LAURA CAMILA CABANZO OLARTE

Estudo sobre respostas comportamentais à infecção no anfíbio anuro

Proceratophrys boiei

A study of behavioral responses to infection in the anuran amphibian

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São Paulo

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Dedicatória

À minha família, pelo amor, incentivo, suporte e sempre ser o
porto mais seguro.

Andrea, Matias, Hilda, Rafael e Chucho.

Epígrafe

“Después de todo, ¿qué es un científico entonces? Es un Hombre curioso que mira a través del ojo de una cerradura, la cerradura de la naturaleza, tratando de saber qué es lo que sucede.”

Jacques Yves Cousteau
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Resumo geral

Esta tese tem como tema central o conceito de febre comportamental, que tem-se definido como o aumento da temperatura corporal pela efetivação da mudança nas temperaturas preferidas devido ao reconhecimento, por parte do corpo, de uma infecção ou patógeno. O trabalho está composto por três partes. Na Introdução Geral é discutida a febre comportamental em sete pontos fundamentais no entendimento desta resposta dentro da Ecofisiologia, começando pela definição, as pesquisas de laboratório e campo existente até o momento, até as limitações ecológicas dos indivíduos e as implicações do tema na conservação. No capítulo 1, com formato de texto científico, apresentamos a pesquisa na qual estudamos o comportamento e a preferência termal da espécie *Proceratophrys boiei* em condições experimentais com indivíduos injetados com lipopolisacáridos (LPS), para simular uma infecção, e indivíduos intactos (injetados com salina, grupo controle). Nessa pesquisa consideramos duas alternativas de respostas no comportamento, tal como discutido na introdução geral: a) febre comportamental, que é caracterizada por um aumento da temperatura corporal dos indivíduos pela mudança nas temperaturas preferidas dentro de uma paisagem termal; b) comportamento de doente, que no contexto do desenho experimental seria reconhecido pela diminuição da atividade dos indivíduos. Assim, registramos os seguintes tratamentos durante 24 horas com uma câmara termográfica: 1) indivíduos intactos no gradiente termal desligado, 2) indivíduos intactos no gradiente termal ligado, 3) indivíduos injetados com salina, no gradiente termal ligado 4) indivíduos injetados com LPS no gradiente termal ligado. Para cada um dos tratamentos foi registrada a distância de locomoção e as preferências termais, junto com outros detalhes do comportamento e as preferências termais. A partir de nossos resultados, concluímos que os indivíduos de *P. boiei* apresentam comportamento de doente como resposta dominante ao ser injetados com LPS, e que as preferências termais destes são consequência do comportamento de doente e não da termorregulação comportamental. Finalmente, a discussão geral explica como o capítulo 1 contribui na discussão de cada um dos sete pontos tratados na introdução geral tentando propor metodologias e estudos mais completos para manter o diálogo entre a fisiologia e a ecologia dos indivíduos no contexto de infecção e doenças.

Abstract

The central theme of this thesis is the concept of behavioral fever, which has been defined as the increase of body temperature by effecting the change in preferred temperatures due to the recognition by the body of an infection or pathogen. The thesis is composed of three parts. In the General Introduction, behavioral fever is presented around seven fundamental points to the understanding of this response within Ecophysiology, starting with the definition and the laboratory and field research until now, to the ecological limitations of individuals and the implications of this theme in conservation. In the first chapter, with scientific text format, we present the research in which we studied the behavior and thermal preference of *Proceratophrys boiei* species under experimental conditions in individuals injected with lipopolysaccharides (LPS), to simulate an infection, and in intact individuals (injected with Saline, a control group). In this research we considered two alternatives of behavioral responses, as discussed in the General Introduction: a) behavioral fever, which is characterized by an increase in the individuals body temperature by changing the preferred temperatures within a thermal landscape; B) patient behavior, which, in the context of experimental design, would be recognized by the decrease in the activity of individuals. Thus, we recorded the following treatments for 24 hours with a thermographic camera: 1) intact individuals in the thermal gradient switched off, 2) intact individuals in the connected thermal gradient, 3) individuals injected with saline, in the bound thermal gradient 4) individuals injected with LPS in the thermal gradient on. For each of the treatments it was recorded the locomotion distance and the thermal preferences, along with other details of the behavior and the thermal preferences. From our results, we conclude that the individuals of *P. boiei* present a patient's behavior as a dominant response when injected with LPS and their thermal preferences are a consequence of patient behavior and not behavioral thermoregulation. Finally, the general discussion explains how chapter 1 contributes to the discussion of each of the seven points highlighted in the general introduction attempting to propose a complete methodology and studies to maintain the dialogue between the physiology and the ecology of individuals in the context of infection and disease.

Introdução Geral

1. Conceito de Febre

Febre é o incremento regulado da temperatura corporal associado ao incremento do “*set point*” do hipotálamo. O “*set point*” é a temperatura limite onde a perda e a produção de energia são iguais. No caso da febre, a temperatura a ser regulada por esse *set point* aumentaria, levando a temperatura do corpo a níveis mais apropriados para a defesa contra um patógeno (Kluger, 1991; Bicego et al., 2000, Guyton and Hall, 2006; Blatteis, 2006).

A resposta febril requer uma série de ajustes fisiológicos e comportamentais que podem variar dependendo da linhagem e dos mecanismos termorregulatórios que a caracterizam (Bicego et al., 2007). Em mamíferos e aves por exemplo, o termo apresenta pouca ambiguidade e os mecanismos que levam à febre são conhecidos. O processo envolve as células de Kupffer no fígado, que são consideradas como o ponto central para a liberação de prostaglandinas (PGE₂), ativando a produção e liberação no sangue de agentes pirogênicos, como as citosinas. Este processo inicia uma cascata de respostas fisiológicas que agem no cérebro e terminam por ativar efetores que levam ao aumento da temperatura corporal (Kluger, 1991; Kluger et al., 1995; Blatteis, 2006). Entre mamíferos e aves existem exemplos de ativação de mecanismos fisiológicos capazes de elevar a temperatura, reduzir a perda de energia ou auxiliar na regulação, como o tremor e o aumento do fluxo sanguíneo regional (Bicego et al., 2007). Esses processos são principalmente fisiológicos mas envolvem também comportamento por mecanismos que também são controlados pelo sistema nervoso central (Stitt, 1973; D'Alecy & Kluger, 1975; Bicego et al., 2007). No entanto, uma diferença entre grupos de vertebrados é que mecanismos comportamentais dominam estados febris em peixes, lagartos e anfíbios. Sabe-se que a área preóptica (POA) pode influenciar o comportamento termoregulatório de animais desses grupos, e para o desenvolvimento da febre tem-se considerado que esta região cerebral possa ser um centro termorregulador susceptível de ser influenciado por processos patológicos (Bicego et al., 2007). Por exemplo, em anuros, a ativação do mecanismo da febre é via a cyclooxygenase (COX), via a produção e liberação das prostaglandinas (PGE₂) (Bicego & Branco, 2002; Bicego et al., 2002). Ainda nesse táxon, a arginina vasotocina (AVT) atua como agente antipirético no sistema nervoso central diminuindo o “set

point” termoregulatório (Bicego et al., 2000). Em algumas espécies de lagartos, anfíbios e peixes, processos como os descritos acima podem levar a uma resposta comportamental que modula a temperatura do corpo aparentemente como defesa à uma infecção ou patógeno, que é chamada febre comportamental (Bicego et al., 2000; Bicego & Branco, 2002).

2. O que é Febre comportamental?

O conceito de febre comportamental tem-se definido como o aumento da temperatura corporal pela efetivação da mudança nas temperaturas preferidas comportamentalmente devido ao reconhecimento de uma infecção ou patógeno (Demas and Nelson, 2011). Portanto, a febre é definida comparando essas temperaturas preferidas em estado de infecção com aquelas em condições de normotermia (Kluger et al., 1975; Boltaña et al, 2013). Neste contexto, normotermia poderia ser definida como o estado no qual a temperatura central e o *set point* são a mesma, mas sem aumento prolongado da temperatura preferencial e corporal (Kluger, 1991; Bicego et al., 2007), e quando se consideram animais não infectados. Como exemplo, o aumento da temperatura corporal, presumidamente modulado pelo hipotálamo, poderia envolver a seleção de microhábitats com temperaturas maiores ao normal. Portanto, a febre é definida em função de contraste com uma condição de *normalidade*. Todavia, tal condição não seria simples de definir. No caso de animais como répteis, peixes e anfíbios que termoregulam, as temperaturas normais seriam tendências comportamentais que se efetivadas no campo, levam a intervalos de temperatura do corpo na qual indivíduos possam desenvolver adequadamente suas funções fisiológicas. Este ponto será retomado no item 4.

3. Febre comportamental no Laboratório

Em condições experimentais, a febre comportamental é estudada utilizando diferentes metodologias de acordo com as perguntas e hipóteses. A literatura apresenta resultados para variadas questões referentes às vias de sinalização do mecanismo, localização de áreas reguladoras e o uso de pirógenos endógenos em contexto experimental. Tais agentes (e.g. prostaglandinas) promovem febre comportamental, como registrado para diferentes linhagens animais, tais como mamíferos e aves , ocorrendo um aumento até de dois graus na temperatura corporal após injetado por pirógenos endógenos (Stitt, 1973; Lipton & Fossler, 1974; D'Alecy & Kluger, 1975;

Blatteis, 1976; Van Miert et al., 1977; Berendt et al., 1980; Van Miert et al., 1986; Kluger, 1991; Gregorut et al., 1992; Van Miert et al., 1992; Johnson et al., 1993; Roth et al., 1994; Sehic et al., 1996; Maloney & Gray, 1998; Koutsos & Klasing 2001; Rudaya et al., 2005; Blatteis, 2006; Mahmoud et al., 2007; De Boever et al., 2010; Nakamura & Morrison, 2011; Sköld et al., 2015; Dantonio et al., 2016). Répteis, peixes, artrópodes, e anfíbios apresentam variadas temperaturas febris quando estudados num gradiente termal (Vaughn et al., 1974; Bernheim & Kluger, 1976a e b; Casterlin & Reynolds, 1977a e b; Casterlin & Reynolds, 1980; Cabanac & Leguelte, 1980; Firth et al., 1980; Bronstein & Conner, 1984; Muchlinski, 1985; Reynolds & Casterlin, 1976; Covert & Reynolds, 1977; Kluger, 1977; Myhre et al., 1977; Reynolds et al., 1978a e b; Hutchison & Erskine, 1981; Louis et al., 1986; Boorstein & Ewald, 1987; Muchlinski et al., 1989; Hallman et al., 1990; Ramos et al., 1993; McClain et al., 1988; Lefcort & Blaustein, 1995; Sherman & Stephens, 1998; Bicego-Nahas et al., 2000; Deen & Hutchinson, 2001; Bicego & Branco, 2002; Bicego et al., 2002; Do amaral et al., 2002; Cabanac & Cabanac, 2004; Merchant et al., 2007; Hunt et al., 2011; Lewellyn et al., 2011). Neste tipo de estudos geralmente se comparam dois grupos de animais. O primeiro é tratado experimentalmente mediante aplicação de pirógenos endógenos (e.g. prostaglandinas; Stitt, 1973; Deen & Hutchinson, 2001; Cabanac & Cabanac, 2004) ou de endotoxinas (lipopolissacárideos, LPS) de uma bactéria gram-negativa. O segundo, o controle, envolve animais injetados com solução salina. Os tratamentos são desenvolvidos numa sala com condições de umidade e temperatura controlada e os animais são colocados em um gradiente termal que permite efetivar o comportamento de febre.

Os tetrápodes ectotérmicos têm sido considerados um modelo apropriado para a pesquisa de febre comportamental porque linhagens bem estudadas apresentam diversos tipos de relação entre a temperatura corporal e a temperatura do habitat. Assim, combinações de linhagens e condições experimentais historicamente facilitaram o estudo da febre em no laboratório e o entendimento de suas implicações para os organismos (Kluger, 1975; 1979). Nesse contexto, os lagartos foram usados para testar a ideia que a febre poderia ser benéfica frente a infecções, e estudos pioneiros demonstraram que o aumento da temperatura mediado pela febre comportamental aumenta a sobrevivência. Estudos com uso de fármacos e taxas de sobrevivência apoiaram a ideia da febre como comportamento com valor adaptativo,

inclusive em outras linhagens (Bernheim & Kluger, 1976a; Bernheim & Kluger, 1976b), mas atualmente se tem apresentado duas novas hipóteses: 1) a febre comportamental é moldada pelo sistema nervoso central coordenada pela febre; 2) a febre comportamental é uma exaptação da febre metabólica (Shepard et al., 2016). Entre os “répteis”, pesquisas posteriores foram efetuadas em serpentes, crocodilos e tartarugas (Burns, 1991; Laburn et al, 1981; Merchant et al., 2007; Zurovsky et al., 1987a; Zurovsky et al., 1987b), e no geral corroboraram a ideia de que a febre comportamental era um mecanismo presente (ou pelo menos mais evidente) só nos lagartos das famílias iguanidae e teiidae (Hallman et al., 1990). No entanto, Goessling e colaboradores (2017) encontraram que a tartaruga *Gopherus polyphemus* apresentou febre comportamental ao mesmo tempo que a resposta imune (diminuição de ferro no plasma sanguíneo e aumento na capacidade bactericida) o que propõe um compromisso entre duas características fisiológicas.

Como visto nesta discussão, o estudo da febre comportamental derivou-se do estudo de febre metabólica (aumento de temperatura central produzida pelo aumento no “set point” do hipotálamo). O tema da febre comportamental em vertebrados ectotérmicos foi retomado intermitentemente ao longo dos anos principalmente em contexto puramente fisiológico. Em outras palavras, o objetivo central desses estudos era responder questões associadas aos mecanismos associados ao processo (quais são os efetores, vias, receptores, ajustes comportamentais, etc.), com mais discussão do que experimentação no relativo ao valor adaptativo da febre. Este último ponto importa, pois aparentemente a febre comportamental é uma resposta amplamente distribuída, mas não necessariamente ubíqua entre os vertebrados ectotérmicos (Kluger et al., 1975; Bernheim & Kluger, 1977; Kluger, 1979). Como é evidente pela ausência de trabalhos realizados na natureza, ou sequer sob condições seminaturais, faltam estudos que facilitem a interpretação mais ecológica da febre. Além disso, faltam estudos incorporando a situação que acontece na natureza, assim como da interação entre diversas funções fisiológicas e o contexto patológico (Lefcort & Eiger, 1993; Karavlan & Venesky, 2016), ou entre os comportamentos de febre e outros comportamentos, como o antidepredatório (Lefcort & Eiger, 1993; Lefcort & Blaustein, 1995).

4. Escopo e temperatura base da Febre comportamental

Em vertebrados endotérmicos a febre pode ser quantificada com relativa facilidade mediante a comparação de uma linha base com uma situação febril (por exemplo 39°C e 41°C seriam temperaturas de febre para mamíferos e aves típicos, respectivamente; Macari et al., 1993). Todavia, nos vertebrados ectotérmicos a quantificação da febre envolve complicações, pois o escopo termal da febre não é único nem absoluto. Além disso as temperaturas preferenciais e de campo variam notoriamente, inclusive entre espécies próximas filogeneticamente (Seebacher & Alford, 2002; Meek, 2011). Em répteis tem-se registrado vários valores de temperaturas corporais considerados como indicadores de febre comportamental (Deen & Hutchinson, 2001), e tais valores podem variar entre os 39°C a 41°C. Entretanto, além da variação filogenética, o escopo termal da febre poderia estar influenciado pela temperatura na qual são colocados os animais no início do experimento, ou até pela massa corporal do animal. De fato, a massa tem sido discutida como um fator importante influenciando a quantidade de reservas energéticas, o que afetaria a resposta termorregulatória quando o animal está infectado apresentando uma resposta alternativa (e.g. hipotermia; Deen & Hutchinson, 2001) e não febre comportamental. Em insetos e peixes as temperaturas febris também podem variar de acordo com o contexto filogenético, fisiológico ou experimental, sendo registrada febre comportamental acima dos 28°C. Os trabalhos sobre essas duas linhagens apresentam o escopo termal da febre de forma similar e o que varia é a forma de ser registrada (gráfico com delta de graus aumentados após o tratamento) (Reynold et al., 1978; Boorstein & Ewald, 1987).

A mudança de temperatura corporal desde a temperatura considerada “normal” ou base, para um novo estado chamado febre comportamental, tem diversas interpretações e costuma envolver faixas de temperaturas mais do que um valor absoluto. Uma complicação é que a maioria dos trabalhos sobre febre comportamental usam a temperatura corporal do tratamento com salina como a temperatura base. Ou seja, o controle serve como ponto de referência para determinar a temperatura febril, mesmo se tal controle envolve com frequência uma punção. Este desenho experimental, mesmo se muito frequente, deixa de lado a temperatura corporal preferida no sistema experimental na ausência de qualquer manipulação invasiva. Como a febre é determinada sempre em relação a uma temperatura base, a escolha de tal temperatura

base termina sendo determinante na interpretação dos resultados. Além disso, tratamentos sutilmente diferentes (por exemplo injeção versus aplicação tópica) podem complicar a interpretação de um conjunto de dados mais abrangente e associado a diferenças nas respostas observadas. Dentro de muitos contextos (e.g. doenças) ter a temperatura corporal base antes de qualquer manipulação seria fundamental para avaliar a febre comportamental principalmente quando a pergunta de pesquisa é relacionada com as respostas de regulação comportamental da temperatura do corpo.

Finalmente, nos ectotermos, as famílias estudadas são restritas (e.g. teiidae, iguanidae) e muitas vezes são usadas mais de uma espécie cujas características podem ser similares, o que deixa vazios sobre o tema dentro da diversidade sistemática. Dentro do grupo dos anfíbios, os anuros são o principal modelo testado, e as espécies estudadas representam um pequeno grupo da diversidade sistemática conhecida (ver tabela 1).

5. Limitações térmicas na natureza

Nos animais ectotérmicos existe uma dependência pelas fontes externas para aumentar a temperatura corporal e termorregular de forma comportamental, seja por tigmotermia ou heliotermia (Hertz et al., 1993, Hutchinson & Dupré, 1992; Navas, 1996). Assim, na natureza, a efetivação de processos termorregulatórios resulta da interação entre a fisiologia e o ambiente, levando em consideração que “fisiologia” neste caso, envolve também os processos neurais que levam a seleção de microhabitats. Por outra parte, mesmo animais com forte inclinação para termorregular podem ser incapazes de efetivar tal comportamento se existirem limitações ecológicas. Assim, nos animais ectotérmicos, limitações podem surgir devido a complexa relação que existe entre a temperatura do corpo e as condições biofísicas do ambiente (Gates, 1980). Em lagartos tem-se registrado limitações térmicas e no ganho energético no tempo derivadas do ambiente (Grant & Dunham; 1988; Angilleta, 2001). As restrições termais e estruturais impostas pelos habitats afetam o uso de microhabitats, e o aproveitamento desses microhabitats por parte dos lagartos é devido ao seu comportamento termoregulatório (Adolph, 1990).

No caso dos anfíbios a biologia termal se caracteriza pela associação entre a regulação da temperatura, balanço hídrico e a morfo-fisiologia da pele (Navas et al., 2008). Essa fisiologia termal pode apresentar divergência e depende da ecologia das espécies (e.g. em anuros Navas et al., 2008). Assim, a temperatura corporal pode ser

influenciada por fatores múltiplos como por exemplo, tempo de atividade e uso de microhábitats (Navas, 1996). A paisagem termal (amplitude das temperaturas de um habitat) é essencial para a termorregulação quando são consideradas em escalas compatíveis com o tamanho corporal do indivíduo (Navas et al., 2013). Em alguns casos esta paisagem termal pode limitar as preferências da temperatura corporal durante as estações (Fontenot e Lutterschmidt, 2011).

6. Febre comportamental no campo

Em 2008, Hetem e colaboradores estudaram a febre comportamental em indivíduos do antílope kudu *Tragelaphus strepsiceros* em condições naturais. Os indivíduos doentes (broncopneumonia bacteriana) desenvolveram os ajustes típicos da febre (vasoconstrição e esfriamento seletivo no cérebro) junto com os comportamentais, apresentando preferência por ambientes quentes e redução do 60% na atividade, diferentemente dos indivíduos não doentes. Atualmente não existem estudos em outros grupos animais que façam um seguimento do desenvolvimento da febre comportamental na natureza como o comentado. No entanto, em anuros, a febre comportamental no campo tem sido proposta com base na observação de indivíduos na natureza, o que tem sido relacionado com presença e prevalência do patógeno *Batrachochytrium dendrobatidis* (Bd). Richards-Zawaski (2010) propõe que a mudança na temperatura corporal de indivíduos de *Atelopus zeteki* afeta a vulnerabilidade dos indivíduos anuros às infecções do fungo durante a época reprodutiva, sugerindo que a história termal do indivíduo pode afetar a relação patógeno – hospedeiro. Assim, a autora conclui que fatores ambientais podem influenciar essa relação patógeno – hospedeiro. Estudos nessas linhas, mas em escala mais fina e com abordagem experimental poderiam acrescentar maior entendimento da importância da febre comportamental na natureza. Estudos dos fatores ambientais, visando entender oportunidade para febre comportamental, podem ser um importante primeiro passo nesta direção (Ortega & Navas, dados sem publicar). Esses estudos podem, além de analisar a paisagem termal, modelar comportamentos diferentes e estudar as consequências termais de diferentes opções comportamentais. Assim, o contexto ecológico das escolhas comportamentais na paisagem termal poderia ser melhor entendido (Helmouth et al., 2016).

7. Implicações da Febre comportamental na Conservação em tetrápodes ectotérmicos

Ao longo dos trabalhos já mencionados a febre comportamental é uma resposta termoregulatória abrangente entre diversas linhagens. Indivíduos infectados por um patógeno (bactéria ou fungo) desenvolvem essa resposta como parte da sua biologia termal quando não se considera o estudo da biologia termal dos indivíduos utilizados e só se inclui um grupo controle, com a injeção de solução salina. Assim, precisa-se corrigir ou confirmar esse tipo de afirmação dirigindo atenção à biologia termal dos indivíduos da espécie, à ecologia termal do indivíduo e ao ambiente (no sentido de se os microhábitats disponíveis permitem desenvolver a febre comportamental).

A partir de todo o conhecimento sobre a febre comportamental em campo e em condições experimentais podemos pensar nas implicações que o tema tem sobre estudos de conservação. Primeiramente é necessário reconsiderar a hipótese de generalidade da resposta mediante o teste de espécies diferentes das comerciais ou comuns em estudos sobre febre comportamental. Isso permitiria conhecer as respostas (seja termoregulatórias ou de doente) que espécies tropicais possam ter, levando em consideração que a diversidade termal dentro de hábitats é muito diversa, e tende a ser menor em florestas tropicais tais como a Mata Atlântica. Portanto, cabe considerar que espécies de áreas fechadas podem apresentar respostas dominantes de comportamento de doente e não de febre. Segundo, é necessário trabalhos de campo abordando esse mesmo cenário (indivíduos infectados por um patógeno) para verificar se as respostas obtidas no laboratório refletem as respostas no campo. As condições de campo são multidimensionais e complexas, muito mais do que as de um gradiente termal, e no momento não temos argumentos para generalizar a todos os grupos de vertebrados ectotérmicos (sequer nos tetrápodes) que a escolha em laboratório de uma variável sob condições controladas sinalize a escolha da mesma variável no campo. Por último, conhecer a resposta dominante (comportamento de doente ou febre comportamental) que uma espécie tropical apresentaria quando infectada, daria um maior entendimento do que aconteceria com as espécies em campo num contexto de doenças emergentes, o que daria aos trabalhos sobre conservação de tetrápodes ectotérmicos um maior valor e significado.

Outra implicação seria considerar a definição de febre comportamental desde o ponto da biologia termal do indivíduo em estudos que tem registrado respostas termorregulatórias ou comportamentos anormais em campo quando estão infectados por um patógeno (Berger et al., 2004). Por fim, o estabelecimento de metodologias padrão na hora de definir febre comportamental no laboratório em espécies que na natureza são afetadas por infecções ou doenças e que se tem acreditado que podem desenvolver esse mecanismo (Woodhams et al., 2003).

No seguinte capítulo abordaremos o estudo das respostas comportamentais de indivíduos da espécie de anuro *Proceratophrys boiei* frente eventos de infecção. Consideramos que podem existir duas alternativas de respostas no comportamento: 1) febre comportamental, caracterizada por um aumento da temperatura corporal dos indivíduos putativamente causada pela mudança nas temperaturas preferidas, e efetivas dentro de uma paisagem termal experimental; 2) comportamento de doente, que é reconhecido pela diminuição da atividade dos indivíduos e uma consequente diminuição da variância termal. Nesse trabalho diferenciamos a resposta dominante em condições experimentais num gradiente termal e comparamos especificamente preferências termais, comportamento dentro do sistema e a distância percorrida entre indivíduos intactos, indivíduos injetados com solução salina e indivíduos tratados com uma injeção de lipopolisacáridos (LPS).

Capítulo 1

A study of behavioral responses to infection in the anuran amphibian

Proceratophrys boiei

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Abstract

Behavioral fever in infected individuals is the increase of body temperature mediated by modified thermal preferences, when the selection of higher temperatures is possible. In ectothermic vertebrates, behavioral fever is often studied through an injection of endotoxins of gram-negative bacteria, followed by protocols registering body temperatures in a thermal gradient. Usually, the results of such tests are compared with those of a control (e.g. Saline injections) to determine body temperatures under “normal” and “febrile” situations. This technique necessarily involves ambiguity in determining fever temperatures, even more given the limits of information on tropical anuran lineages. Within tropical anurans, species from open environments (and presumably naturally exposed to diverse thermal environments) have received most attention, whereas forest species have been neglected. Hence herein we tested the hypothesis that individuals of the species *Proceratophrys boiei* (Odontophrynidae) from the Atlantic forest of Brazil, respond behaviorally to simulated infection. There are two possible response: fever and sick behavior (characterized by reduced mobility and low thermal variance). To test this hypothesis, *P. boiei*'s individuals were placed in a thermal gradient and their body temperature (assumed to be preferred) was recorded for 24h in each experiment. To simulate an infection we injected systemically lipopolysaccharide (LPS) injection with a dose of 2 mg/kg in *P. boiei*'s individuals. Our results suggest that individual *P. boiei* reduce activity after LPS injections, and that they prefer the extremes of a gradient (perhaps as a refuge), with no thermal preferences. Thus, sickness behavior is a dominant response, and given the overall results, the presence of individuals in the hot extreme of the gradient, when evident, resulted from inactivity and not from thermoregulation. A diverse thermal landscape theoretically allowing for thermoregulation was not sufficient to elicit a behavioral

fever response. This may be a trend in other anuran species, possibly more common in forest forms.

Keywords: Amphibians, Fever, Infrared images (FLIR camera), Lipopolysaccharide (LPS), Sickness behavior, Temperature regulation,

1. Introduction

Fever is the regulated increase of body temperature associated with a change in thermoregulatory set point (Kluger, 1991). The thermoregulatory activity during a fever is driven either by metabolic physiology or by behavior. In the case of physiologically-driven fever, relevant processes vary across lineages, but generally encompass increased metabolic and heart rate and reduced loss of body heat, mediated by signaling cascades involving thyroid hormones (Bicego et al., 2007). In contrast, the behavioral course for fever exploits the thermal diversity of the environment (if available) under modified patterns of neural physiology. During pathogen infection, real or simulated by an endotoxin injection, several ectothermic animal species increase body temperature by means of such modified behavior, which is called behavioral fever (Reynolds et al., 1976; Kluger, 1977; Bicego et al., 2000; Bicego and Branco, 2002; Demas and Nelson, 2011; Bolltaña et al., 2013). Behavioral fever can be elicited under experimental conditions that make thermal gradients available for treated animals. Under such circumstances, behavioral fever is defined as the selection of higher than *normal* temperatures, mediated by shifts in temperature preferences (Firth et al., 1980; Muchlinski, 1985).

Interpreting fever as a behavioral trait is not obvious because preferred temperatures may be naturally variable (Brattstrom 1979; Lillywhite, 1970; 1974; Navas, 1997; Sanabria et al., 2012). This is of importance for a body temperature “*higher than normal*” makes sense only when the “*normal*” temperature is unambiguously defined. This is a complex issue, though, dominant behavioral inclinations in the field may vary for many reasons, and thermal preferences do vary in nature in the absence of infection and due to shifts in physiological states. Anuran body temperature is influenced by time of year, photoperiod and time of day, and temperature and humidity of the environment (Schwarzkopf & Alford, 1996; Seebacher et al., 2002). However, whereas some amphibian species thermoregulate behaviorally

(Brattstrom, 1963) and exploit thermal resources in their microhabitat (Tracy & Christian, 1986), others thermoconform (Iturra-Cid et al., 2014; Rodriguez et al., 2016), prioritize hydoregulation (Cruz & Galindo, 2017; Navas et al., 2007) or even develop hypothermia under dry conditions. Additional complications are that thermoregulation in the laboratory not necessarily reflects field thermal ecology (Feder, 1982), and that thermal preferences may vary substantially. In experimental systems, animals with no manipulation at all are very rarely explored, so that punctured controls are considered as a normal thermoregulation pattern, or that such pattern must come from the literature. In the latter case, studies may differ in the thermoregulatory responses elicited (Sherman et al., 1991).

Behavioral fever was originally reported for amphibians under experimental frameworks. Early treatments involved pathogens (Gram-negative bacteria, endotoxin LPS, fungus, etc.) or pyrogenic agents (e.g. prostaglandins, PGE, a known activator of fever) and some of them elicited behavioral fever (Hutchinson & Erskine; 1981). Studies also focused on thermoregulatory responses to drugs (Capsaicin, prostaglandin E₁, melatonin, and chlorpromazine), that is believed to stimulate regulation of temperature through behavioral responses (Hutchison, 1981; Hutchison & Erskine, 1981; Hutchison & Spriestersbach, 1986). Salamanders were the target of groundbreaking studies that demonstrated, for example, that *Necturus maculosus* increases body temperature (behavioral fever) when injected with prostaglandin E₁ into the third ventricle of the brain, although nowadays anurans receive most attention regarding behavioral fever (Bicego & Branco, 2002 see Table 1). However, fever is not the only possible response to infection in animals, and anurans are no exception. Sickness behavior is technically not mutually exclusive with fever, and is a syndromic response to pathogenic states usually characterized by lethargy and anorexia (Hart, 1988; Larson & Dunn, 2001; Johnson, 2002). In ectothermic tetrapods this behavior has received less attention than it deserves, for behavioral fever requires active thermoregulation. Therefore, the activity involved in behavioral thermoregulation may indeed be mutually exclusive with lethargy, so that behavioral fever and sickness behavior are unlikely to be dominant trends simultaneously. It is of particular significance that sickness behavior and not behavioral fever may dictate patterns of activity in infected individuals of some anuran species, even those known to thermoregulate in thermal gradients.

A key example of complex responses occurs in the toad *Rhinella marina*, a species able of multifaceted regulation of body temperature through behavior (Malvin & Wood, 1991, Sievert, 1991). Yet, despite this ability, individuals injected with lipopolysaccharide (LPS) may either display or not a thermoregulation response, apparently via perception of the thermal environment. Therefore, the observed thermoregulatory response to pyrogens depends on the initial position of toads in a thermal gradient, and animals may abandon thermoregulation and displaying sickness behavior as a dominant response (Llewellyn et al., 2011). Consequently, behavioral fever may help individual amphibians against infection, but needs not to be a universal response, and dominant responses may be influenced by the thermal settings of environments. This is a key issue linking amphibian disease and conservation, for emergent disease is a putative cause of amphibian extinction in numerous contexts (e.g. *Batrachochytrium dendrobatidis*, Bd, Woodhams et al., 2003; Berger et al., 2004; Pitrowski et al., 2004; Forrest et al., 2011; Karavlan & Venesky, 2016). Finally, understanding the role of fever remains ambiguous, because studies testing behavioral fever as a thermoregulatory response in nature are lacking, and responses are not obvious even if no ambiguity exists regarding the physiological ability to develop fever in the laboratory. This information is essential to validate proposals of correlational studies suggesting that changes in body temperature can influence chances of pathogen infection in nature (Richards-Zawacki, 2010; Rowley et al., 2013).

Alternative limitations of the available data on anuran fever include both a systematic restriction and an emphasis on species that thrive in open environments, hence more studies are needed with forests species, particularly in tropical and subtropical species. Forest species are usually exposed to comparatively less variable thermal landscapes than those from open environments, and this may preclude or constrain behavioral fever. Accordingly, we investigated thermal responses to simulated infection in one anuran species from the Brazilian Atlantic forest under two conditions of thermal landscape, one with diversified temperatures (complex) and another at constant room temperature (flat). Given feasibility and good knowledge on natural history by our group, we centered our study on the species *Proceratophrys boiei* (Odontophrynidae). Our study aimed to answer and test the following questions and hypotheses: 1) Which response between behavioral fever and sickness behavior becomes dominant after LPS injection in individual *P. boiei*? We hypothesized that

given the forest thermal landscapes in which this species thrives; sickness behavior could be expressed, perhaps simultaneously with febrile responses (across individuals). A corollary of the previous question is that sickness behavior, if evident, would manifest in reduced activity of infected frogs. If shifts in activity do occur after infection we ask 2) whether these shifts are affected by the structure of thermal landscapes. Our expectation was that diverse thermal landscapes could favor febrile responses whereas flatter thermal landscapes would favor sickness behavior.

2. Material and methods

2.1 Species information

Proceratophrys boiei is an anuran amphibian species endemic to the Atlantic Forest of Brazil, occurring from the state of Espírito Santo to the state of Santa Catarina and can inhabit the Cerrado transitional areas above 1200m in the states of Minas Gerais, São Paulo, and Rio de Janeiro. This species has cryptozoic habits, and when inactive remains hidden in excavated or natural cavities in the soil (Giaretta et al., 1999). They are regarded as nocturnal (Haddad et al., 2013), but can be active at the end of the afternoon (17h) and during the day. The reproductive season runs from September to January, with a higher presence of males vocalizing at the end of the rainy season (Bertolucci, 1998; Haddad et al., 2013). The males call mostly at night (Pombal, 1997) but also in the morning (CC pers. obs) on the ground near small streams or lakes (Bertolucci & Rodrigues, 2002). Females lay eggs in swamps or in the stream (AmphibiaWeb, 2015). Tadpoles are benthic (Izecksohn et al. 1979), and metamorphs are more commonly seen in February (Prado & Pombal 2008). Typical body size is 40-62 mm SVL in adult males and 40-74 mm SVL in females and the mean body mass is 9.53 g (Cochran, 1954; Prado and Pombal, 2008; AmphibiaWeb, 2015).

2.2 Maintenance of Individuals in captivity

Frogs were kept in groups of two to five individuals and maintained in glass terrariums provided with moist litter for hiding. Filtered water was supplied and changed twice a week. Frogs were fed cockroaches *ad libitum*. Maintenance temperature was influenced by environmental temperature, under a 12-hour photoperiod.

2.3 Measurement of body temperature in the system (preferred T_b)

To evaluate body temperature in the laboratory we used a thermal system composed by a water bath, with the aluminum surface of 1.10 m x 0.30 m x 0.50 m. We established the extreme temperatures as 5°C and 35°C, which did not harm the animals, for some frogs may die when approaching drastic gradient extremes (CA Navas pers. obs). The soil of the system was covered with Petri dishes full of water and by a 5 mm layer of vermiculite to offer proper refuge to the animals as recommended in other studies (Spotila, 1982). This layer was also important for lethargic behaviors to be elicited. We used thermography to determine relevant thermal parameters (body temperature, substrate [vermiculite] temperature and overall structure of the thermal gradient). For more accurate measures we calculated the emissivity of vermiculite from thermography software (Flir R & D Software 3.3) using an object of known emissivity (0.95) as reference. The emissivity of frog skin was considered as 0.95, according to the calibration established by Tattersall et al. (2004).

2.4 Experimental background

Experiments were developed between April and May of 2016 when the reported air temperatures in Intervalles Park were 19.2°C and 14.4°C, respectively (Intervalles climatological station). Given permits and field availability, we limited this research to 22 individuals captured in October of 2015 and adjusted control options to this N value. We are aware that captivity itself can be a confounding variable, and designed a reciprocal treatment control as to maximize the amount of information collected from the number of animals available. For control, we split all animals into two groups, one to be injected with LPS and another with saline solution (early season) and measured thermal parameters. After two weeks, the converse treatments were performed, that is, each group received the reciprocal treatment. Individuals were fasted 48 hours before the experiments to avoid any influence of feeding on thermoregulation.

It turned out that preliminary analyses pointed out to subtle yet relevant differences in the results among experiments performed in April and May, so we decided not to pool this data. It must be clear that animals were studied (but not collected) in different seasons, and the differences here highlighted apply to two phases of the same set of animals measured at different time of year. For example, in our reciprocal design, Late

Season individuals injected with LPS, in Early Season received a saline injection, and vice versa (see details below). The groups generated are hereafter referred to as Early Season (1) and Late Season (2) frogs. We name these groups after season for this species has proven very resilient to captivity, but the timing of captivity in this experiment encompassed the most relevant climatic seasonal transition in nature, which occurs from autumn to early winter (Databank, hydro-meteorological – Climatological station at Parque Estadual Intervales). In addition, we had learned from field observations that this frog shifts behavioral patterns between the reproductive (males call near water) and post-reproductive seasons (individuals found far from water sources), and therefore field behavior changes from April to May.

We quantified behavior on a flat thermal landscape (thermal gradient off, room temperature, Early season only) and a complex thermal landscape (thermal gradient on, both seasons). Under these two conditions, we compared intact individuals, individuals injected with saline and individuals injected with LPS. We did not measure actual distances moved to limit disturbance to animals, but deduced activity from changes in position along the gradient, which was split in five areas 1 (warm) to 5 (cold), so that area 3 was in the middle of the gradient. We quantified distances moved based on the location of individual frogs along the image sequences during the procedure. Each treatment started in Area 3 at 18h and then frogs were left free to move during 24 hours. We thermographed the gradient every 10 minutes during those 24 hours (in previous testing, we showed that individual frogs could be observed from the substrate in thermographs). We produced various measures of central tendency and dispersion of body temperature and quantified movement with what we called “time series” variables. These variables were associated to changes across time and included both movement (central tendency and dispersion) and direction of shifts in body temperature (increase, decrease or none). Details of this pool of variables are available in Table 2.

2.5 Calculations and statistical analysis

To detect eventual preferences for any position in the gradient (each of the five areas) we contrasted frequencies of location in a flat thermal landscape (room temperature) against a uniform distribution (equal frequencies among areas, i.e., no preferences) using a Chi-square test (Zar, 1998).

We combined all data on body temperature (central tendency and dispersion) and activity with Principal Component Analyses (PCA) composed in all cases by the 13 variables related in Table 2. These PCAs were performed emphasizing the best possible data for each experimental contrast; given that the data set had gaps (e.g. Early season animals only) (see Table 3). The conditions and amount of data differed slightly across treatments, so that a global analysis was not possible. These PCA's differed in details, but globally the three main components displayed highest loads for similar arrangements of variables (example in Table 4). In all cases the data generated a first component with high loads for temperature central tendency, a second component highlighting thermal variance, and a third component reflecting "time series" (mainly a quantification of shifts in position or body temperature through time).

One-way ANOVAs were performed with each of the component scores chosen of the PCA performed to determine whether the time of the year or the treatments had influence in the temperature preferred by the individuals. For treatments performed in different seasons, we did this test separately to see if there is a difference in each of the component score chosen between the early and late season. A Kruskal-Wallis was performed in the cases where the assumptions of normality did not apply.

To evaluate if there was a decrease or increase in distance moved that is associated with sickness behavior and behavioral fever, we contrasted treatments with main effects ANOVA over total distance moved.

3. Results

3.1 Spatial selection in flat and complex thermal landscapes

In a flat thermal landscape, frogs were not neutral regarding their position in the system and distributed with some preference for areas 1 and 5 ($X^2_{(4, 0.05)} = 995.91$, $p < 0.001$, Fig. 1A). In a complex thermal landscape, Early Season individuals also preferred areas 1 and 5 despite their more extreme temperatures, but shifted preferences in favor of area 5 (coldest) ($X^2_{(4, 0.05)} = 4492.18$; $p < 0.001$) (Fig. 1B) reaching a pattern resembling that of Late Season individuals ($X^2_{(4, 0.05)} = 324.15$; $p < 0.001$) (Fig. 1C). The injection of saline solution modified preference pattern, yet still within a context of preferences for extreme areas. As in the flat thermal landscape (first experiment), areas 1 and 5 were preferred by frogs collected either Early ($X^2_{(4, 0.05)} = 847.93$, p

<0.001) or Late Season ($X^2_{(4, 0.05)} = 846.91$, $p < 0.001$), although not necessarily with same frequencies (Figure 2A-B). Individuals injected with LPS, selected mostly Area 1 (hot) in the Early Season ($X^2_{(4, 0.05)} = 160.62$, $p < 0.001$) and Area 5 (cold) in Late Season ($X^2_{(4, 0.05)} = 502.52$, $p < 0.001$) (Figure 2C-D).

3.2 Thermal and behavioral variation

3.2.1 Movement and exploration in a flat and complex thermal landscapes (Component 3)

Intact frogs moved more than any other experimental group (Fig. 3). Among intact frogs movement was slightly higher in flat (Go) than in complex thermal landscape (To) (Fig. 3A), ($F_{(1, 0.05)} = 79.101$, $p = 0.00001$). This exploration and movement by frogs in a flat thermal landscape changed with the injection of saline, causing a reduction in movement evident in complex thermal landscape (S) ($F_{(1, 0.05)} = 21.053$, $p = 0.00001$) (Fig. 3B). Similarly, LPS reduced movement in a complex thermal landscape ($F_{(1, 0.05)} = 17.122$, $p = 0.0001$) (Fig. 3C). However, the groups called Early and Late season displayed some differences. When exposed to complex thermal landscape, intact frogs measured in Early season moved less than those measured in the Late Season ($F_{(1, 0.05)} = 25.201$, $p = 0.00001$) (Fig 3A). The same occur with the saline injection: Early season frogs moved more in a complex thermal landscape than Late Season Frogs ($F_{(1, 0.05)} = 6.684$, $p = 0.011$). But when compared to the season frogs from LPS treatment, Early season frogs did not differ in movement pattern compared to Late Season frogs ($F_{(1, 0.05)} = 3.573$, $p = 0.061$).

When exposed to complex thermal landscapes, frogs injected with saline (S) moved less compared to intact frogs (To) ($F_{(1, 0.05)} = 8.681$, $p = 0.004$), and in this context Early and Late Season frogs were comparable ($F_{(1, 0.05)} = 1.141$, $p = 0.287$, Fig. 4A). Once injected with LPS (L) frogs moved less than intact frogs (To) ($F_{(1, 0.05)} = 4.966$, $p = 0.027$) (Fig. 4B) from both season groups, and this reduction was clear only in the complex thermal landscape (comparison between intact frogs (To) and frogs injected with LPS (L) ($F_{(1, 0.05)} = 2.290$, $p = 0.132$).

In complex thermal landscapes, frogs injected with either saline (S) or LPS (L) displayed comparable movement ($F_{(1, 0.05)} = 0.061$, $p = 0.805$) with no differences between Early and Late Season frogs ($F_{(1, 0.05)} = 0.065$, $p = 0.799$). Details of movement

results in this comparison of treatments are presented in the supplementary material, Figure S1.

3.2.2 Body Temperatures: Central tendency measures (Component 1) and Dispersion and variance of the temperature data (Component 2)

Frogs in a flat thermal landscape (Go) were forced to a body temperature equal to room temperature. Therefore, similarities in mean, median and mode body temperatures between flat and complex landscapes ($F_{(1, 0.05)} = 0.295$ $p = 0.587$) reflect a choice of extreme temperatures when the gradient was on, and the pattern remained independently of season ($F_{(1, 0.05)} = 0.245$ $p = 0.621$). Naturally, this trend on central tendency was not parallel by measures of dispersion in body temperature. In a complex thermal landscape, indicators of dispersion of body temperature were led by the temperature/area preferences, which were influenced by time of year (Early versus Late season frogs). As expected, frogs in a flat thermal landscape displayed comparatively narrow variances in temperature ($F_{(1, 0.05)} = 62.579$, $p = 0.00001$). Differences in thermal central tendency variables occurred also when comparing intact frogs in a flat thermal landscape with frogs injected with saline ($F_{(1, 0.05)} = 17.671$ $p = 0.00001$) and with frogs injected with LPS ($F_{(1, 0.05)} = 27.586$, $p = 0.00001$) in a complex thermal landscape. Intact frogs in a flat thermal landscape presented about the narrowest variances for body temperature for being constrained to room temperature. So their dispersion of body temperatures was lower than those in a complex thermal landscape, exposed to a saline injection (S) ($F_{(1, 0.05)} = 35.593$, $p = 0.00001$) or a LPS injection (L) ($F_{(1, 0.05)} = 35.822$, $p = 0.00001$). Details of all central tendency and dispersion results in these treatments are presented in the supplementary material, figures S2 A to C and figure S3 A to C, Supplementary data.

In complex thermal landscapes (To) mean body temperatures compared among intact frogs and frogs with saline injection (S) ($F_{(1, 0.05)} = 1.793$, $p = 0.182$), showed no differences between Early and Late season frogs ($F_{(1, 0.05)} = 2.514$, $p = 0.114$). Dispersion was also comparable in this context (To, S) ($F_{(1, 0.05)} = 0.043$, $p = 0.836$), as it was comparing Early and Late Season frogs ($F_{(1, 0.05)} = 0.124$, $p = 0.725$). Details of all central tendency and dispersion results in these treatments are presented in the supplementary material, figure S4A and figure S5A.

When complex thermal landscapes (To) were available, intact and LPS injected (L) frogs were compared regarding their mean body temperature ($F_{(1, 0.05)} = 0.967$, $p = 0.327$). However, some differences existed in measures taken at different time of year, for Early Season frogs displayed higher mean body temperature than Late Season frogs in both experiments (1, 2, $F_{(1, 0.05)} = 16.850$, $p = 0.00001$) (Fig. 5).

Overall, intact and LPS injected frogs displayed similar trends in thermal dispersion indicators ($F_{(1, 0.05)} = 2.402$ $p = 0.123$), and these similarities remained independently of time of year (Early versus Late season frogs, $F_{(1, 0.05)} = 1.043$ $p = 0.308$). Similarly, under complex thermal landscapes both saline injected (S) and LPS (L) injected frogs were comparable regarding central tendency indicators ($F_{(1, 0.05)} = 1.668$, $p = 0.199$). Also, these two groups (S, L) of frogs displayed similar dispersion indicators of the body temperature ($F_{(1, 0.05)} = 0.136$ $p = 0.713$). Early and Late Season frogs also displayed comparable values ($F_{(1, 0.05)} = 0.642$ $p = 0.425$). Details of all central tendency and dispersion results in these treatments are presented in the supplementary material, figure S4B and figures S5 B to C, Supplementary data.

3.3 Comparison between time of the year: Early vs Late Season frogs (1,2)

Intact frogs in a complex thermal landscape (To) in Early and Late Season

Early season frogs compared to Late Season frogs in a complex thermal landscape regarding the three principal components: the two groups presented a similar outline in central tendency ($F_{(1, 0.05)} = 1.601$, $p = 0.208$) and dispersion of frogs body temperature ($F_{(1, 0.05)} = 0.929$, $p = 0.337$), and in movement and exploration ($F_{(1, 0.05)} = 1.301$, $p = 0.256$). Details of all central tendency, dispersion and movement results in this treatment are presented in the supplementary material, figures S6 A to C.

Frogs with saline injection in a complex thermal landscape (S) in Early and Late Season

Early Season frogs matched thermal central tendency indicators of Late Season frogs ($F_{(1, 0.05)} = 0.481$, $p = 0.490$), and dispersion was also similar ($F_{(1, 0.05)} = 0.607$, $p = 0.438$). Regarding movement of frogs, Early Season frogs presented different median movement but with no significant differences ($F_{(1, 0.05)} = 0.034$, $p = 0.853$) compared to Late Season frogs. Details of all central tendency, dispersion and movement results

in this treatment are presented in the supplementary material, figures S7 A to C, Supplementary data.

Frogs with LPS injection in a complex thermal landscape (L) in Early and Late Season

Early season and Late Season frogs showed slight, yet not significant differences in central tendency of body temperature ($F_{(1, 0.05)} = 0.117$, $p = 0.734$), variance ($F_{(1, 0.05)} = 0.117$, $p = 0.734$) or exploration and movement ($F_{(1, 0.05)} = 1.192$, $p = 0.279$). The median movement was the same and behavior similar between these two groups of frogs. Details of all central tendency, dispersion and movement results in this treatment are presented in the supplementary material, figures S8 A to C, Supplementary data.

3.4 Distance moved in the treatments

All groups differed in distance movement around the gradient ($F_{(3, 0.05)} = 26.208$, $p = 0.00001$). Intact frogs in a flat thermal landscape moved more compared to intact frogs, saline injected frogs, and LPS injected frogs in a complex thermal landscape. Overall, frogs injected with saline and LPS moved less (Fig. 6).

4. Discussion

Most studies aiming behavioral responses to infection in amphibians focus on body temperature, especially both mean and variance data (Kluger, 1977; Bicego et al., 2002). In this article, we used an experimental design contrasting two alternative responses, fever and sick behavior, which conveys a more complex dimension of the problem. In the laboratory, a typical fever response involves moving in a gradient as to raise body temperature compared to indicators typical of non-infected animals (Kluger et al., 1975; Reynolds & Casterlin, 1976; Hunt et al., 2011). Under laboratory conditions, such response is unambiguously clear in some anuran species (Kluger, 1977; Bicego et al., 2002; Cabanac & Cabanac, 2004), but does not need to be a universal. Indeed, we show that *P.boiei* injected with LPS decreases movement, and that the dominant response to infection is lethargy (but not prostration), not fever. LPS reduced movement and exploration, and to some degree thermal variance, with impact of time of year. Therefore, the exploratory behavior across thermal landscapes may vary with the climatic transitions that occur through the year in the area they inhabit. Anyhow, we suppose that our results indicate more altered movement patterns (a

correlate of thermal variance in complex thermal landscapes) and not an active thermoregulatory response in the strict sense. Overall, under our experimental conditions *P. boiei* did not behave as a thermoregulating species but as a thermoconformers, and fever was not a dominant behavioral response.

Given that behavioral fever in ectothermic tetrapods is seen as a state of altered thermoregulation, and that thermoregulation was not evident in our study, sickness behavior was the dominant pattern. This behavioral response has been considered as a first line defense (Hart, 1988) before other physiological responses of the immune system (Braga, 2013; Llewellyn et al., 2011). Fever and Sickness behavior are not mutually exclusive in endothermic tetrapods; even more for these two responses have the same activation pathways and mediators (e.g. cytokines; Hart, 1988; Dantzer, 2001). In birds for example, after LPS injection, individuals became feverish and reduced activity being somnolent and increasing anorexia (Johnson et al., 1993). However, in ectothermic tetrapod, like anurans, these responses may fail to manifest simultaneously, or may emerge only under some circumstances according to perception of thermal landscapes. Llewellyn and colleagues (2011) showed that toads reduce activity after simulated infection, apparently also responding with sickness behavior more than behavioral fever. In that study, shifts in mean temperature seem a consequence of reduced activity like in *P. boiei*. Indeed, in the experimental designed by Llewellyn and colleagues the body temperature of cane toads depends on the place of release, so that thermoregulation, if occurred at all, was an opportunistic response. An important contrast between our study and that by Llewellyn and colleagues is that not infected cane toads are known to thermoregulate in thermal gradient (Malvin & Wood, 1991; Sievert, 1991) whereas *P. boiei* does not seem to do so under any tested circumstance. Rather, *P. boiei* explore and move around when not infected (e.g. flat and complex thermal landscape). Another contrast between cane toad and *P. boiei* is that the latter species inhabits transition and fully-grown forests (Haddad et al., 2013; Prado & Pombal, 2008; Bertoluci & Rodrigues, 2002), thus the evolution of their thermoregulatory behavior may be influenced by lack of opportunity for thermoregulation, as in the case of some lizards (Ruibal, 1961).

The infection-induced lethargy observed by both Llewellyn and colleagues (2011) and us is characteristic of sickness behavior (Hart, 1988; Inui, 2001), a syndrome

encompassing inactivity, anorexia, and change in the diel cycle of body temperatures (Hart, 1988), which is common in vertebrates as part of the immune system response (Hart, 1988; Inui, 2001) and may concur with refuge seeking (Llewellyn et al., 2011). We interpreted the preferences for the narrower borders of the system as a refuge-seeking behavior emerging under our experimental conditions, and this is something to be tested formally in future studies. A similar behavioral response was reported in anuran tadpoles, which swim to gradient borders and remain there even if no established gradient exist or any other treatment, and there remain for several hours (the same experimental period) (Lucas & Reynolds, 1967).

A typical protocol in the study of fever is to use saline injection as a control for LPS injection (Casterlin & Reynolds, 1977; Bicego et al., 2002; Llewellyn et al., 2011). Our results demonstrated that this may not be an adequate procedure in some cases as *P. boiei*, because the impact of the injection is perceivable in terms of activity and position, and regarding this aspect produces results closer than those with LPS treatment. This result of change in behavior because of saline injection points to a refuge-seeking behavior as mentioned above. Also, whereas controls to analyze behavior in the system are rare, they are possibly important. In the absence of such controls, it would have been difficult for us to tell apart preferences for a position within a system, and preferred temperatures, thus leading to misinterpreted results.

Although we report here dominant behavioral trends across the study, we must point out the high inter-individual variability that characterizes results across the study. Such variability has implications. On one hand, responses across individuals are not homogeneous, and the lack of an obvious thermoregulatory trend likely enhanced observed thermal variances. On the other hand, under these conditions it becomes impossible to define a “normal” thermal situation to anchor a definition of behavioral fever. This observation, often overlooked, is not new. Kluger (1977) had noticed this for the anuran *Hyla cinerea*, and commented on the high variability in the body temperature present in both, control and treatment groups, pointing out that this could be a characteristic of amphibians perhaps related to alternative behavioral drives to regulate salt and water. Deen & Hutchinson (2001) reported a similar response on juveniles of the specie *Iguana iguana* with LPS injection displaying two different thermoregulatory responses, fever and hypothermia. The inter-individual variability of

the two behavioral responses was apparently affected by acclimation temperature and magnitude of energetic reserves.

Regarding season, we emphasize again that the differences of the data collected at different times of year came as an unexpected result, and do not constitute a formal study of seasonality. However, as turned out, the data do support the idea that dominant behavioral responses to infection could vary according physiological differences by time of year. We do not know of studies testing these hypotheses, but it seems plausible for thermoregulatory patterns may change across the year in anurans (Sievert, 1991; Bicego et al., 2001; Noronha de Souza et al., 2016). Seasonality may be important because in nature seasons affect the average temperatures and the structure of thermal landscapes (Ortega & Navas, in preparation). Thus, individuals, due to ecological restrictions, physiological shifts, or both, may change thermal preferences including context disease (Schwaner, 1989). Finally, differences in fever response relate to the dose, site injection and kind of endotoxin applied (Kluger, 1991), so we cannot postulate that *P. boiei* will respond as here described under any sort of natural or experimental infection. However, we show unambiguously that behavioral fever may not be a dominant response under some circumstances that we interpret as compatible with ecology. On the same lines, behavioral responses to infection could vary with the time of day at which endotoxins are applied. In birds, for example, variation in the behavioral response is context-dependent and regulate according to the needs and time of the infection (Skold et al., 2015). In anurans, variation in preferred body temperatures occur across seasons (Bicego et al., 2001; Noronha de Souza et al., 2016). More studies about this topic need to corroborate this hypothesis for anurans.

5. Conclusions

Proceratophrys boiei did not develop behavioral fever when LPS was injected. In individuals of *P. boiei*, LPS injection produces a change in behavior with a reduction in activity and decrease in body temperature variation, characteristics of sickness behavior. This response was dominant behavior in all individuals. Our study present sickness behavior as an alternative response for a tropical species giving us a better understanding of the potential of thermoregulatory and behavioral responses of forest species when infected.

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8. Figures and Tables

Figure 1. Position preferences in the system. **A.** Intact frogs in a flat thermal system. **B.** Intact frogs on a complex thermal landscape in the Early season, **C.** Intact frogs on a complex thermal landscape in the Late season. The numbers 1-5 are the areas on the thermal gradient in which 1 is hot and 5 is cold.

Figure 2. Temperature/position preferences in the system. **A.** Frogs with saline injection on a complex thermal landscape in the Early Season. **B.** Frogs with saline injection on a complex thermal landscape in the Late season. **C.** Frogs with LPS injection on a complex thermal landscape in the Early season. **D.** Frogs with LPS injection on a complex thermal landscape in Late season. The numbers 1-5 are the divided areas on the thermal gradient, one is hot and five is cold.

Figure 3. Boxplot of component 3 of the PCA performed (movement and exploration on the thermal gradient). **A.** Intact frogs in a flat thermal landscape (Go) vs. intact frogs in a complex thermal landscape (To). **B.** Intact frogs in a flat thermal landscape (Go) vs. frogs injected with saline in a complex thermal landscape (S). **C.** Intact frogs in a flat thermal landscape (Go) vs. frogs injected with LPS in a complex thermal landscape (L). Blue and green colors are the Early and Late season (respectively) when the experiments were developed.

Figure 4. Boxplot of component 3 of the PCA performed (movement and exploration on the thermal gradient). **A.** Intact frogs (To) vs. frogs injected with saline (S) in a complex thermal gradient. **B.** Intact frogs (To) vs. frogs injected with LPS (L) in a complex thermal landscape. Blue and green colors are the Early and Late season (respectively) when the experiments were developed.

Figure 5. Boxplot graphic of component 1 (central tendency measures of the temperatures registered) from the PCA performed. Intact frogs (To) and frogs injected with LPS (L) in a complex thermal landscape. Blue and green colors are the Early and Late season (respectively) when the experiments were developed

Figure 6. Distance moved in each experiment. Intact frogs in a flat thermal landscape (Go), complex thermal landscape (To) and frogs with saline injection (S) and frogs with LPS injection (L) in the complex thermal landscape. Values represents mean with 95% confidence interval.

Table 1. Species that thermoregulates by thigmothermy, thermal preference and behavioral fever temperature registered in literature.

MODEL	BEHAVIOR	THERMAL PREFERENCE (°C) (CONTROL)	FEVER (°C)
<i>Hyla cinerea</i>	Tigmotermia	25.5	27
<i>Rhinella marina</i>	Tigmotermia	25	32
<i>Rhinella schneideri</i>	Tigmotermia	23	31
<i>Bombina bombina</i>	Tigmotermia	20.3	24.3
<i>Pelophylax esculentus</i>	Tigmotermia	26	32.5
<i>Lithobates catesbeiana</i>	Tigmotermia	25.5	28.1
<i>Lithobates pipiens</i>	Tigmotermia	28.5	31.2
<i>Phyllodites maculosus</i>	Tigmotermia	13.5	18.5

Table 2. Description of the 13 variables measured from thermography recorded in the treatments.

VARIABLE (UNITS)	DESCRIPTION
	CENTRAL TENDENCY
Mean (°C)	Mean of the temperature measured for each individual in each treatment.
Median (°C)	Median of the temperature measured for each individual in each treatment.
Moda (°C)	Moda of the temperature measured for each individual in each treatment.
	DISPERSION
Variance	Variance of the temperature measured for each individual in each treatment.
Standard Deviation	The standard deviation of the temperature measured for each individual in each treatment.
Minimum (°C)	Minimum of the temperature measured for each individual in each treatment.
Maximum (°C)	Maximum of the temperature measured for each individual in each treatment.
Range (°C)	Minimum minus maximum value for temperature measured in each individual for each treatment.
Mean Change (°C)	Mean of the degrees change (difference between previous and next temperature registration) of the temperature measured for each individual in each treatment.
Variance Change	Variance of the mean change.
	TIME SERIES
Mean Direction	Mean of the direction (0, did not change; 1 increase temperature; -1 decrease temperature) of the temperature measured for each individual in each treatment.
Mean Movement	Mean of the moves done between areas of the system for each individual in each treatment.
Variance Movement	Variance of the mean movement for each individual in each treatment.

Table 3. Pairs of experiments for the PCA analysis.

Intact frogs in a flat thermal landscape (Go) vs. Intact frogs in a complex thermal landscape (To)

Intact frogs in a flat thermal landscape (Go) vs. Frogs injected with saline in a complex thermal gradient (S)

Intact frogs in a flat thermal landscape (Go) vs. Frogs injected with LPS in a complex thermal landscape (L)

Intact frogs in a complex thermal landscape (To) vs. Frogs injected with saline in a complex thermal gradient (S)

Intact frogs in a complex thermal landscape (To) vs. Frogs injected with LPS in a complex thermal landscape (L)

Frogs injected with saline in complex thermal gradient (S) vs. Frogs injected with LPS in complex thermal landscape (L)

Intact frogs in a complex thermal landscape 1 vs. Intact frogs in a complex thermal landscape 2 (To)

Frogs with saline injection in a complex thermal landscape 1 vs. Frogs with saline injection in a complex thermal landscape 2 (S)

Frogs with LPS injection in a complex thermal landscape 1 vs. Frogs with LPS injection in a complex thermal landscape 2 (L)

Table 4. Example of the three principal components chosen and its loads in each PCA of a pair of experiments. The matrix of components correspond to injected saline frogs vs. injected LPS frogs in a complex thermal landscape.

Matrix of components ^a			
	Componente		
	1	2	3
Mean	,971	,129	,025
Median	,958	,150	,020
Moda	,933	,216	,001
Min	,904	-,307	,005
Max	,799	,538	,039
Variancechange	,725	-,226	,402
Meanchange	-,557	,334	-,528
range	,010	,943	,042
Variance	,012	,896	,075
StandDev	,157	,856	,148
Meanmov	-,142	,400	,755
Variancemov	-,088	,427	,743
Meandirection	-,139	,067	-,373

9. Supplementary Data

Figure S1. Boxplot of component 3 of the PCA performed (movement and exploration on the thermal gradient). Frogs injected with saline (S) vs. Frogs injected with LPS (L) in complex thermal landscape. Blue and green colors are the Early and Late season (respectively) when the experiments were developed.

Figure S2. Boxplot graphic of component 1 (central tendency measures of the temperatures registered) from the PCA performed. **A.** Intact frogs (Go) in a flat thermal landscape vs. Intact frogs in a complex landscape (To). **B.** Intact frogs (Go) in a flat thermal landscape vs. Frogs injected with saline (S) in a complex thermal gradient. **C.** Intact frogs (Go) in a flat thermal landscape vs. Frogs injected with LPS (L) in a complex thermal landscape. Blue and green colors are the Early and Late season (respectively) when the experiments were developed.

Figure S3. Boxplot of component 2 (dispersion and variance of the data) of the PCA performed. **A.** Intact frogs (Go) in a flat thermal landscape vs. Intact frogs (To) in a complex landscape. **B.** Intact frogs in a flat thermal landscape (Go) vs. Frogs injected with saline (S) in a complex thermal gradient. **C.** Intact frogs in a flat thermal landscape (Go) vs. Frogs injected with LPS (L) in a complex thermal landscape. Blue and green colors are the Early and Late season (respectively) when the experiments were developed.

Figure S4. Boxplot graphic of component 1 (central tendency measures of the temperatures registered) from the PCA performed. **A.** Intact frogs (To) vs. Frogs injected with saline (S) in a complex thermal gradient. **B.** Frogs injected with saline (S) vs. Frogs injected with LPS (L) in complex thermal landscape. Blue and green colors are the Early and Late season (respectively) when the experiments were developed.

Figure S5. Boxplot of component 2 (dispersion and variance of the data) of the PCA performed. **A.** Intact frogs (To) vs. Frogs injected with saline (S) in a complex thermal landscape. **B.** Intact frogs (To) vs. Frogs injected with LPS (L) in a complex thermal landscape. **C.** Frogs injected with LPS (L) vs. Frogs injected with saline (S) in complex thermal landscape. Blue and green colors are the Early and Late season (respectively) when the experiments were developed.

Figure S6. Boxplot graphic of PCA performed for intact frogs in a complex thermal landscape (To). **A.** Central tendency measures of the temperatures registered (Component 1) **B.** Dispersion and variance of the data (Component 2) **C.** Movement and exploration on the thermal landscape (Component 3). Blue and green colors are the Early and Late season (respectively) when the experiments were developed.

Figure S7. Boxplot graphic of PCA performed for frogs injected with saline (S) in a complex thermal landscape. **A.** Central tendency measures of the temperatures registered (Component 1) **B.** Dispersion and variance of the data (Component 2) **C.** Movement and exploration on the thermal landscape (Component 3). Blue and green colors are the Early and Late season (respectively) when the experiments were developed.

Figure S8. Boxplot graphic of PCA performed for frogs injected with LPS (L) in a complex thermal landscape. **A.** Central tendency measures of the temperatures registered (Component 1) **B.** Dispersion and variance of the data (Component 2) **C.** Movement and exploration on the thermal landscape (Component 3). Blue and green colors are the Early and Late season (respectively) when the experiments were developed.

Discussão Geral e Conclusões

A definição de febre tem sido discutida em múltiplos trabalhos ao longo dos anos e a discussão tem sido dominada pela pesquisa voltada a se entender respostas fisiológicas. Os trabalhos realizados até o momento envolvem diferentes linhagens de aves, mamíferos, répteis, peixes e insetos (D'Alecy & Kluger, 1975; Kluger, 1975; Casterlin & Reynolds, 1977; Reynolds et al., 1978) e no geral mostram a ação de endógenos pirógenos (e.g. prostaglandinas) na mudança da temperatura corporal quando injetado um pirógeno exógeno (e.g. LPS) em condições experimentais. Embora, para o caso dos anfíbios a informação é pouca sobre os processos e respostas fisiológicas desenvolvidos durante a febre (Hutchison & Erksine, 1981; Bicego e Branco, 2002; Bicego et al., 2002) é necessário mais trabalhos que combinem o estudo das respostas fisiológicas associados a termorregulação no contexto de espécies doentes.

No contexto de respostas à infecção, a termorregulação modificada conhecida como febre comportamental é apresentada como dominante nos anfíbios, mas estudos como do Llewellyn e colaboradores (2011) e o nosso estudo com *P. boiei* apresentam uma resposta alternativa como dominante, o comportamento de doente. A simultaneidade dessas duas respostas é registrada para mamíferos (Hart, 1988) e aves (Johnson et al., 1993), em condições experimentais. Entretanto, para o caso específico dos animais que termorregulam ativamente, o comportamento de doente poderia ser incompatível com a resposta febril. Nesse contexto, as condições experimentais na pesquisa sobre o tema devem considerar as duas respostas, fisiológica e/ou comportamental. Estudos que considerem o desenvolvimento das diferentes respostas face uma infecção ou patógeno (febre comportamental, comportamento de doente, hipotermia, etc), levaria a metodologias mais standardizadas e aplicáveis para qualquer linhagem. A abordagem que usamos para estudar a febre comportamental levando em consideração o ponto de partida no sistema e o deslocamento (atividade), inclusive antes de manipulação invasiva dos indivíduos, amplia o panorama de análise. Uma vantagem é que tal abordagem leva em consideração respostas variadas, sejam termorregulatórias ou letárgicas. Uma metodologia similar foi desenvolvida por Llewellyn et al. (2011) que estudaram a alimentação, movimentação, atividade e

termorregulação de indivíduos de *Rhinella marina* e demonstraram que os perfis termais após a infecção dos indivíduos estudados eram consequência da inatividade como resposta à injeção de LPS. Assim, tanto esse como nosso trabalho corroboram a importância do comportamento doente em anuros.

Nosso estudo demonstrou a mudança no comportamento de *P. boiei* como principal resposta, diminuindo a exploração e movimento sobre o gradiente quando foram injetados com LPS. Assim a variação observada na temperatura do corpo foi uma consequência da mudança no comportamento que parece atribuível ao comportamento de doente e não a uma resposta febril. Por isso, concluímos também que definir febre comportamental a partir da observação das temperaturas preferidas como única variável estudada pode levar a interpretações erradas. O erro não estaria associado à habilidade de desenvolver febre em si, mas à dominância da resposta, pois uma vertente (comportamento de doente) poderia ser inibido pelo sistema experimental. Estes estudos sobre presença ou ausência de febre devem ser realizados em paralelo com outros de cunho mecanicista sobre os mecanismos que levam a febre comportamental em tetrápodes ectotérmicos (Kluger, 1975; Bernheim & Kluger, 1976; Bicego et al., 2000, Bicego & Branco, 2002, Bicego et al., 2002).

Diferente dos trabalhos clássicos sobre febre comportamental em vertebrados (Kluger, 1977; Casterlin & Reynolds, 1977b; Kluger, 1979; Kluger, 1991), no presente estudo houve grande variabilidade inter-individual, o que dificultou a identificação de um padrão de termorregulação modificada durante a injeção de LPS. A variabilidade interindividual neste contexto pode ter diversas causas, e não é particularmente surpreendente mas deixa claro que respostas termorregulatórias homogêneas não caracterizam a população. Segundo alguns estudos, esse tipo de variação de resposta pode ser influenciado pela temperatura anterior (aclimatação), pelas reservas energéticas do animal (Deen & Hutchinson, 2001) ou, como hipótese pelo compromisso existente com a regulação de água e sais (Kluger, 1977). Análises mais específicas que permitam avaliar essa variabilidade interindividual permitirá maior entendimento sobre as respostas que os indivíduos podem apresentar no laboratório.

A febre comportamental na literatura apresenta variados escopos termais de febre ao longo das linhagens de tetrápodes ectotérmicos que representam uma faixa de temperaturas e não um valor absoluto como já comentado. Mais do que um escopo

termal dos indivíduos injetados com LPS, o resultado obtido sobre a temperatura corporal em *P. boiei* permite estabelecer modelos sobre a distribuição das temperaturas com o tratamento de LPS. Aliás, essa distribuição de temperaturas corporais tanto nos controles quanto nos tratamentos no laboratório oferecem um panorama do potencial de *P. boiei* para o desenvolvimento das respostas de febre comportamental e comportamento de doente no campo.

Sabemos que a estrutura da paisagem termal afeta a seleção de microhabitats nos animais ectotérmicos (Adolf, 1990), mas para os anuros sabemos ainda pouco sobre este tema, ainda no contexto da termoregulação convencional. Obviamente, sabemos menos ainda sobre termoregulação modificada no contexto de febre comportamental e seu impacto sobre o escopo individual ou populacional de doenças. Estudos com escalas finas no campo que considerem o tamanho corporal e fatores microclimáticos (Helmuth et al., 2016), especialmente quando um indivíduo está doente melhorariam nosso entendimento sobre o universo global de respostas fisiológicas e comportamentais contra patógenos.

Estudos de campo como o do Richards-Zawacki (2010) ou de laboratório como do Karavlan & Venesky (2016) que associam as respostas termorregulatórias dos anuros estudados a presença do patógeno *Batrachochytrium dendrobatidis* (Bd) permitem entender que a temperatura, de alguma maneira, poderia influenciar a relação anuro-patógeno e a prevalência do Bd nos indivíduos. No entanto, as variáveis de comportamento, de exploração e movimento que usamos no nosso trabalho salientam a associação do comportamento com a resposta termoregulatória quando os indivíduos estão doentes. Nessas linhas, futuros estudos tanto de laboratório quanto de campo, deveriam focar em perguntas como: Quais respostas comportamentais e/ou termoregulatorias estão sendo desenvolvidas? As respostas variam entre contextos ecológicos e de infecção? Se sim, qual resposta seria dominante? O universo de respostas é compatível com as observações de ecologia termal? A inclusão deste tipo de perguntas nos estudos permitiria manter o vínculo entre a fisiologia termal, a ecologia termal e a conservação, no contexto das respostas dos anfíbios às doenças emergentes.

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Anexos e Apêndices

Anexo A. Aprovação da Comissão de Ética no Uso de Animais

www.ib.usp.br

CERTIFICADO

Certificamos que o Projeto intitulado **"Identificação de estados febris a partir do uso experimental de lipopolisacáridos (LPS) no anuro *Proceratophrys boiei*"**, protocolo nº 240/2015, sob a responsabilidade do Prof. Dr. Carlos Arturo Navas Iannini (Colaboradora: Laura Camila Cabanzo Olarte), que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009 e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais – CEUA do Instituto de Biociências, em reunião de 12 de fevereiro de 2016.

Qualquer alteração e/ou intercorrência deverá ser comunicada a CEUA-IB.

Vigência de Projeto: 10/02/2016 a 10/02/2017

Nº da solicitação ou autorização SISBIO: 2474595

Atividade(s): busca ativa - captura manual de *Proceratophrys boiei* (sapo de chifres)

Espécies/Grupos Taxonômicos: Anfíbio

Local: Parque Estadual Intervales

Profa. Dra. Mariz Vainzof
Coordenadora da Comissão de Ética no Uso de Animais

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