with LPS;

2) The preferred body temperature before treatment is higher than operative body temperature during the active period in the field;

3) The preferred body temperature of infected toads is higher than preferred body temperature of higid toads.

4) The metabolic rates before (standard) and after treatments are positively associated with the temperature tested;

5) Infection simulation by LPS increases metabolic rates at all temperatures tested;

6) The metabolic response is reduced at fever body temperature of the toads.

Materials and methods

Experimental animals and laboratory maintenance

Rhinella icterica is a large Bufonidae from the *Rhinella marina* species group, found throughout the south and southeastern Brazil Atlantic Rainforest, from Rio de Janeiro State to Uruguay (Stevaux 2002; Pramuk et al. 2008). We collected the animals in 2014 during reproductive season at two localities. We captured 25 adult males of *R. icterica* at the Botanical Garden of Universidade Estadual Paulista (Botucatu-SP/Brazil) (22°53'38.01" S; 48°30'7.79" W) between July and August, and 17 at the Botanical Garden of São Paulo (São Paulo-SP/Brazil) (23°38'19.3" S 46°37'27.7" W) in September. The toads were located by visual inspection and captured by the hands. Individuals were then transported to the laboratory in individual plastic containers for later additional data collection. In laboratory, the toads were kept in individual plastic containers (0.40 m x 0.30 m x 0.25 m) and exposed to natural light/dark cycles and temperature, with water freely available and a piece of PVC pipe to be used as a shelter. The toads were regularly fed with cockroaches at each 05 days. Their containers were cleaned every day in order to prevent parasite autoinfection. The toads had fasted for 3 days before each trial test of preferred temperature in the thermal gradient and metabolic rate measurements (Andrade et al., 2005). All measurements in the laboratory were carried out between July and November of 2014.

Operative temperature in the field

Temperature sensors attached to data logger (HOBO) was put inside four agar models (4.7%) of two different masses (~20g and 40g) in microhabitats used by the toads during their reproductive activity. Two agar models of each mass were put in contact with the soil and the other two in contact with the water. Data were recorded

during 24 hours in intervals of 10 minutes. Only data recorded during the period of activity in the field were used for posterior analyses (from 18:00h to 05:00h).

Preferred body temperature set-point range in the thermal gradient

The preferred body temperature of toads was determined in a thermal gradient located in a room with natural cycles of temperature and photoperiod conditions. The thermal gradient used is composed of an aluminum floor (1.10 m long, 0.30 m height and 0.50 m wide) with the surface covered with adhesive contact paper. This procedure reduced infrared radiation reflectance of the aluminum and provided a surface characterized by the same emissivity of toad skin (Kastberger & Stachl, 2003; Tattersall et al. 2004). One end of the thermal gradient floor was cooled at 10°C with a metal streamer immersed in a water bath, and the other end was heated to 40°C with an electrical resistor immersed in another water bath (Eletrolab). A third water bath connecting the two ends kept the temperature along the gradient more homogeneous. To avoid toad dehydration and allow access to water at all temperatures, 12 petri dishes (r = 2.25cm) filled with water were placed throughout the gradient at each 9.0 centimeters.

The body temperature was determined as the superficial body temperature of the toads measured with a non-invasive method by infrared radiation (IR) thermal images obtained from a camera sensitive to IR (FLIR Systems SC660 equipped with IR lens 24 mm) (Figure 01). This device detects electromagnetic radiation of wavelength infrared (7.5 - 13 µm) emitted by objects, produces a 12-bit image (640x480 pixels) and stores the temperature information of each pixel at a resolution of 0.1°C. We assumed an emissivity of 0.95 for the toad skin, which is a reasonable estimate for biological tissues according to methodology of Tattersall and collaborators (2004).

To standardize the use of the IR thermal images, a preliminary test was performed using a thermocouple (Thercouple T, Omega, USA) inserted and secured 2cm inside the cloaca of the toads (N = 2). The thermocouples were connected to data loggers (HOBO, Onset) that recorded body temperature each 10 minutes for 20 hours. During the same period, IR thermal images were taken and recorded at each 10 minutes. We performed independent t-test to analyses difference between the body temperature recorded from thermocouple that from IR thermal images. The cloacal temperatures recorded did not differ from the superficial body temperatures derived from the thermographic camera records of the toads (Figure 02; t_1>-3.56, *P*>0.07).

The thermal gradient was divided in the middle with a styrofoam plate (1.10 m long, 0.30 m of height, 0.05 m thickness). This procedure enabled to perform measurements of two toads at each time. The animals were placed in the middle of each side of the thermal gradient at 14:00h and records started at 16:00h. Thermal images were taken and recorded at 10 minute-intervals for 24 hours. After this period, toads were randomly divided in two groups. One of the groups (N=7) was injected with100µL of saline (NaCl 0.9%), while the other group (N=8) was injected with 2.0 mg/kg body weight of LPS (from *Escherichia coli*, serotype 0127:B8, Sigma-Aldrich Chemical) diluted in 100µL of saline. Injections were performed at the dorsal lymph sac of the animals (Bicego et al., 2002), and preferred body temperature was monitored for additional 24h. IR thermal images were analyzed using professional analyzing software (FLIR ResearchIR).

The range between the lower and upper quartiles was used to calculate the set-point range of preferred body temperature of the toads (Hertz et al., 1993; Noronha-de-Souza et al., 2015). The means of: A) operative temperature in the field; B) preferred body temperature before treatments; and C) preferred body temperature during

manifested behavioral fever (for a period of 12 hours starting 2-hours after treatments) were chosen to measure the metabolic rates of the toads before and after treatments with saline or LPS (for procedure details see *Metabolic Rate* section).

Metabolic Rates (MR)

The MR of thirty males of *R. icterica* were determined as the oxygen consumption rates obtained by intermittent flow respirometry (Bartholomew and Lighton, 1986; Gomes et al, 2004). Toads were randomly divided in three groups, and measurements of oxygen consumption rates at standard conditions were conducted at the temperatures of 17°C, 22°C, and 26°C. After measurements of MR at standard conditions, toads within each temperature were randomly assigned to injections of 100µL of saline (NaCl 0.9%) (N=5) or 2.0 mg/kg body weight of LPS (from *Escherichia coli*, serotype 0127:B8, Sigma-Aldrich Chemical) diluted in 100µL of saline (N=5). The injections were performed into the dorsal lymph sac of the animals (Bicego et al., 2002), and the rates of oxygen consumption were monitored for additional 72h at 17°C, and 48h at 22°C and 26°C. Before the measurements, the toad´s containers were placed for two hours inside an environmental cabinet (Model 011, Eletrolab), where the set for respirometry was placed. This cabinet was set to the test temperature and at photoperiod similar to the natural conditions (12D:12N). Posteriorly, the toads were individually placed inside a transparent cylindrical metabolic chambers (r = 5.5cm; h = 7.0cm, vol = 660ml) and remained for additional two hours before recording the oxygen consumption, in order to recover from handling stress, assume a resting posture and achieve experimental body temperature (Gomes et al., 2004). During this period, humidified air was pumped (350 $mL.min^{-1}$) into the metabolic chambers to prevent dehydration of animals and to prevent occurrence of hypoxia and/or

hypercapnia. Moistened cotton was also placed inside the metabolic chambers to guarantee hydration state during measurements (Gomes et al., 2004).

At the beginning of the data collection, the metabolic chambers were connected to the respirometry set. A pump with controlled flow mass (SS3-Sable System; Henderson, NV, USA) sent, sequentially for 10 minutes, constant air flow (350 $mL.min^{-1}$) to the metabolic chambers. After this 10 minutes period the air flow to the metabolic chambers sequentially were closed for 80 minutes at temperature test of 17°C and 60 minutes at temperature test of 22°C and 26°C, by using a control system of air flow with multiple channels (V3-Sable Multiplexer Systems; Henderson, NV, USA). After this period, chambers were sequentially opened for 10minutes, and the air flowed at the same flux to an oxygen analyzer (FC-10a O2 analyzer-Sable Systems; Henderson, NV, USA). This period of 10 minutes was enough for a complete volume air renovation of each chamber and to formation of a baseline for the next chamber to be opened. Data from the O_2 analyzer was recorded on a computer that was equipped with EXPEDATA 1.1.15 software (Sable Systems), via an UI2 interface (Sable Systems). We scrubbed air from the chambers of both water vapor and CO_2 via a column containing drierite/ascarite/drierite prior to gas analysis. We calculated $\dot{V}O_2$std as the derived oxygen consumption from the deflection area of a curve of oxygen concentration versus time after full washing of the chamber (Bartholomew & Lighton, 1996; Gomes et al., 2004). We measured body mass (0.01g) immediately before and after the tests, and the mean of these values was used to express $\dot{V}O_2$std as $mLO_2.g^{-1}.h^{-1}$. All of the respirometry tests were videotaped and only the moments when the toads were at rest were considered for the calculations.

The metabolic response to the simulated infection was calculated as the ratio between LPS:Saline metabolic rates at 17°C, 22°C and 26°C for the period of manifested behavioral fever (12 hours starting from 2-hours after treatments).

Statistical analyses

The variables were submitted to descriptive analyses and then log_{10}-transformed to increase normality of the data before statistical analyses. We used unpaired t-test to analyze differences between toads assigned to injection with saline and LPS before and after the treatment on the measurements of (1) preferred body temperature on the thermal gradient, and (2) metabolic rates in each of the temperatures tested. ANOVA followed by Bonferroni *post hoc* tests was used to analyze the effect of temperature on metabolic rates before and after treatments. ANOVA followed by Bonferroni *post hoc* tests also was used to analyze the effect of temperature on metabolic rates and metabolic response to LPS or saline injection for the period of manifested behavioral fever. In all analyses we considered $P<0.05$ as significant.

Results

Operative temperature in the field and preferred body temperatures in the thermal gradient

The mean temperatures that the animal can achieve thermoconforming in the field during the night and thermoregulating in the heterothermal environment in laboratory conditions were different [Table 01; $F(2, 287) = 3120.5$, $P<0.001$] The operative set-point range temperature of the toads in the field was lower than the preferred body temperature set-point range in the thermal gradient before treatments ($P<0.01$), and both were lower than the preferred body temperature set-point range in the thermal gradient after LPS treatment ($P<0.01$, Figure 03). Toads injected with LPS showed higher preferred body temperature than toads injected with saline in the thermal gradient (Figure 04; $t_{13} < -2.17$, $P<0.05$).

Effect of temperature on MR

The temperature increased the MR of the toads before treatments [Figure 05; $F(2,27) = 62.13$, $P<0.001$], after saline injection [Figure 05; $F(2,27) = 92.69$, $P<0.001$], and after LPS injection [Figure 05; $F(2,27) = 89.55$, $P<0.001$]. MR differ between 17°C, 22°C and 26°C in all situations ($P<0.01$).

Effect of treatments (saline versus LPS) on MR at 17°C, 22°C and 26°C

Toads exhibited increased MR for a period of 12 hours after 2 hours of the injection of LPS at 22°C when compared to toads injected with Saline (Figure 06B; $t_7 < -3.53$; $P<0.04$). MR of toads injected with LPS and saline did not differ at 17°C (Figure 06A; $t_8 > -1.61$; $P>0.10$) and at 26°C (Figure 06C; $t_8 > -2.19$; $P>0.08$).

Effect of temperature on MR during period of manifested behavioral fever

The temperature increased MR of the toads for 12 hours 2-hours after saline injection [Figure 07; $F(2,27) = 30.80$, $P<0.001$]. However, the MR of saline injected toads at 17°C and 22°C are similar ($P>0.07$) and lower than MR at 26°C ($P<0.001$) during this period. The temperature also increased MR of the toads for 12 hours 2-hours after LPS injection [Figure 07; $F(2,27) = 113.94$, $P<0.001$]. However, the MR at 26°C and 22°C are similar ($P>0.16$) and higher than MR at 17°C ($P<0.001$) during this period.

The temperature influenced metabolic response to simulated infection, but not linearly [Figure 08; $F(2,27) = 59.11$, $P<0.001$]. The metabolic response to infection simulation was higher at 22°C than at 26°C and 17°C ($P<0.01$)

Discussion

The Cururu toads (*R. icterica*) exposed to simulated infection have the ability to manifest behavioral fever when exposed to a heterothermal environment, corroborating our predictions and results from previous studies that exposed other anuran species to exogenous pyrogens like inactivated bacteria (*Aeromonas hydrophila*,and frogs Mycobacterium) and LPS in laboratory conditions (Kluger, 1977; Myhre et al., 1977; Sherman et al, 1991; Bicego et al., 2002). Although males of *R. icterica* have the ability to manifest behavioral fever in a thermal gradient, our data show that the environmental temperatures available during their active nocturnal phase are well below the temperatures preferred by toads that are higid and exposed to simulated infection. Nocturnal activity may be an important constraint to body temperature regulation in anurans, given that it restricts the possibility of heat gain from the environment (Hutchison & Dupré, 1992; Noronha-de-Souza et al., 2015). This statement has been corroborated by an study conducted in semi-captivity conditions with a phylogenetically close species (*R. schneideri*), even considering that the period of reproductive activity is largely limited to seasons with a higher thermal quality for this species (Noronha-de-Souza et al., 2015).One possibility to infected nocturnal toads to slightly elevate and stabilize body temperatures might be to bask in water bodies that have gained heat during the day (Lillywhite, 1970; Lillywhite, 1971; Hutchison and Dupré, 1992). Previous studies have shown that these toads maintain mean body temperatures during their active nocturnal phase in the field (18.8°C) closer to the water temperature (19.7°C) and higher than air temperatures (15.9) (Moretti, 2011). This is mostly due to the fact that these males use to call within or in contact with large water bodies (Moretti, 2011). These results suggest that, although nocturnal activity largely constrains body temperature regulation in *R. icterica*, their calling sites might offer an opportunity to

restricted thermoregulation. However, few studies have shown the ability and ecological relevance of the behavioral fever in the nature. Llewellyn and collaborators (2012), for example, showed that cane toads (*R. marina*) manifested behavioral fever in the thermal gradient and outdoor enclosure conditions, but did not manifest behavioral fever in natural condition. These limited observations of manifested behavioral fever by amphibians in the field may reflect more the lack of appropriate thermal diversity of the environment than lack of ability (Hutchison and Dupré, 1992; Llewellyn et al., 2012).

Although the maintenance of higher body temperatures can promote beneficial effects associated to immune response and enhanced survival, it may also increases the metabolic cost to the host (Kluger, 1991; McNab, 2002; Ashley et al, 2012). The metabolic cost is associated to the effect of increase body temperature set-point, and also should reflect the activation and maintenance of immune system to combat infection (Kluger, 1991; Sherman and Stephens, 1998; Lochmiller and Deerember, 2000; Llewellyn et al., 2011; Ashley et al., 2012). As expected, our results show that temperature influences the metabolic rates of the toads. Consequently, the metabolic rate is higher at preferred body temperature of infected toads. Moreover, our results indicate that toads should sustain this increase for at least the period of 12 hours. As consequence, infected toads could deplete energetic reserves and compromise others ATP-requiring process (Clarke and Fraser, 2004; Llewellyn et al., 2012). However, balances between increased energy expenditure with mechanisms to save energy (e.g.: sickness behavior), should regulate the physiological process involved in the combat to infection (Adelman and Martin, 2009; Llewellyn et al., 2012). Additionally, it is suggested that if the energy reserves are not sufficient to sustain regulated elevation of body temperature, the infected individual may resort to hypothermia, another way of save energy in severe infection (Deen and Hutchison, 2001; Steiner and Romanovsky,

2007; Krall et al., 2010; Liu et al., 2012; Sköld-Chiriac et al., 2015). However, future investigations are necessary to better understand the effect of infection severity in the energetic balance in *R. icterica*.

Unlike our predictions, the results show that additional metabolic cost associated to immune response occur only at preferred body temperature of the higid toads, and seemed irrelevant at operative and fever body temperatures. A possible explanation for these results is that immune system kept inactive when stimulated at low body temperature after infection simulation (Avtalion et al., 1973). On the other hand, at the higher body temperatures of infected toads, the immune response might exhibit lower energetic cost for activation than at preferred body temperature of higid toads, decreasing the metabolic scope of activation. Another possibility is that, at the higher preferred temperatures, the individuals might have reached a metabolic ceiling (Clarke and Fraser, 2004). However, this hypothesis is very improbable, given that metabolic rates during aerobic locomotor performance in Bufonids exceed more than 2.5 times the metabolic rate during rest (Gatten, Jr. et al., 1992) and our results show that metabolic rate of infected toads at 26°C is only 40% higher than metabolic rate of higid toads at same temperature. However, more tests are necessary to understanding better the effect of temperature on parameters associated to metabolic costs of immunological response to infection in *R. icterica*.

Besides the fact that LPS-infected toads increase the set-point of thermal regulation, metabolic rates of LPS-injected toads are similar at preferred and fever body temperatures. Given that standard metabolic rates are higher at the fever body temperatures, these results indicate that the metabolic scope associated exclusively with the immune processes involved in the inflammation response should decrease at the higher body temperatures associated with behavioral fever in *R. icterica*. One possible

explanation for this result is that controlled rises in body temperature coevolved with optimized immunological processes. Several studies have proposed that, despite the increased energy expenditure, the regulation and effectors of infection combat improve in elevated body temperatures (Kluger, 1991; Hasday 2000; Blatteis, 2003; Boltaña et al., 2013; Nord et al., 2013). Studies in both mice and fish have shown that fever is essential to optimize immunological response to combat infection, increasing the survival of the hosts (Jiang et al., 2000; Boltaña et al., 2013). In this way, metabolic scope associated with the response to the infection might be also optimized. On the other hand, at operative body temperature, metabolic cost and metabolic response to infection is irrelevant. Additional studies are necessary to investigate the effectiveness of immune reaction at these different temperatures, as well as its relationships with the metabolic costs of infection reaction at these temperatures.

In conclusion, our results show that Cururu toads (*R. icterica*) injected with LPS manifested behavioral fever when exposed to heterothermal environment. Additionally, the operative body temperature in the period of reproductive activity is well below the preferred body temperature of the toads, and the metabolic rate not increases in response to infection at this temperature. Our results also show that the metabolic rate is higher at preferred body temperature of infected toads. Moreover, metabolic costs associated to the response to infection are lower at the fever body temperature when compared to preferred body temperature of higid toads.

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Table 01. Descriptive analyses of the operative temperature with agar model in the field and the preferred body temperature set-point range in the gradient thermic independent of ecological costs and constrains (before treatments) and the set-point range of the body temperature after infection simulation with saline or LPS (after treatments).

Variable		N	Mean	Standard deviation	Minimum	Maximmun	Confidence interval -95.0%	Confidence interval +95.0%
Body temperature (°C)	Agar model	2 (66)	16.7	0.2	15.5	17.8	16.6	16.7
	Before treatment	15 (144)	22.7	2.6	11.3	30.0	1.9	4.2
	Saline during behavioral fever	7 (66)	22.8	2.1	15.3	28.9	1.3	4.5
	LPS during behavioral fever	8 (66)	25.7	1.3	20.3	30.6	0.9	2.8
Metabolic rate at 17°C ($mLO_2.g^{-1}.h^{-1}$)	Before treatment	10	0.013	0.003	0.003	0.037	0.012	0.013
	After saline treatment	5	0.013	0.002	0.004	0.027	0.012	0.013
	After LPS treatment	5	0.011	0.002	0.002	0.030	0.011	0.012
Metabolic rate at 22°C ($mLO_2.g^{-1}.h^{-1}$)	Before treatment	10	0.024	0.010	0.004	0.054	0.023	0.025
	After saline treatment	5	0.019	0.008	0.005	0.057	0.018	0.021
	After LPS treatment	5	0.030	0.009	0.012	0.082	0.029	0.032
Metabolic rate at 26°C ($mLO_2.g^{-1}.h^{-1}$)	Before treatment	10	0.027	0.009	0.008	0.050	0.027	0.028
	After saline treatment	5	0.026	0.010	0.012	0.049	0.025	0.028
	After LPS treatment	5	0.036	0.011	0.018	0.069	0.034	0.037

Figure 01. Superficial body temperature of the toads measured by infrared thermal images obtained from a camera sensitive to infrared radiation (FLIR Systems SC660 equipped with IR lens 24 mm). Sp1 and Sp2 indicate the superficial body temperature of the toads. The color vertical square indicate temperature range of the thermal gradient.

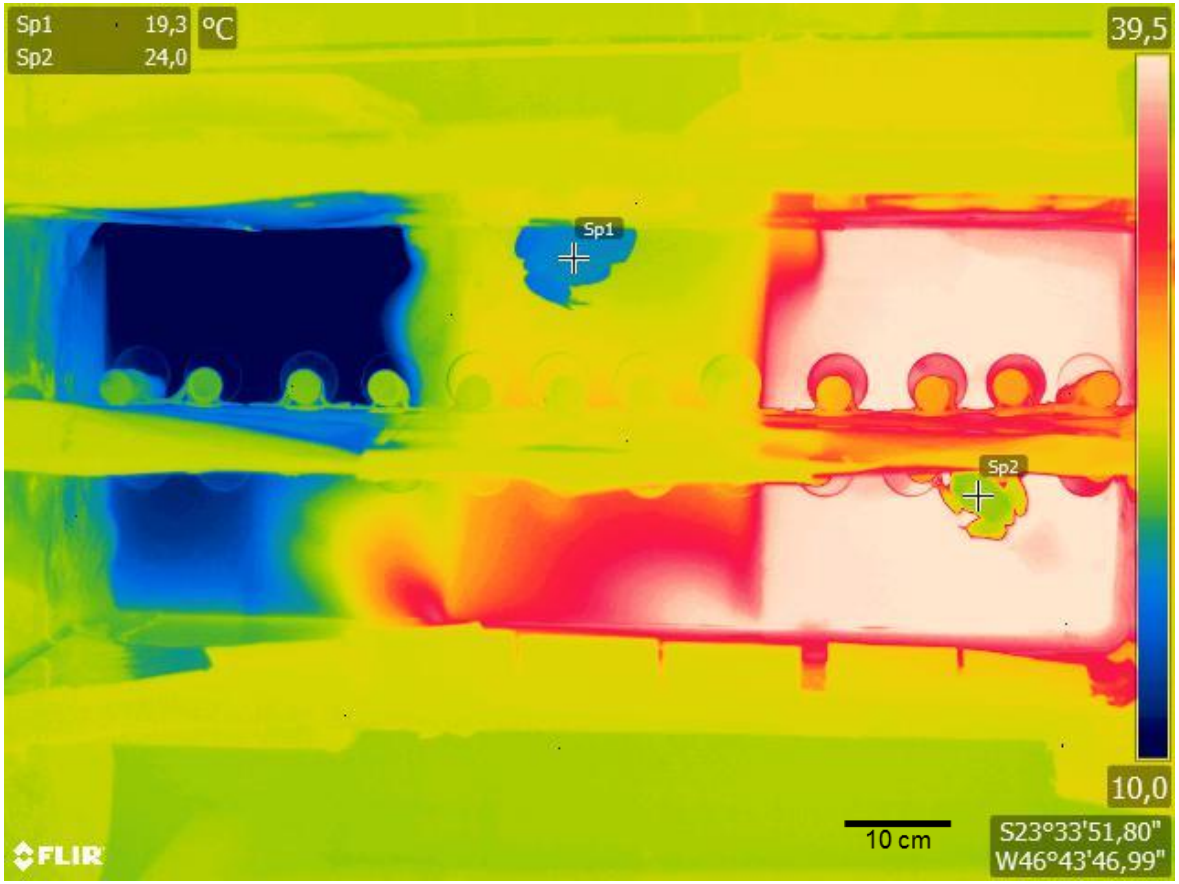


Figure 02. Body temperature of toads (N=2) recorded with the thermographic camera (filled line) and, at the same time, body temperature of toads recorded with a thermopar (Model T), secured 2cm in the cloaca of the toad (dashed line). Values are means ± standard deviation. Body temperature of the toads measured with the thermographic camera did not differ from the body temperature measured with thermopar (t-test; *P*>0.49).

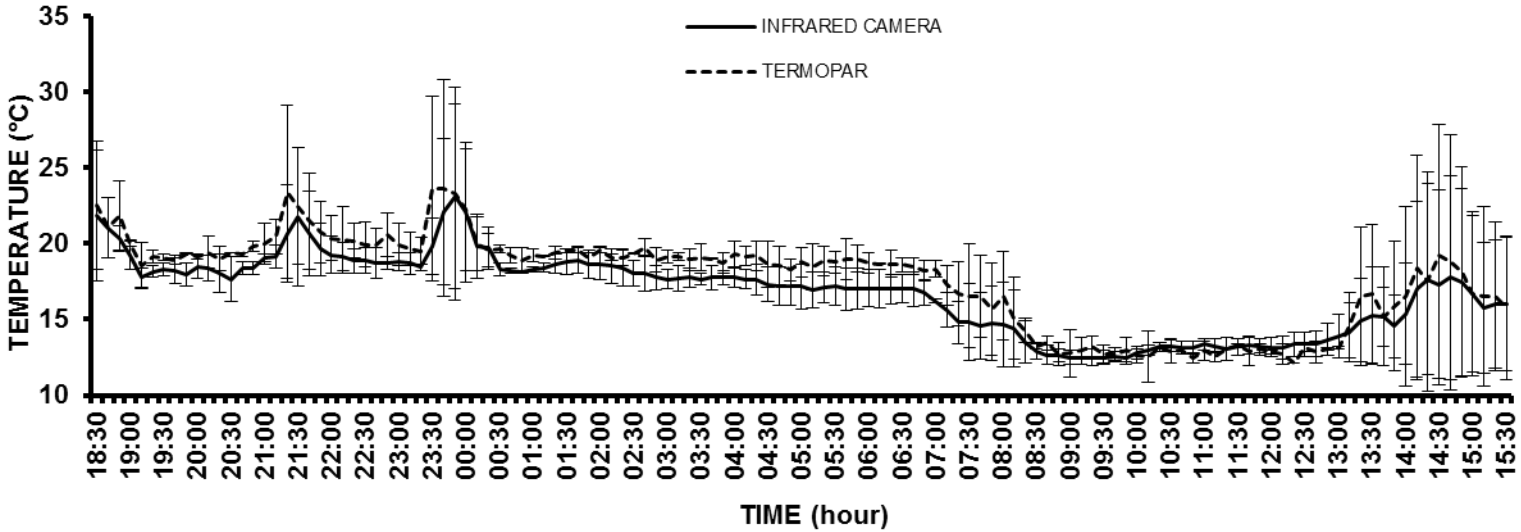


Figure 03. Frequency distribution of the operative temperature with agar model in the field and the preferred body temperature set-point range in the thermal gradient independent of ecological costs and constrains (before treatments) and the set-point range of the body temperature after infection simulation with saline or LPS. Vertical dashed line show temperature set-point range.

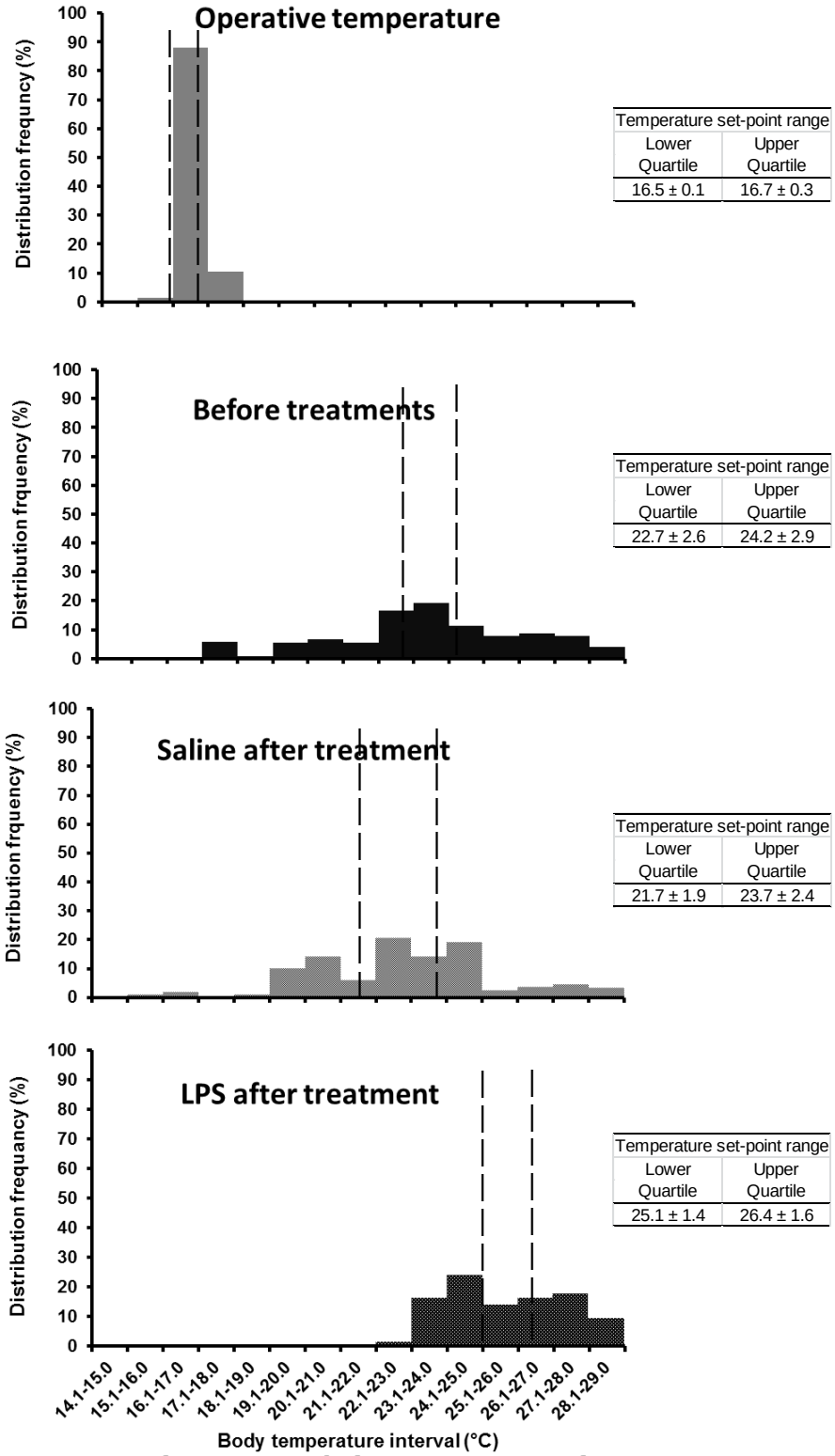


Figure 04. Preferred body temperature of the toads in the thermal gradient for the period of 24 hours before treatments, and 24 hours after saline (bold line) or LPS (gray line) injections. Arrow head indicate the injection hour of saline or LPS. White square (day) or black square (night) below the coordinate axis indicate photoperiod phase. The values are means ± standard deviation. *P<0.05 (unpaired t-tests, $-4.13 < t_{13} < -2.17$).

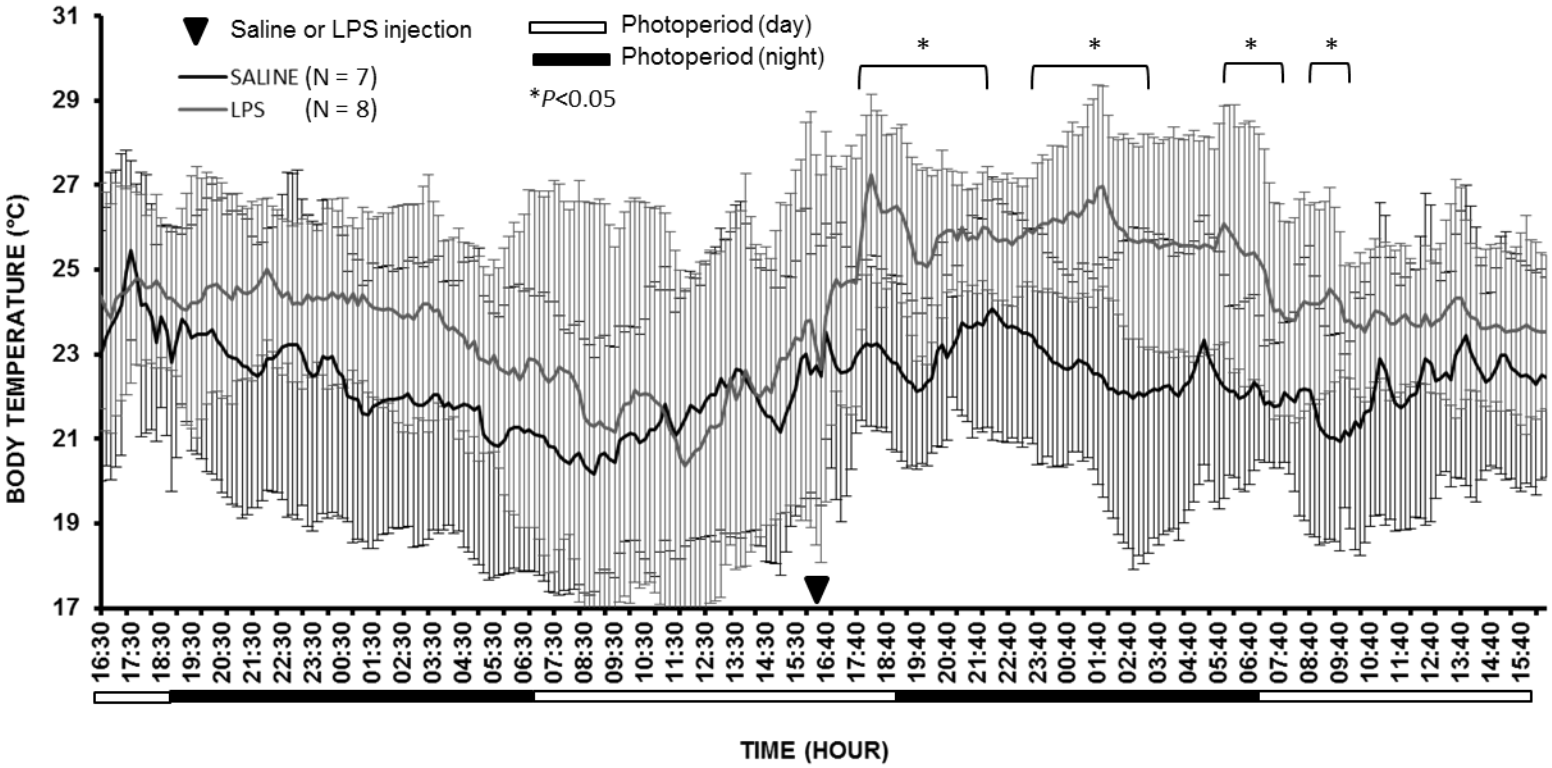


Figure 05. Effect of temperature on metabolic rates (MR) of the toads: before treatments; after saline injection; and after LPS injection. MR increase with temperature before treatments [Anova; $F(2,27) = 62.13$, $P<0.001$], after saline injection [Anova; $F(2,27) = 92.69$, $P<0.001$], and after LPS injection [Anova; $F(2,27) = 89.55$, $P<0.001$]. Values are means ± standard deviation. Different letters indicate differences in the MR at different temperatures ($P<0.01$, Bonferroni *post hoc* test). $\dot{V}O_2$ = rate of oxygen consumption.

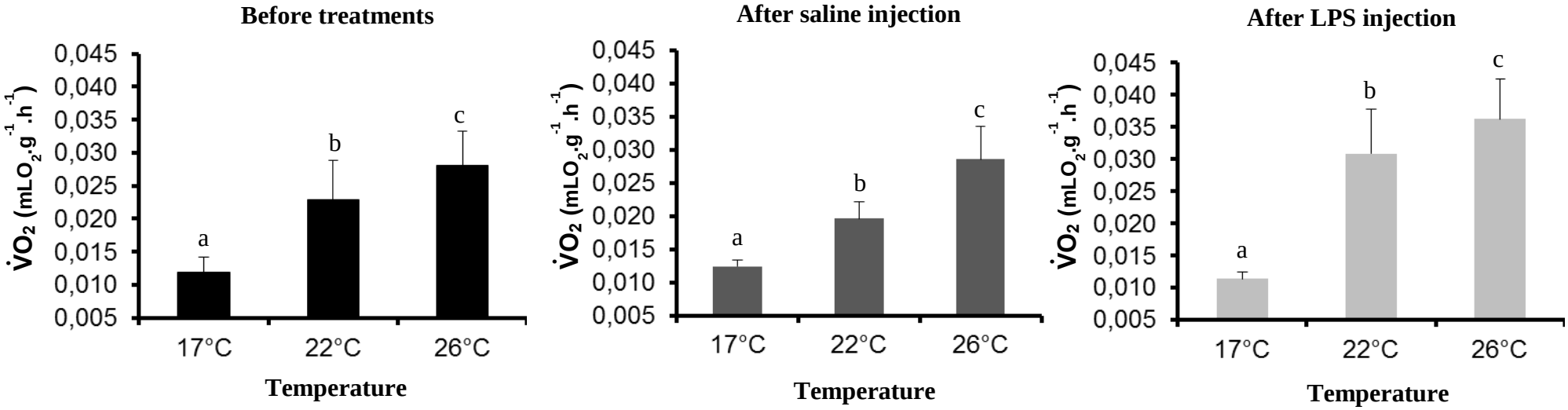
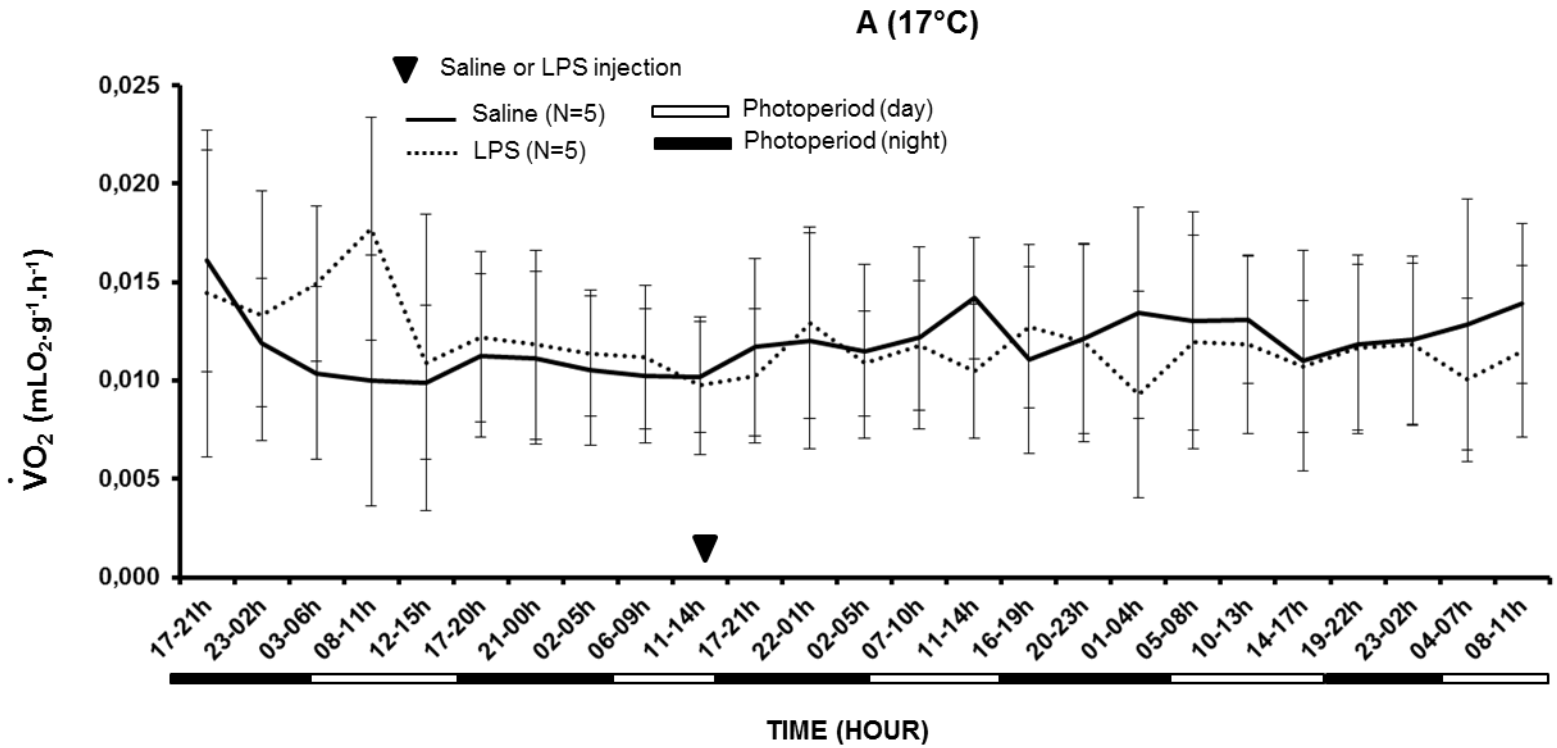
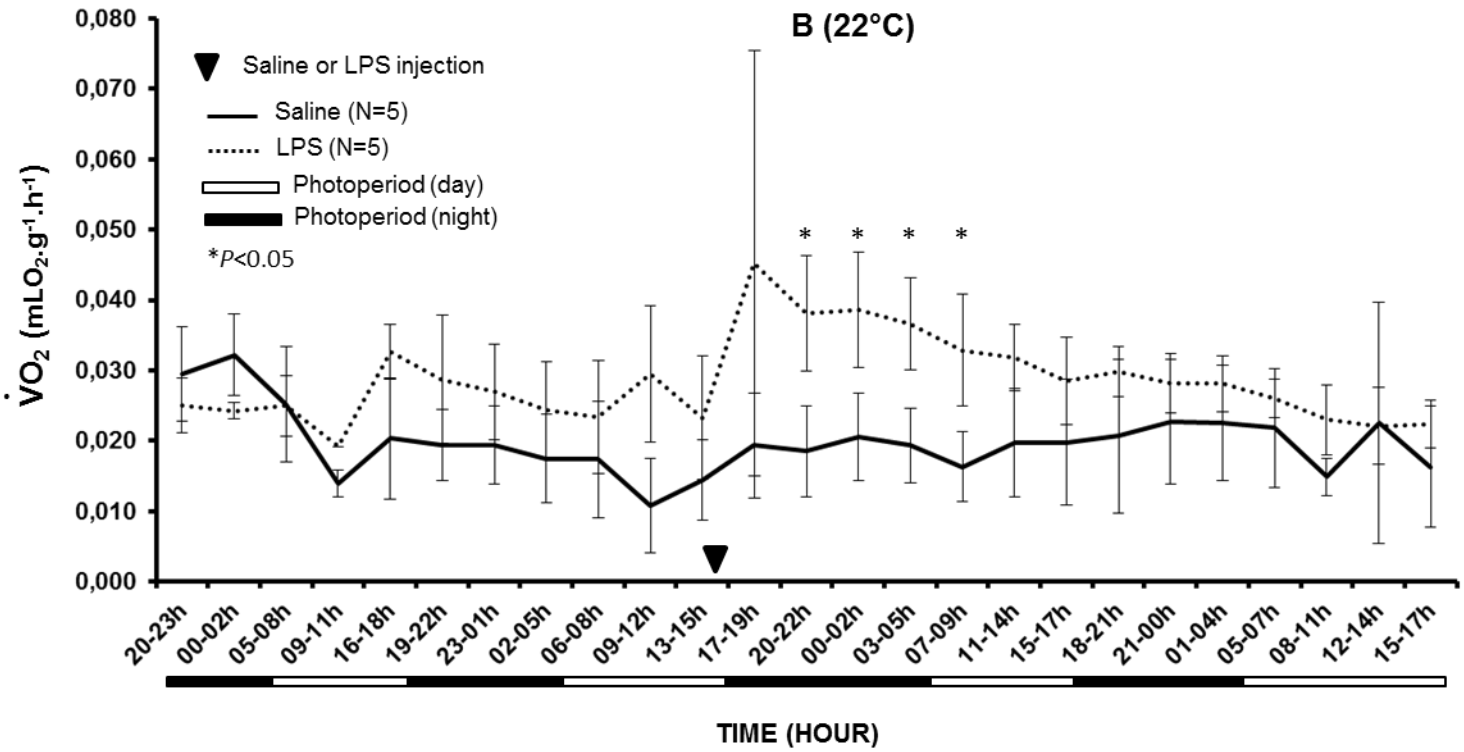


Figure 06. Metabolic rates of the toads before treatments and after saline (filled line) or LPS (dashed line) injections at: 17°C (A), 22°C (B) and 26°C (C). Arrow head indicate the injection hour of saline or LPS. White square (day) or black square (night) below the coordinate axis indicate photoperiod phase. The values are means ± standard deviation. *P<0.05 (unpaired t-test, -4.36 < t_8 < 1.80). $\dot{V}O_2$ = rates of oxygen consumption.




B (22°C)
Saline or LPS injection
Saline (N=5)
LPS (N=5)
Photoperiod (day)
Photoperiod (night)
*P<0.05
V̇O2 (mLO2.g-1.h-1)
0,080
0,070
0,060
0,050
0,040
0,030
0,020
0,010
0,000
20-23h
00-02h
05-08h
09-11h
16-18h
19-22h
23-01h
02-05h
06-08h
09-12h
13-15h
17-19h
20-22h
00-02h
03-05h
07-09h
11-14h
15-17h
18-21h
21-00h
01-04h
05-07h
08-11h
12-14h
15-17h
TIME (HOUR)

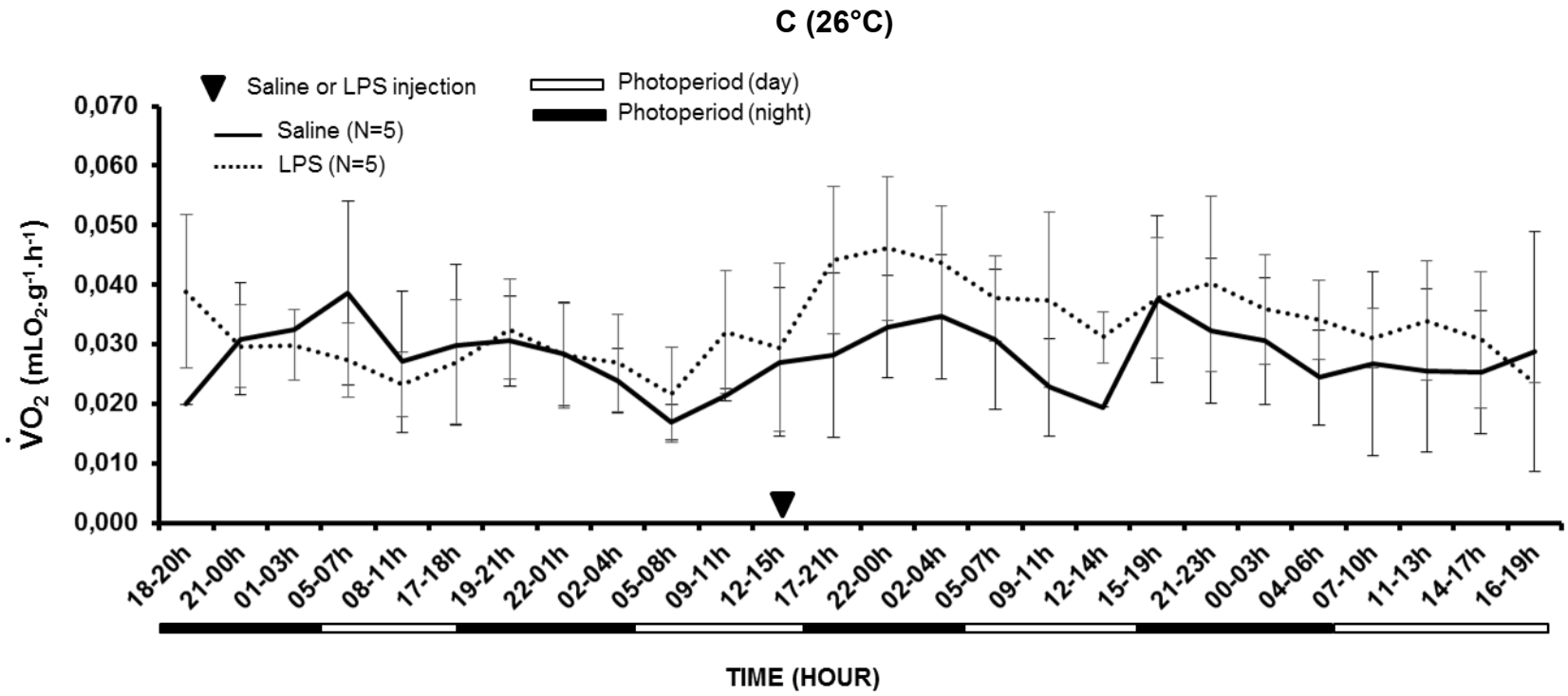

C (26°C)
Saline or LPS injection
Photoperiod (day)
Photoperiod (night)
Saline (N=5)
LPS (N=5)
0,070
0,060
0,050
0,040
0,030
0,020
0,010
0,000
$\dot{V}O_2$ (mLO$_2$.g^{-1}.h^{-1})
18-20h
21-00h
01-03h
05-07h
08-11h
17-18h
19-21h
22-01h
02-04h
05-08h
09-11h
12-15h
17-21h
22-00h
02-04h
05-07h
09-11h
12-14h
15-19h
21-23h
00-03h
04-06h
07-10h
11-13h
14-17h
16-19h
TIME (HOUR)

Figure 07. Effect of temperature on metabolic rates (MR) of the toads during 12 hours period 2-hours after treatments: with saline or LPS. MR increase with temperature after saline injection [Anova; $F(2,27) = 30.80$, $P<0.001$], and after LPS injection [Anova; $F(2,27) = 113.94$, $P<0.001$]. Values are means ± standard deviation. Different letters indicate difference in the MR at different temperatures ($P<0.01$, Bonferroni *post hoc* test). $\dot{V}O_2$ = rates of oxygen consumption.

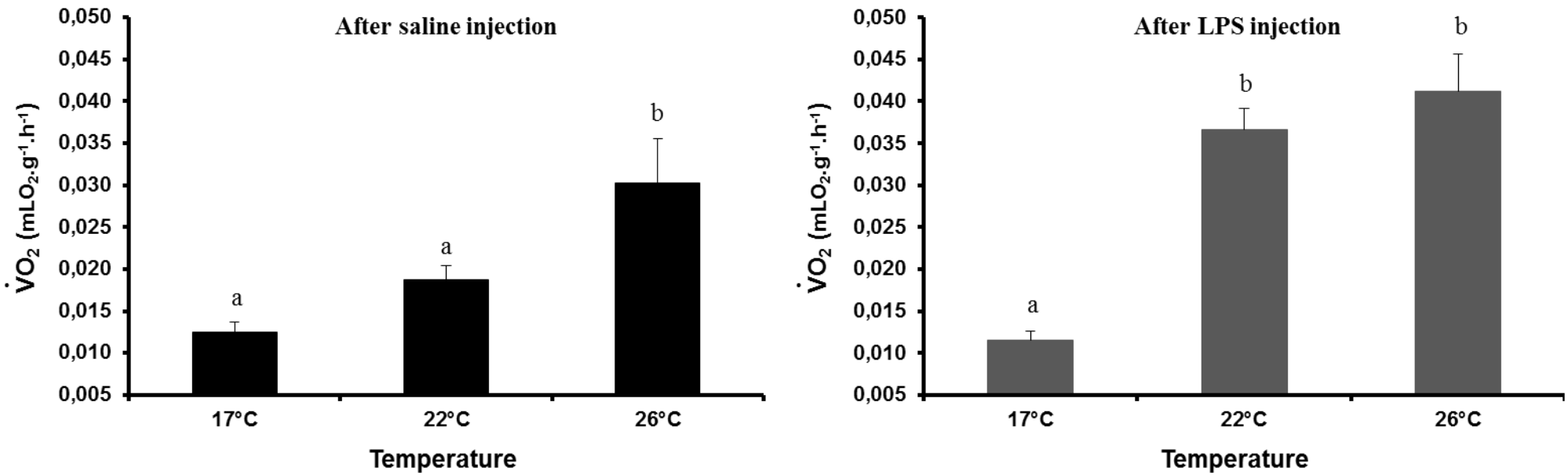
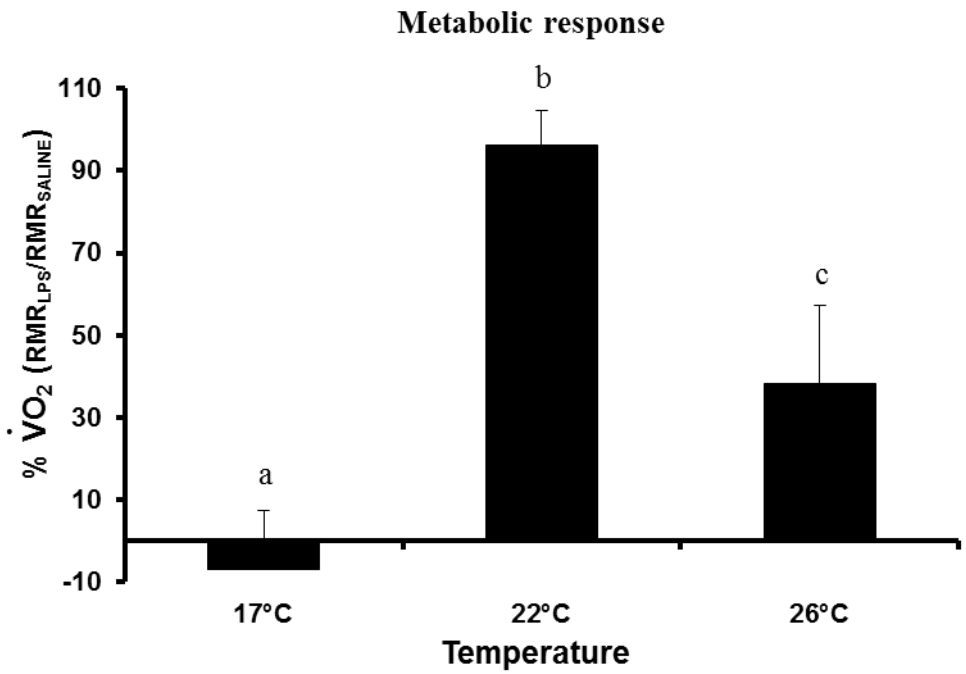


Figure 08. Metabolic response of the toads to infection simulation (LPS:Salina ratio) for 12 hours period 2-hours after treatments at 17°C, 22°C and 26°C. Metabolic response differ at different temperatures [Anova; $F(2,27) = 59.11$, $P<0.001$]. Values are means ± standard deviation.

Different letters indicate difference in metabolic response at different temperatures ($P<0.01$, Bonferroni *post hoc* test). $\dot{V}O_2$ = oxygen consumption rate.



Conclusões Gerais

Conclusões gerais

Nosso trabalho mostrou, como esperado, que o sapo Cururu é capaz de manifestar febre comportamental em ambientes de alta qualidade termal. Entretanto, a temperatura operacional no campo, durante o período que os sapos estão em atividade reprodutiva, é muito menor que a temperatura corpórea dos sapos hígidos, indicando uma restrição à capacidade de elevar e estabilizar a temperatura corpórea nestas condições. Contudo, não ocorre aumento de taxa metabólica em resposta à infecção simulada nessa temperatura.

Mostramos também, como previsto, que a taxa metabólica é maior na temperatura preferencial dos sapos infectados. Contudo, ao contrário do que esperávamos, mostramos que a simulação de infecção por LPS aumenta a taxa metabólica somente na temperatura preferencial dos sapos hígidos, sendo este efeito irrelevante na temperatura operacional e na temperatura preferencial dos sapos infectados. Talvez, não haja ativação do sistema imune na temperatura operacional. Por outro lado, o custo energético de ativação imunitária parece ser menor na temperatura preferencial dos sapos infectados do que na temperatura preferencial dos sapos hígidos, diminuindo o escopo metabólico da ativação do sistema imune. Estes resultados indicam uma provável coevolução entre o aumento controlado do ponto de ajuste "*set-point*" da temperatura corpórea com custo energético otimizado da resposta imune.