Thaysa Gomes de Oliveira

Efeitos de uma infecção simulada no comportamento alimentar e locomotor de anuros

Effects of a simulated infection on feeding and locomotor behavior of anurans

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Dedico esta pesquisa a minha família, em especial a minha mãe, a todos professores e colegas do departamento de Fisiologia, e a toda sociedade que apoia e acredita na pesquisa e na ciência.

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"Os benefícios da ciência não são para os cientistas, e sim para humanidade."

Louis Pasteur
(Tradução livre)

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Resumo

O tema central dessa pesquisa é o efeito da ativação do sistema imune no comportamento de anuros. Salientamos que as respostas imunes, a alimentação e a locomoção demandam recursos metabólicos e energéticos, e geralmente ocorrem em paralelo, o que pode gerar um *trade-off*. Além disso, quando um animal está doente as respostas imunológicas podem levar a depressão do comportamento, o que pode estar associada à economia destes recursos. Em anfíbios, foi observado que uma infecção simulada pode induzir uma depressão comportamental, com redução da alimentação e locomoção. No entanto, estudos com espécies invasoras têm demonstrado que estes animais mantem sua capacidade de dispersão (comportamento locomotor) mesmo doentes. Assim, esta dissertação é composta por cinco partes. A introdução geral apresenta a interface entre o comportamento alimentar e locomotor de anfíbios, as respostas imunológicas, os mecanismos atrelados a infecção simulada por LPS, e os custos associados aos dois comportamentos e a ativação do sistema imune. No capítulo 1, estudamos o comportamento alimentar e locomotor de anuros da espécie *Aquarana catesbeiana* após infecção simulada por injeções de LPS. O tratamento com LPS reduziu a alimentação e a locomoção dos indivíduos, evidenciando a depressão comportamental nesta espécie. No capítulo 2, investigamos o impacto da infecção simulada por injeções de LPS no comportamento locomotor de anuros machos e fêmeas da espécie *Xenopus laevis*. O tratamento com LPS reduziu o desempenho locomotor dos animais, mas não teve efeito sobre os movimentos voluntários e não demonstrou ser diferente entre os sexos na maioria das variáveis observadas. No capítulo 3, investigamos o impacto da infecção simulada por injeções de LPS no comportamento locomotor de anuros da espécie *Xenopus laevis* (espécie invasora) e de anuros da espécie *Xenopus allofraseri* (espécie não invasora). A infecção simulada reduziu o desempenho locomotor em ambas as espécies, no entanto a espécie invasora foi menos afetada. Finalmente, a discussão geral explica a integração das pesquisas realizadas nos três capítulos e como estes contribuem para a discussão de cada ponto abordado na introdução geral, mostrando um diálogo entre fisiologia e ecologia de anfíbios no contexto de doenças.

Abstract

The central theme of this research is the effect of immune system activation on anuran behavior. We emphasize that immune responses, food and locomotion demand metabolic and energy resources, and usually occur in parallel, which can generate a trade-off. In addition, when an animal is sick, the immune responses can lead to behavioral depression, which may be associated with the economy of these resources. In amphibians, it has been observed that a simulated infection can induce behavioral depression, with reduced feeding and locomotion. However, studies with invasive species have shown that these animals maintain their ability to disperse (locomotor behavior) even when they are sick. Thus, this dissertation is composed of five parts. The general introduction presents the interface between the feeding and locomotor behavior of amphibians, the immune responses, the mechanisms linked to simulated infections by LPS, and the costs associated with both behaviors and the activation of the immune system. In chapter 1, we studied the feeding and locomotor behavior of anurans of the species *Aquarana catesbeiana* after simulated infection by LPS injections. The treatment with LPS reduced the feeding and locomotion of the individuals, evidencing the behavioral depression in this species. In chapter 2, we investigated the impact of simulated infection by LPS injections on the locomotor behavior of male and female anurans of the species *Xenopus laevis*. Treatment with LPS reduced the locomotor performance of the animals, but had no effect on voluntary movements and did not show to be different between the sexes, in most of the variables observed. In chapter 3, we investigated the impact of simulated infection by LPS injections on the locomotor behavior of anurans of the species *Xenopus laevis* (invasive species) and anurans of the species *Xenopus allofraseri* (non-invasive species). The simulated infection reduced locomotor performance in both species, however the invasive species was less affected. Finally, the general discussion explains the integration of research carried out in the three chapters and how they contribute to the discussion of each point covered in the general introduction, showing a dialogue between amphibian physiology and ecology in the context of diseases.

Introdução Geral

1.Interface entre o comportamento alimentar e locomotor de vertebrados

A alimentação e a locomoção são atividades básicas na maioria dos vertebrados, sendo interdependentes e complementares. A ingestão de alimentos fornece energia para a atividade locomotora, e a locomoção permite ao animal ter acesso a fontes alimentares (Nielsen- Schimdt, 1975; Stehouwer, 1992; Randall *et al*., 2000; Pough *et al*., 2008). O comportamento alimentar ocorre através de diferentes componentes, que em conjunto demandam a utilização de energia e metabólitos, eventualmente com impacto no orçamento destes recursos (Nielsen- Schimdt, 1975; Stehouwer, 1992; Pough *et al*., 2008), e também difere dependendo do estilo de forrageamento de um animal. Por exemplo, em animais móveis e associados ecologicamente a fontes de alimento dispersas, particularmente no caso de predadores, a detecção de uma presa está associada a motivação comportamental envolvendo a procura de alimento, deslocamento direcionado (uma vez que o alimento é detectado), tentativa de captura e ingestão (Nielsen-Schimdt, 1975; Taigen & Pough, 1983; Taigen & Pough, 1985; Randall *et al*., 2000; Pough, 2008; Hill *et al*., 2012). Esse tipo de ecologia, relacionado ao comportamento alimentar, acarreta maiores gastos de recursos energéticos e metabólicos, dado a maior movimentação e locomoção necessária para obter o alimento (Mayr 1963; Krebs & Davies, 1987; Taigen & Pough, 1983; Taigen & Pough, 1985). No entanto, há também predadores com hábitos "sit-and-wait" que apresentam forrageamento passivo, onde o animal espera que presas se aproximem e então realiza o ataque e a captura através de um movimento rápido (Mayr 1963; Taigen & Pough, 1981; Taigen & Pough, 1983; Taigen & Pough, 1985; Krebs & Davies, 1987; Pough, 2008; Lowe, 2009; Hill *et al*., 2012).

1.1 Controle fisiológico do comportamento alimentar

A alimentação e a locomoção possuem diferente controle fisiológico no organismo. O controle da alimentação envolve principalmente o hipotálamo e o trato gastrointestinal, que se comunicam e levam, de forma geral, a três principais componentes da alimentação, a ingestão alimentar e fome, a saciedade, e a digestão e absorção de nutrientes (Schwartz *et al.*, 2000; Valassi *et al.*, 2008; Shin *et al*., 2009; Cegla *et al*., 2010; Cavalcante, 2016). A ingestão alimentar que leva a sensação de fome é controlada principalmente por substâncias orexígenas que desencadeiam o apetite, como hormônios secretados pelo estômago e intestino. Um

exemplo destes hormônios é a grelina que é produzida principalmente no estômago quando vazio e gera sinais que vão agir no sistema nervoso central (SNC). O cérebro interpreta e integra estes sinais e promove uma resposta reguladora coordenada levando a sensação de fome e busca por alimento (Matisuda & Keisuke, 2007; Valassi *et al.,* 2008; Cegla *et al*., 2010; Hill *et al*., 2012; Alvarez-Leite *et al*., 2016; Bender *et al.,* 2018).

Por outro lado, a saciedade ocorre principalmente pela ação de neurônios anorexígenos que vão agir para inibir a fome através da secreção de hormônios anorexígenos, que podem ser de curto ou longo prazo. Estes hormônios vão agir principalmente via nervo vago sinalizando ao SNC para desencadear a sensação de saciedade (Valassi *et al.,* 2008; Cegla *et al*., 2010; Cavalcante, 2016; Alvarez-Leite *et al*., 2016; Bender *et al.,* 2018). Os hormônios de curto prazo são produzidos quando o bolo alimentar chega no intestino delgado, um exemplo é a insulina, produzida pelo pâncreas em resposta ao aumento dos níveis de glicose no sangue após uma refeição. A insulina é capaz de inibir a ingestão alimentar através da estimulação de neurônios anorexígenos e inibição de orexígenos (Baskin *et al.,* 1999; Valassi *et al.,* 2008; Cegla *et al*., 2010; Cavalcante, 2016; Alvarez-Leite *et al*., 2016; Bender *et al.,* 2018). Os hormônios anorexígenos de longo prazo compreendem principalmente a leptina, um hormônio peptídico produzido pelos adipócitos no tecido adiposo que será liberado na corrente sanguínea. A leptina vai agir nos núcleos do hipotálamo estimulando neurônios anorexígenos que então vão inibir neurônios orexígenos de se expressarem, o que reduz a ingestão alimentar levando a sensação de saciedade (Baskin *et al.,* 1999; Richard & Wlaker, 2002; Matisuda & Keisuke, 2007; Valassi *et al.,* 2008; Cegla *et al*., 2010; Hill *et al*., 2012; Cavalcante, 2016; Alvarez-Leite *et al*., 2016; Bender *et al.,* 2018).

A digestão pode ser modulada por reflexos curtos e longos. Os reflexos curtos ocorrem através do sistema nervoso entérico que reveste o trato gastrointestinal e que ao receber o alimento gera diretamente uma resposta sem a participação do SNC. Os reflexos longos ocorrem através do nervo vago que está presente em todo trato gastrointestinal e o conecta com o SNC para gerar uma resposta (Valassi *et al.,* 2008; Cegla *et al*., 2010; Hill *et al*., 2012; Alvarez-Leite *et al*., 2016; Bender *et al.,* 2018). O controle da digestão envolve três fases, a primeira relacionada a motivação, chamada fase cefálica, que compreende a percepção do alimento que vai ser ingerido através dos órgãos do sentido e estimula, através do nervo vago, a glândula salivar e motilidade gástrica que leva a uma preparação do organismo a chegada do alimento, em seguida ocorre os processos de mastigação (em algumas espécies) e deglutição. A segunda fase compreende a fase gástrica que acontece quando o alimento chega até o

estômago e estimula células endócrinas e parietais a produzirem substâncias ácidas responsáveis pela digestão. A terceira fase compreende a fase intestinal quando o bolo alimentar é liberado para o duodeno, esta fase depende da regulação neural e humoral que vai liberar substâncias como o suco pancreático e bile para o intestino delgado que vão dar início ao último processo de digestão (Valassi *et al.*, 2008; Cegla *et al*., 2010; Hill *et al*., 2012; Alvarez-Leite *et al*., 2016; Bender *et al.*, 2018).

Após a digestão, proteínas, carboidratos e gorduras geram compostos como aminoácidos, açucares e ácidos graxos que são oxidados e produzem quase toda a energia química necessária ao organismo para a realização de atividades. Para que exista equilíbrio relativo à utilização de energia, o gasto energético total e a ingestão de alimentos devem ocorrer na mesma proporção, levando ao balanço energético do organismo e homeostase (Nielsen-Schimdt, 1975; Shin *et al*., 2009). Na maioria dos indivíduos, a ingestão é bem ajustada ao gasto de energia, e se o gasto de energia for aumentado ou diminuído a ingestão alimentar tende a ser regulada de acordo com as necessidades energéticas (Nielsen-Schimdt, 1975; Jensen, 2000; Crespi & Denver, 2005; Asarian & Langhans, 2005; Shin *et al*., 2009; Alvarez-Leite *et al*., 2016; Cavalcante, 2016; Bender *et al.*, 2018).

O estudo do comportamento alimentar e da alimentação pode ser realizado em laboratório, e sob condições laboratoriais costumam ter um baixo custo energético (salvo em casos de restrição alimentar) em relação ao animal na natureza, independentemente do tipo de hábito alimentar. Portanto, realizar metodologias que tentem incluir o que o indivíduo enfrenta no ambiente natural para se alimentar são necessárias, para que seja possível, a partir de testes experimentais, fazer inferências sobre estes animais na natureza. A alimentação pode ser analisada através do tamanho de cada refeição (e.g. número de presas ingeridas) em cada episódio alimentar e pela frequência e busca do alimento (interesse pelo alimento). Dessa forma, mudanças na ingestão alimentar podem ter reflexo em variáveis quantificáveis, por exemplo na observação da diminuição do tamanho e/ou na frequência de refeições, que ser refere ao número de vezes que o indivíduo se alimenta em um determinado período (Asarian & Langhans, 2005). Portanto, o padrão de refeições é informativo na análise do comportamento alimentar e pode permitir verificar se um animal está se alimentando normalmente em laboratório ou se houve diminuição da alimentação (Asarian & Langhans, 2005).

1.2 Controle fisiológico do comportamento locomotor

A locomoção é uma ação motora que leva um animal de uma localização para outra, sendo provavelmente a atividade diária mais comum entre os animais. O comportamento locomotor geralmente está associado a motivação neurofisiológica para se deslocar, o que pode envolver inúmeros fatores, como exploração, fuga de predadores, procura de parceiros para reprodução, procura por fontes hídricas e a busca por alimentos (Nielsen-Schimdt, 1975; Randall *et al*., 1989; Pough, 2008). O tipo de locomoção realizada por um animal está intimamente relacionado a evolução da morfologia e da fisiologia, que por sua vez estão relacionadas ao tipo de habitat e substratos do local onde o indivíduo habita (Zug, 1972; Nielsen-Schimdt, 1975; Dickinson *et al.*, 2000). A locomoção está associada a um sistema locomotor que de certas perspectivas é conservado nos vertebrados, apesar da enorme diversidade funcional manifesta em tipos de locomoção tão diversos como rastejar, nadar, saltar, voar, andar e correr (Nielsen-Schimdt, 1975; Randall *et al*., 1989; Pough, 2008; Hill *et al*., 2012). Os anuros exemplificam esta situação, pois se trata de uma linhagem que, mesmo com um tipo corpóreo relativamente conservado, têm características morfológicas associadas ao tipo de habitat e à diversidade comportamental (Zug, 1972; Seymour, 1973; Emerson, 1978; Emerson, 1985; Edamura *et al*., 1992; Marsh, 1994; Gomes *et al*., 2009; Ottano, 2011).

Em termos mecanicistas, a regulação fisiológica da locomoção envolve a interação de componentes como o controle supra espinhal do tronco encefálico, medula espinhal, gerador padrão central do movimento (GPC) e *feedback* sensorial (Busches & Manira, 1998; Grillner *et al*., 1998; Basso, 2005; Barrièrre *et al*, 2007; Katz, 2015; Dutta *et al*., 2019). A regulação da locomoção se inicia quando a informação sobre a intenção de se realizar um movimento é transmitida por estímulos emitidos do córtex para a medula espinhal através de fibras nervosas. Esses estímulos ativam neurônios do centro locomotor, o GPC localizado na medula espinhal, que vai acionar os grupos musculares efetores que após o início da locomoção mantém uma atividade rítmica dos neurônios que comandam a atividade muscular (Grillner, 1975; Nielsen-Schimdt, 1975; Edamura, 1992; Busches & Manira, 1998; Grillner *et al*., 1998; Barrièrre *et al*, 2007; Kandel, 2014; Albuqerque, 2015; Katz, 2015; Dutta *et al*., 2019). A locomoção, na maioria dos casos, é baseada no movimento de ritmicidade dos membros e fundamentada na contração e relaxamento alternado de músculos extensores e flexores em cada membro (Grillner, 1975; Nielsen-Schimdt, 1975; Grillner *et al*., 1998; Basso, 2005; Barrièrre *et al*, 2007; Kandel, 2014; Katz, 2015; Dutta *et al*., 2019). Durante a locomoção o organismo também recebe, através de receptores sensoriais, *feedback* aferente sobre o movimento realizado e sobre

o substrato que está sendo percorrido. Esse *feedback* permite ao animal processar informações sobre o ambiente em que está se locomovendo, por exemplo obstáculos ao longo do percurso e mudança de solo, e, com isso, adequar os movimentos do corpo para cada situação (Grillner *et al*., 1998; Barrièrre *et al*, 2007; Hill *et al*., 2012; Katz, 2015).

2. Comportamento alimentar e locomotor de anfíbios

O comportamento alimentar e locomotor de anfíbios pode variar dentro de cada grupo e ao longo do desenvolvimento do indivíduo, sendo que alimentação e locomoção podem ter sua ocorrência correlacionada. Mesmo entre formas adultas, existe enorme variação na ecologia da alimentação e uma variação correspondente na fisiologia da locomoção de anfíbios. Assim, dependendo do tipo e hábito alimentar e locomotor, uma espécie de anfíbio irá apresentar um determinado comportamento associado à obtenção de alimentos, o que também está relacionado ao tipo de habitat e ao tipo de presa (Bennett & Licht, 1973; Emerson, 1976; Taigen & Pough, 1981; Taigen & Pough, 1983; Taigen & Pough, 1982; Taigen & Pough, 1985; Deban *et al.*, 1992; Deban *et al.*, 2001; Pough *et al*., 2008). Isso pode ser observado nos anuros que passam por diferentes fases de desenvolvimento, com variação no comportamento alimentar e locomotor.

2.1 Controle fisiológico do comportamento alimentar de anfíbios

A passagem de anuros da vida aquática para a terrestre envolve a metamorfose, uma mudança profunda no plano organizacional do animal que inclui, entre outros fatores, o tipo de dieta e locomoção (Zug, 1972; Pough, 1984; Hourdry *et al*., 1996). Os diferentes tipos de comportamento alimentar dos anuros estão relacionados às mudanças morfológicas que acontecem durante o crescimento e ao tipo de alimento que o animal consegue ter acesso em cada ambiente, nos diferentes estágios de vida (Emerson, 1976; Taigen & Pough, 1981; Taigen & Pough, 1983; Taigen & Pough, 1985; Deban *et al.*, 2001; Pough *et al*., 2008). No estágio larval os girinos são primariamente herbívoros filtradores enquanto no estágio pós-metamórfico a maior parte dos anuros são exclusivamente predadores (Shinn & Doles, 1978; Toft, 1980; Dole *et al.*, 1981; Pough & Kamel, 1984; Deban *et al.*, 2001; Crespi & Denver 2005). A percepção do alimento pelos girinos ocorre através de sinais químicos e mecânicos, e a maioria compartilha os mesmos mecanismos básicos de alimentação por filtragem e bombeamento ou sucção do alimento (Orton, 1953; Sokol, 1962; Ewert, 1987; Mcdiarmid & Altig, 1999; Deban *et al*., 2001). Nos anuros adultos, pistas olfativas e táteis, o desenvolvimento de novas conexões

neuronais ópticas e a aquisição da visão estereoscópica facilitam a percepção e captura do alimento (Keating & Chung, 1974; Duellman & Trueb, 1986; Nishikawa & Roth, 1991; Deban *et al.*, 2001).

A regulação fisiológica do comportamento alimentar de anuros adultos ocorre, em geral, através dos mesmos mecanismos comentados anteriormente (tópico 1.1) com mudanças decorrentes dos diferentes estágios de vida e hábito alimentar. Essas mudanças acontecem durante e após a metamorfose e envolvem às transformações do trato digestivo que passa a receber diferentes tipos de alimentos e é completamente modificado. Por exemplo, ocorre o desaparecimento do aparelho de filtragem da faringe e o desenvolvimento de um estômago que passa a receber presas maiores (Nielsen-Schimdt, 1975; Pough & Kamel, 1984; Randall *et al*., 1989; Hourdry *et al*., 1996; Hill *et al*., 2012). A alimentação de anuros adultos, particularmente o comportamento predatório, está relacionada com a morfologia do animal, sendo a maioria capaz de capturar e ingerir insetos e animais compatíveis com seu tamanho corpóreo (Bury & Whelan, 1984; Hourdry *et al*., 1996; Almeida, 2010). O hábito alimentar de anuros adultos pode diferir entre as linhagens, existindo dois tipos de predadores, ativos e do tipo "sit and wait", que geralmente apresentam comportamento sedentário. No primeiro caso, envolve a busca ativa por presas, o que utiliza como componente principal a locomoção na procura de alimento, dentre este tipo de predador há indivíduos que consomem presas menores e em maior quantidade, como formigas que vivem em colônias, por exemplo (Bennett & Licht, 1973; Taigen & Pough, 1981; Taigen *et al*, 1982; Taigen & Pough, 1985). No caso de predadores sedentários ("sit and wait") a obtenção de alimento ocorre de forma passiva, onde os animais aguardam pela aproximação da presa, estes animais geralmente se alimentam em menor quantidade, porém de presas maiores, como grandes artrópodes (Bennett & Licht, 1973; Taigen & Pough, 1981; Taigen *et al*, 1982; Taigen & Pough, 1985).

2.2 Controle fisiológico do comportamento locomotor de anfíbios

A atividade locomotora de anuros apresenta modificações em seu padrão geral, ainda mais no contexto da enorme diversidade na história natural deste grupo que tem correlações entre ecologia, fisiologia, morfologia e comportamento (Zug, 1972; Seymour 1973; Bennett & Licht 1973; Emerson 1976; Toft 1980; Taigen *et al.* 1982; Pough & Taigen, 1990; Stehouwer, 1992). A relação estreita entre forma e função tem sido sugerida como resultado de processos adaptativos, dado que a locomoção é relevante para a aptidão, pois permite ao animal ser capaz de se mover no ambiente e realizar atividades que dependem de deslocamento, como a procura

por alimentos. Portanto, a locomoção pode afetar a alimentação, a fuga de predadores, a reprodução, a exploração ambiental e, com isso, o balanço de energia do organismo (Zug, 1972; Duellman & Trueb, 1986; Bennett & Licht 1973; Edamura *et al*., 1992; Stehouwer, 1992; Dickinson *et al*., 2000; Gomes *et al*., 2009).

O tipo de locomoção está ligado a fase de desenvolvimento e ao tipo corpóreo, que por sua vez está relacionado ao tipo de hábito alimentar. Por exemplo, no estágio larval os girinos se movimentam através de batimentos da cauda na água o que permite a filtração. Já nas formas adultas a locomoção envolve sempre quatro membros bem desenvolvidos, com membros traseiros alongadas e um corpo curto e inflexível (Nielsen- Schmidt, 1975; Pough, 1984; Randall *et al*., 1989; Pough *et al*., 1992; Hill *et al*., 2012). Em geral, as patas traseiras são utilizadas para se locomover, saltar ou nadar, enquanto os membros anteriores são utilizados durante a alimentação para manipular o alimento (Shinn & Dole, 1978; Toft, 1980; Dole *et al*., 1981; Pough, 1984; Deban *et al*., 2001). As espécies que possuem pernas curtas se movem através de pequenos saltos e são, em sua maioria, predadores ativos, já espécies com pernas alongadas que se movem por grandes saltos são, geralmente, predadores do tipo senta e espera (Zug, 1972; Taigen *et al*., 1982; Pough *et al*., 2008). Ainda, entre as linhagens de anuros, quando adultos, os tipos de locomoção podem ser diferentes entre as espécies, devido as adaptações morfológicas que levam a diferentes formas do corpo, que geralmente está associado ao ambiente de habitação (Emerson, 1978; Zug, 1978). Por exemplo, em linhagens terrestres a locomoção ocorre, predominantemente, através de saltos ou de caminhada, em linhagens aquáticas a locomoção ocorre exclusivamente através da natação, e em linhagens arbóreas a locomoção ocorre através de saltos e o animal tende a ficar em árvores onde se fixa através de fricção ou aderência no tronco pelos discos presentes nos dedos (Emerson, 1978; Zug, 1978).

O controle fisiológico da locomoção dos anfíbios também ocorre através do GPC localizado na medula espinhal que recebe sinais neuronais para se locomover. Estes sinais são iniciados no córtex cerebral gerando o batimento da cauda na água em anuros no estágio larval, e o caminhar, saltar e nadar de anuros em fase adulta (Stehouwer & Farel, 1980; Stehouwer & Farel, 1981; Stehouwer, 1992). Isso é possível devido as diferenças intrínsecas na organização da medula espinhal nos diferentes estágios de vida, as mudanças morfológicas e de hábitat, e pelos *feedbacks* que o animal recebe através de informações do ambiente em cada fase do desenvolvimento, passando da água para a terra (Stehouwer & Farel, 1980; Stehouwer & Farel, 1981; Stehouwer, 1992). A locomoção dos girinos através da cauda é mediada por conexões

monossinápticas entre as células de Mauthner (definido como um neurônio que é capaz de conduzir um comportamento específico sozinho), do tronco encefálico, de motoneurônios espinhais e GPCs (Stehouwer & Farel, 1980; Stehouwer & Farel, 1981; Stehouwer, 1992). Em anuros pós-metamórficos que se locomovem através de caminhada ou de saltos nas formas terrestres, e do nado nas formas aquáticas, a locomoção é controlada através de GPCs. A locomoção se inicia através de comandos dos GPCs para motoneurônios que vão enviar impulsos alternados, bilateralmente simétricos e sincronizados para os músculos dos membros posteriores do animal que desencadeiam o movimento rítmico de locomoção (Stehouwer & Farel, 1984).

3. Custos e benefícios do comportamento alimentar e locomotor de anfíbios

A alimentação e a locomoção são atividades básicas em animais vertebrados que demandam recursos internos do organismo animal, como energia química e metabólitos (Bennet, 1978; Taigen & Pough, 1981; Taigen & Pough, 1985). Nos animais móveis, a alimentação e a locomoção geralmente ocorrem associadas com variação no gasto destes recursos dependendo do tipo de ambiente, a distância média das fontes de alimento, o tipo de locomoção, o formato da busca e captura de presas e os processos associados à digestão (Bennett & Licht, 1973; Emerson, 1976; Taigen & Pough, 1981; Taigen & Pough, 1983; Pough, 1984; Taigen & Pough, 1985; Randall *et al..,* 1989; Pough *et al*., 1992; Pough *et al*., 2008). O sucesso reprodutivo do indivíduo depende de um comportamento alimentar que, em média, garanta ganhos ao animal, em termos de energia e nutrientes, relativo aos custos da busca que envolve a locomoção, manipulação, ingestão e digestão. Portanto, é presumível que custos mais altos para obter alimento e para se locomover podem levar a uma menor taxa de alimentação e de realização de outras atividades dependentes da locomoção (Bennett & Licht, 1973; Emerson, 1976; Taigen & Pough, 1981; Taigen & Pough, 1983; Taigen & Pough, 1985; Davis *et al*., 2010; Begon *et al*., 2017).

Os diferentes tipos de comportamento alimentar e locomotor de anfíbios levam a diferenças significativas nas capacidades metabólicas entre as espécies correlacionadas com os hábitos alimentares (Bennett & Licht, 1973; Bennett, 1978; Taigen *et al.,* 1982; Aspey & Lustick, 1983; Taigen & Pough, 1983; Taigen & Pough, 1995; Pough & Taigen, 1990; Ottano, 2011; Braga, 2013). Sendo que os tipos de forrageamento têm associações metabólicas, onde as espécies mais ativas apresentam taxas metabólicas mais altas e se caracterizam por uma elevada capacidade aeróbia associada ao movimento extensivo na busca por presas (Bennett &

Licht, 1973; Emerson, 1976; Taigen *et al*., 1982; Taigen & Pough, 1983). Ainda, dependendo do tipo de presa a ser consumida, e, com isso, do tipo de captura realizada, vai existir distinção na capacidade aeróbia. Por exemplo, um predador que consome presas capturadas apenas mediante um movimento rápido da língua, tende a apresentar gastos metabólicos e energéticos mais baixos neste contexto, visto que a captura geralmente não necessita de grandes esforços. Ao contrário, um predador que consome presas que exigem manipulação, na qual a captura envolve luta e fuga, apresentam maiores gastos energéticos e metabólicos (Bennett & Licht, 1973; Bennett, 1978; Taigen *et al.*, 1982; Taigen & Pough, 1983; Taigen & Pough, 1995; Pough & Taigen, 1990; Ottano, 2011). Já predadores do tipo "sit and wait", apresentam taxas metabólicas mais baixas e também baixa capacidade aeróbica, pois não realizam movimentos extensivos de busca por presas, e alta capacidade anaeróbica pois, a captura de presas grandes e móveis envolve uma investida rápida através de um alto esforço (Bennett, 1978; Taigen *et al.*, 1982; Taigen & Pough, 1983; Taigen & Pough, 1995; Pough & Taigen, 1990; Ottano, 2011).

Neste contexto, dependendo da ecologia e fisiologia do forrageio, as demandas de energia e metabólitos ligados à alimentação e locomoção podem impor restrições a outros sistemas fisiológicos que também guardam correlação com ecologia e utilização de recursos, como a ativação do sistema imune e as repostas imunológicas (Aspey & Lustick, 1983; Larson & Dunn, 2001; Braga, 2013). Cenários em que a ocorrência da alimentação e da locomoção ocorrem em paralelo com a ativação do sistema imune não são improváveis, e poderiam, relativo à utilização de recursos, gerar um tipo de compromisso chamado de *trade-off*. Em anuros estudos na temática apresentada oferecem a oportunidade de entender como as características metabólicas e energéticas estão relacionadas com o desempenho em determinados tipos de atividade, dada à diversidade comportamental inter e intraespecífica do grupo. Além disso, a relação entre a variação funcional dos sistemas energéticos e metabólicos com a variação comportamental dos anuros, principalmente da perspectiva da alocação de recursos que permite a ocorrência da alimentação e locomoção juntamente com outras atividades ainda não estão bem esclarecidas neste grupo.

4. Influência de respostas imunológicas no comportamento alimentar e locomotor

As respostas imunológicas estimuladas por patógenos ou parasitas fazem parte da história natural de todos os vertebrados (Hart, 1988; Aubert, 1999; Larson & Dunn, 2001). Microrganismos como bactérias, em certos contextos, podem gerar patogêneses quando se instalam em locais do organismo no qual estimulam respostas imunes, incluindo processos

inflamatórios (Hart, 1988; Aubert, 1999; Larson & Dunn, 2001). O eixo hipotálamo-hipófise-adrenal (HPA) é responsável por modular a resposta imune e inflamatória, mantendo a capacidade do organismo de responder a estímulos e estresse (Hart, 1988; Aubert, 1999; Sapolsky, *et al*., 2000; Larson & Dunn, 2001; Dhabhar 2014). Quando uma bactéria é reconhecida como patogênica pelo organismo, o sistema imune é ativado e gera respostas imunológicas que formam uma linha de defesa com o objetivo de eliminar o patógeno e/ou células infectadas. As respostas imunológicas iniciais são conhecidas como resposta de fase aguda que incluem febre, inflamação, liberação de proteínas defensivas no fígado e aumento da função de leucócitos (Casterlin & Reynolds, 1977; Hart, 1988; Aubert, 1999; Adelman & Martin, 2009). Essas respostas envolvem diferentes tipos celulares, como fagócitos, linfócitos e células endoteliais que agem mediados por citocinas. As citocinas pró-inflamatórias, como IL-1, IL-6 e TNF-α, são sintetizadas e liberadas por células imunocompetentes como macrófagos durante o curso do processo infeccioso (Hart, 1988; Dantizer, 2000; Dantizer, 2004; Dantizer, 2006; Adelman & Martin, 2009; Abbas, *et al*., 2013; Madigan, *et al.*, 2016).

A síntese e liberação de citocinas pró-inflamatórias (IL-1, IL-6 e TNF-α) induzidas por endotoxinas patogênicas pode levar a alterações neurológicas, endócrinas, metabólicas e comportamentais (Hart, 1988; Aubert, 1999; Konsman, *et al.*, 1999; Konsman, 2000; Dantizer, 2001; Dantzer, 2006). As repostas comportamentais levam a redução geral da realização de atividades, denominada na literatura como depressão comportamental e caracterizada pela letargia, redução da interação social e da procura por parceiros para reprodução, e redução do comportamento alimentar e locomotor, o que ocorre na maioria dos vertebrados (Casterlin & Reynolds, 1977; Hart, 1988; Lefcort & Eiger, 1993; Aubert, 1999; Dantzer, 2006; Adelman & Martin, 2009; Lewellyn *et al.*, 2011; Braga, 2013). A redução do comportamento alimentar é mediada principalmente pelas interleucinas IL-1 e IL-6 que tem a produção aumentada e levam a perda do apetite, diminuem a quantidade de alimentos ingeridos, e, com isso, o indivíduo apresenta uma menor taxa de alimentação após o desafio imune (Hart 1988; Plata-Salamán, *et al.*, 1988; Larson *et al*., 1996; Kent *et al*., 1944; Inui 2001; Larson *et al*., 2001; Larson & Dunn, 2001).

As respostas imunes demandam recursos (energia e metabólitos) para ocorrer, como comentado. Assim, a redução do comportamento alimentar e locomotor, decorrentes da depressão comportamental durante um desafio imune, pode estar associada à conservação destes recursos, que podem, eventualmente, serem alocados ao sistema imune e respostas imunológicas e, consequentemente, auxiliar na eliminação do patógeno e combate a infecção

(Hart, 1988; Aubert, 1999; Dantzer, 2006). Além disso, a redução da alimentação e locomoção pode levar a menor ingestão de micronutrientes importantes para o crescimento de microrganismos patogênicos (e.g. ferro no sangue), e diminuir o risco de predação, devido a menor taxa de locomoção e consequente menor exposição, o que pode estar associado também ao menor interesse na busca por alimentos por exemplo (Hart, 1988; Aubert, 1999; Dantzer, 2006; Adelman & Martin, 2009). Portanto, a redução do comportamento alimentar e locomotor, que caracteriza em muitos casos os indivíduos doentes, não é um efeito deletério ou indesejável da doença, mas uma estratégia fisiológica que pode ser ecologicamente crítica (Hart 1988).

Estudos sobre as respostas imunológicas desencadeadas pela ativação do sistema imune têm sido realizados principalmente em animais endotérmicos como os mamíferos. No entanto, é provável que muitos dos mecanismos moleculares de ação de citocinas e, consequentemente, suas respostas comportamentais e autonômicas sejam compartilhadas com vertebrados ectotérmicos, como os anfíbios. Em anuros por exemplo, foi observado que após um processo infeccioso simulado algumas espécies apresentam depressão do comportamento com redução das taxas de alimentação e locomoção (Llewellyn *et al.*, 2011; Braga, 2013). Outras espécies de anuros, ao contrário, apresentam um sistema imune deprimido e conseguem manter sua capacidade de realizar atividades mesmo doentes (Llewellyn *et al.*, 2012; Brow & Shine, 2014; Gardner *et al.*, 2020).

5. Ação e efeito do LPS após simulação de infecção

Simulações de infecção podem ser realizadas em laboratório para estudos sobre sua influência em diferentes tipos de atividades realizadas pelos animais. Para isso podem ser utilizadas injeções de lipopolissacarídeo (LPS) da parede celular de bactéria gram-negativas, que são endotoxinas reconhecidas pelo organismo animal como agentes patogênicos. Estes agentes ativam o sistema imune inato e levam a respostas imunológicas de combate à infecção (Sherman *et al*., 1991; Sherman & Stephens, 1998; Ulevitch & Tobias, 1999; Bicego, 2002). A ação do LPS no organismo desencadeia uma resposta imune, pois, promove a mobilização de citocinas (IL-1β, IL-6, interferõns, TNFα). Estas citocinas são chamadas de pirógenos endógenos que induzem, através de vias humorais e neuronais, respostas efetoras que levam, dentre outras, a diminuição de comportamentos como o alimentar e locomotor (Hart, 1988; Aubert, 1999; Dantzer, 2006).

A ação do LPS que desencadeia processos infecciosos e, consequentemente, respostas imunológicas, acontecem pelo lípideo A da molécula de LPS. O lípideo A é um carboidrato

central ligado a um fosfolipídio preso na membrana externa de bactérias gram-negativas, que dentro do organismo é exposto e leva a uma variedade de respostas (Rietschel *et al.*, 1993; Ulevitch & Tobias, 1999). No organismo da maioria dos animais, o receptor para o LPS é composto por Toll (TLR-4), MD-2 (fator de diferenciação mielóide) e CD14, que quando se ligam ao LPS levam a formação de um complexo (LPS-LBP-CD14) que aumenta a atividade de ligação do LPS e leva ao sucesso da infecção. Este complexo vai induzir a transcrição de citocinas pró-inflamatórias por exemplo, IL-1, IL-6, TNF-α, dentre outros, que vão induzir uma cascata neuro-imune-endócrina e desencadear as respostas imunológicas autonômicas e comportamentais já comentadas (RietscheL *et al*., 1993; Rietchel *et al*., 1999; Ulevitch & Tobias, 1999; Takeda & Akira, 2005).

A ação específica do LPS no organismo de anuros é pouco esclarecida em detalhes na literatura, mas já foi observado que anuros injetados com LPS apresentam em seus níveis plasmáticos glicocorticóides e moléculas pró-inflamatórias como as citocinas (IL-1β, IL-6, TNF-α), que desencadeiam uma resposta inflamatória e levam as alterações comportamentais comentadas (Ferreira *et al*., 2021; Junior *et al*., 2021; Bastos *et al*., 2022).

6. Apresentação dos capítulos seguintes

No Capítulo 1 abordamos o estudo das respostas comportamentais, especificamente alimentação e locomoção, em indivíduos da espécie de anuro *Aquarana catesbeiana* (Dubois *et al*., 2021), frente a uma infecção simulada. Consideramos que 1) Infecção simulada por LPS diminui o comportamento alimentar e locomotor dos indivíduos em um ambiente onde o acesso às presas não é imediato e envolve exploração. Neste trabalho diferenciamos as respostas dominantes de alimentação e locomoção em ambiente experimental, analisando os animais antes e depois dos tratamentos com LPS. No Capítulo 2 abordamos o estudo das respostas comportamentais, especificamente desempenho locomotor e movimentos voluntários, em indivíduos machos e fêmeas da espécie de anuro *Xenopus laevis* frente a uma infecção simulada. Consideramos que 1) Infecção simulada por LPS diminui o desempenho locomotor e movimentos voluntários dessa espécie de anuro; 2) Existem diferenças no desempenho locomotor e movimentos voluntários entre machos e fêmeas após a infecção. Neste trabalho foram analisadas a resistência locomotora e força de salto como componentes do desempenho locomotor e os movimentos voluntários, nos animais antes e depois dos tratamentos. No Capítulo 3, as respostas comportamentais de indivíduos da espécie de anuro *Xenopus laevis*, espécie invasora, e de indivíduos da espécie *Xenopus allofraseri*, não invasora, frente a uma

infecção simulada foram analisadas. Consideramos que 1) Infecção simulada por LPS reduz o desempenho locomotor e movimentos voluntários nas duas espécies; 2) Essa redução é menor na espécie invasora *Xenopus laevis* em relação a espécie não invasora. Neste trabalho foram analisadas a resistência locomotora e força de salto, como componentes do desempenho locomotor, e os movimentos voluntários antes e depois dos tratamentos e comparados entre as espécies. Em seguida é apresentada a discussão geral e conclusão final da dissertação, seguida pelas referências e anexos.

Capítulo 1

Food and locomotor response to simulated infection in the anuran *Aquarana catesbeiana* (Shawn, 1802) Dubois *et al.*, 2021. *In preparation.*

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Abstract

Food and locomotion are crucial to survival, as are immune responses. When pathogenic microorganisms activate the immune system, the immune responses include behavioral changes, which can lead to reduced behaviors, such as feeding and locomotion, which is known as behavioral depression. In addition, immune responses and feeding and locomotion require energy and metabolic resources to occur, therefore, the immunological responses and the occurrence of feeding and locomotor behavior can, in certain contexts, be conflicting, generating a trade-off. In this context, the reduction of these behaviors could lead to savings in resources that could eventually be used by the immune system to fight infections. This scenario has already been observed in amphibians, which after a simulated infection, individuals reduced prey ingestion and locomotion. Thus, this research had the objective to analyze whether the feeding and locomotion behaviors of anurans are altered by a simulated infection, mainly in terms of intensity and frequency and, with that, if these animals present lower rates of prey ingestion and indicators of locomotion in an experimental environment where access to prey is not immediate and involves environmental exploration.

Keywords: Amphibians; Behavior; Feeding; Infection; Locomotion; LPS.

1. Introduction

Feeding and locomotion are basic activities in vertebrate animals that demand resources, such as energetic and metabolic. In mobile animals these two activities generally occur in association with variation in resource expenditure depending on the type of environment, the average distance from food sources, the type of locomotion, the format of searching for and capturing prey, and the processes associated with digestion (Bennett and Licht, 1973; Emerson, 1976; Huey and Pianka 1981; Anderson and Karasov 1981; Taigen and Pough, 1981; Taigen and Pough, 1983; Pough, 1984; Taigen and Pough, 1985; Randall *et al..*, 1989; Pough *et al*., 1992; Pough *et al*., 2008). To ensure that the energy budget is balanced, eating behavior must ensure a final gain, in energy and nutrients, in relation to the costs of searching for food (Anderson and Karasov 1981; Davis *et al*., 2012; Begon *et al*., 2017). The costs associated with feeding behavior in amphibians depend on foraging strategies (Bennett and Licht, 1973; Emerson, 1976; Taigen and Pough, 1981; Taigen and Pough, 1983; Taigen and Pough, 1985; Deban *et al.,* 2001; Pough *et al.,* 2008), which display great diversity.

Depending on the ecology and physiology of feeding and locomotion, amphibians species may contrast profoundly in the behavior associated with obtaining food, which is also influenced by habitat and prey type (Bennett and Licht, 1973; Emerson, 1976; Anderson and Karasov 1981; Taigen and Pough, 1981; Taigen and Pough, 1983; Taigen and Pough, 1985; Deban *et al.,* 2001; Pough *et al*., 2008; Davis *et al*., 2012). For example, some anurans species are very active predators that travel great distances to find prey, even if the processes of capture and ingestion are not expensive. This case implies locomotion as a main component of the energy budget associated with foraging and, generally, leads to higher costs related to feeding, when all components are analyzed globally. In contrast, there are sit-and-wait predatory counterparts, typically with sedentary habits, which wait for prey to approach, in which case the cost of food tends to be lower, even if the cost of capturing and handling prey may be comparatively higher (Schimdt-Nielsen, 1975; Anderson and Karasov 1981; Taigen & Pough, 1983; Taigen & Pough, 1985; Randall *et al*., 1989; Pough *et al*., 1992; Pough, 2008; Begon *et al*., 2017). Depending on the ecology and physiology of foraging, its demands may impose restrictions on other physiological systems that require similar metabolic resources, such as immune system (Ader *et al*., 1987; Hill *et al*., 2012).

Scenarios in which the occurrence of feeding and locomotion occur in parallel with the immunological responses are plausible and could lead to the type of compromise known as a trade-off. This is so because the activation of the immune system can occur at any time in the

animal life, usually as a result of pathogenesis caused by microorganisms such as bacteria that, in certain contexts, will stimulate immune responses including inflammatory processes (Ader *et al*., 1987; Hart, 1988; Aubert, 1999). At the onset of an infections process, when a bacterium is recognized as pathogenic, an immune acute phase response occurs, which includes fever, inflammation, release of defensive proteins in the liver, and increased leukocyte function (Ader *et al*., 1987; Hart, 1988; Aubert, 1999; Adelman and Martin, 2009). These responses involve different cell types, such as phagocytes, lymphocytes and endothelial cells that act mediated by cytokines. Pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α, are synthesized and released by immunocompetent cells such as macrophages during the course of the infectious process (Hart, 1988; Dantizer, 2000; Dantizer, 2004; Dantizer, 2006; Adelman and Martin, 2009; Abbas, *et al*., 2013; Madigan, *et al*., 2016). The action of these cytokines can lead to neurological, endocrine, metabolic and behavioral changes, where the sick individual starts to present a behavioral depression characterized by lethargy, hypophagia, decreased social interaction and decreased demand for partners for reproduction. (Hart, 1988; Kent *et al*., 1992; Aubert, 1999; Larson and Dunn, 2001; Adelman and Martin, 2009).

Behavioral depression caused by the immune response may include reduced feeding and locomotor behavior, which has been observed both in endothermic animals (Hart 1988; Kent *et al*., 1992; Hatalski and Lipkin, 1997; Aubert 1999; Larson and Dunn, 2001) and in ectothermic vertebrates such as anurans (see in Llewellyn *et al.,* 2011; Braga, 2013). The reduction of these behaviors can lead to lowered use of energy and metabolic resources, which in turn become available to the immune system for the fighting infection. (Hart, 1988; Aubert, 1999; Dantzer; 2006; Llewellyn *et al.,* 2011). Also, reduced feeding and locomotion reduce the intake of micronutrients thus limiting the populational growth of pathogenic microorganisms (Ellin and Wolf, 1974; Bullen, 1981; Weinberg, 2009), and may also decrease the risk of predation due reclusive behaviors (Hart, 1988; Aubert, 1999; Adelman and Martin, 2009; Llewellyn *et al*., 2011). Therefore, reducing eating and locomotion concomitantly with an infection could be an ecologically critical strategy (Hart, 1988).

Research with amphibians is incipient in the context presented, which motivated this investigation. Overall, we propose that there may be trade-offs between feeding and locomotion with immune responses derived from the activation of the immune system in anurans (Llewellyn *et al.*, 2011; Braga, 2013). Thus, we aim to answer whether a simulated infection reduces the feeding and locomotion of anurans, aspects that would indicate the occurrence of behavioral depression. To simulate infection, we injected lipopolysaccharide (LPS) from the

cell wall of gram-negative bacteria, and to analyze the impact on anuran behavior. We contextualize the question in environments where the cost of food is not minimal (ad libitum feeding), as in nature, and that changes in behavior would be better represented by a system in which access to food requires locomotion, orientation (in short, foraging behavior) and exploration. We hypothesized that simulated infection leads to a reduction in feeding and locomotor behavior in anurans (behavioral depression) when the cost is above the minimum.

2. Methodology

2.1 Experimental model

Individuals of the anuran species *Aquarana catesbeiana* (Shawn, 1802) Dubois *et al*., 2021, are predatory, opportunistic and generalist individuals (Daza and Castro, 1999; Wu *et al.*, 2005). In nature, studies have shown the generality of *A. catesbeiana* for food, with different food types being found in its stomach, from insects, fish, plants and other amphibians (Lima and Agostinho, 1992; Daza and Castro, 1999; Coppo, 2003; Wu *et al.*, 2005; Casali, 2010). *A. catesbeiana* is attracted by the movement of prey through sight, which has a significant importance in feeding (Daza and Castro, 1999; Wu *et al.*, 2005; Almeida, 2016).

2.2 Maintenance of animals in the laboratory

Individuals of the anurans species *A. catesbeiana* were obtained from a frog farm in the state of São Paulo during the months of January/March. Thirty-six juvenile animals were used, weighing between 60-90 grams at the beginning of the experiments. The sex in these individuals was not established because frogs with this size do not have a defined reproductive system, being considered in a juvenile state. The anurans were kept in bioterium for ectothermic vertebrates, of the Department of Physiology of the Institute of Biosciences of the University of São Paulo, during 8 days for habituation to the environment (protocol of ethics in the use of animals nº 369/2020). The animals were kept in terrariums with UV light, temperature of approximately 25ºC, access to water, light/dark cycle of 13h/11h and were fed twice a day with 5 cockroaches and 5 pellets at a time, during the eight days of stay in the bioterium (Brazil - CONCEA, 2016). The individuals received equal food in the bioterium and during the experimental tests (in time and quantity). The weight of the animals was measured with an electronic scale and the snout-cloaca length (CRC) was measured with a caliper before and after the treatments. After the bioterium period, the stages of the experiment were carried out.

2.3 Temperature and photoperiod of experimental tests

The experimental tests were carried out in a climate room programmed with a preferential temperature of 25ºC for the species *A. catesbeiana*, according to Lillywhite (1971). The photoperiod used during the tests was 13 hours of light and 11 hours of darkness provided by lamps and controlled by a timer, from 6:00 am. to 19:00 pm. with the light on and from 19:00 pm. to 6:00 am. in the morning with the light off.

2.4 LPS administration and dosage

To simulate infection in animals, injections of LPS from the cell wall of the gram-negative bacterium *Escherichia coli* (serotype 0127: B8, Sigma-Aldrich Chemical) were prepared and applied to the dorsal lymph sac of the frogs (Bicego *et al*., 2002; Llewellyn *et al.*, 2011; Moretti, 2016). To prepare the doses, a ringer solution for amphibians was first prepared using KCL 25%; 25% CaCL2; NaHCO3 dissolved in 1L of water (final solution with pH 7.2 and autoclaved) (Passier and Mendelson, 2017). Then, an initial LPS solution was prepared, consisting of 10mg of pure powdered LPS diluted in 2ml of ringer solution. The dose I of LPS was prepared for 12 animals at a concentration of 2 mg/kg of the initial LPS solution diluted in ringer solution. The dose II of LPS was prepared for 12 animals at a concentration of 3 mg/kg of the initial LPS solution diluted in ringer solution (based on the work of Bicego *et al*., 2002; Llewellyn *et al.,* 2011). Thus, 12 animals were injected with dose I of LPS (2.0mg/Kg), 12 animals were injected with dose II of LPS (3.0mg/Kg) and 12 animals were injected with Ringer solution (in the proportion of body weight). LPS (treatments) and ringer (controls) injections were always performed at 7:00 am. After 1 hour of the injection, the animals were recorded.

2.5 Experimental design

2.5.1 Experimental arena

The place where the feeding and locomotion experiments were carried out consisted of arenas made with 61-liter white polypropylene boxes (external dimensions 62cm x 39cm x 32cm; internal dimensions 56cm x 36cm x 31cm) and covered with nylon fabric. Inside the arena, 3,5 liters of water (height of water in the arena 2 cm) and a PVC tube were placed (as a shelter for the animal during the test). The place where the food was placed consisted of a dry area composed of a transparent acrylic cube (15x15cm) which had access through a small passage of 5 cm in diameter through which the animal could pass to feed. A camera was placed above the arenas to film the animals. Each animal was tested separately.

2.5.2 Feeding during experimental tests

At the onset of testing 10 cockroaches and 10 pellets of industrial frog food were placed per day inside the acrylic cube, always at the same time and during the four days of the test. The food was placed twice a day, with 5 cockroaches and 5 pellets at 8:00 am. and another 5 cockroaches and 5 pellets at 5:00 pm., following the same times that the frogs were fed in the farm where they were created. The food was placed inside the transparent acrylic cube and, in order to eat, the animal had to find the passage and pass through it to gain access to the food and then return to the water. The frogs were positioned inside the hiding place on the opposite side of the acrylic cube when even the prey was placed.

2.5.3 Experimental groups: Control and treatments

Animals were randomly assigned and tested before (no treatment) for 2 days (48 hours) and after LPS (treatment) or ringer (control) injections for an additional 2 days, totaling 4 consecutive days of testing and filming. Tests were performed with 1 animal assigned to each treatment at a time. Twelve animals were used for each experimental group (LPS dose I, LPS dose II and ringer), totaling 36 animals used in this study.

Under all conditions we evaluated both feeding and locomotion variables (see 2.6). For each experimental group we tested the 12 animals intact (without treatment) and after treatment. Each animal passed for 48 hours of test intact and then a treatment was applied depending of the experimental group (ringer, LPS I, LPS II) and observed for another 48 hours. The same animals were tested for both feeding and locomotion variables.

2.6 Analyzed variables

To understand the locomotion and feeding behavior in anurans, we observed and quantified the following variables:

Feeding Behavior

Quantified variables: A- number of times the animal went to the box with food; B- number of attempts against the box with food; C- number of ingested preys; D- total time (min) that the animal spent to find the passage in the box with food.

Locomotor behavior associated with feeding

Quantified variables: E- total distance covered (m); F- total time with continuous movement (min); G- number of jumps; H- total time in the shelter.

3. Statistical analysis

Statistical Approach

Given the characteristics of data distribution, non-parametric, paired and unpaired tests were used, according to the nature of the data groups obtained.

Statistical analysis of feeding behavior

Comparison of feeding variables (A; B; C; D presented above) between animals before (intact without treatment) vs. afterwards (animals injected with LPS I; animals injected with LPS II and animals injected with ringer) was performed using the Wilcoxon nonparametric test, and the comparison between the experimental groups (ringer vs. LPS I vs. LPS II) was performed using of the Kruskal-Wallis test.

Statistical analysis of locomotor behavior

Comparison of locomotion variables associated with feeding in the experimental environment (E; F; G; H presented above) between animals before (intact without treatment) vs. afterwards (animals injected with LPS I; animals injected with LPS II and animals injected with ringer) was performed using the Wilcoxon nonparametric test, and the comparison between the experimental groups (ringer vs. LPS I vs. LPS II) was performed using of the Kruskal-Wallis test.

Principal component analysis - PCA

A principal components test (PCA) was performed to visualize in general the behavior of the data considering the feeding and locomotion variables between animals of all the treatment groups.

Analysis of the body mass

A *t*-test was performed to compare the weight of animals before treatment (intact) and after animals injected with LPS I, LPS II and ringer.

4. Results

4.1 Feeding Behavior

Intact Animals vs. injected with ringer

The search for food, determined by the number of times the animal went to the box with the prey, increased after the injection of ringer solution (28%), in relation to the control animals (n= 12; Wilcoxon test p= 0.002; Table 1. Fig. 1). Affinity for food, determined by the number of attempts against the box with prey, also increased after ringer injection (31%) compared to

control animals (n=12; Wilcoxon test $p<0.001$; Table 1. Fig. 1). Regarding the number of ingested prey and the total time to find access in the food box, the two groups (control and treatment) were comparable (n=12; Wilcoxon test $p=0.124$ and $p=0.684$ respectively; Table 1. Fig. 1). As well as body mass, which was also not altered by ringer injection (n=12; $t=0.179$; $p=0.859$; Table 5). The feeding behavior of the animals did not change between the test days (Table 1).

Intact animals vs. injected with LPS I (2.0mg/Kg)

The search for food, determined by the number of times the animal went to the box with the prey, reduced after the injection of LPS dose I (36%), compared to control animals (n=12; Wilcoxon test $p<0.001$; Table 1. Fig. 2). Affinity for food, observed through the number of attempts against the box with prey, was reduced after LPS dose I injection (64%), compared to control animals (n=12; Wilcoxon test $p<0.001$; Table 1 Fig. 2). The number of ingested preys was also reduced after LPS dose I injection (27%), compared to control animals (n=12; Wilcoxon test $p<0.003$; Table 1; Fig. 2). Regarding the time to find access in the food, there was an increase in animals injected with LPS dose I (88%) compared to control animals (n=12; Wilcoxon test $p<0.001$; Table 1. Fig. 2). The animals body mass was not modified by the LPS injection, although there was a reduction in feeding (n=12; $t= 0.539$; $p= 0.594$; Table 5). The feeding behavior of the animals did not change between the test days (Table 1).

Intact animals vs. injected with LPS II (3.0mg/Kg)

The search for food, observed by the number of times the animal went to the box with the prey, was not modified by the injection of LPS dose II (n=12; Wilcoxon test $p=0.409$; Table 1). The affinity for the food, determined by the number of attempts against the box with the prey, was reduced after the injection of LPS dose II (22%), compared to control animals (n=12; Wilcoxon test $p<0.001$; Table 1 Fig. 3). As well, as the number of ingested preys, which was also reduced after the injection of LPS dose II (28%), in relation to the control animals (n= 12; Wilcoxon test $p<0.001$; Table 1. Fig. 3). In relation to control animals, the total time to find access in the food increased after LPS dose II injection (97%) (n=12; Wilcoxon test $p<0.001$; Table 1. Fig. 3). Although there was a reduction in feeding after LPS injection, the animals' body mass was not modified (n=12; $t=0.681$; $p=0.496$; Table 5). The feeding behavior of the animals did not change between the test days (Table 1).

Comparison between treatment groups - anurans injected with ringer vs. anurans injected with LPS I vs. anurans injected with LPS II

The search for food, observed by the number of times the animal went to the box with the prey, was reduced after the injection of LPS doses I and II (59% and 56%, respectively), in relation to the animals injected with ringer solution. (n=36; Kruskal-Wallis test $p<0.001$; Table 2; Fig. 4). Affinity for food, determined by the number of attempts against the box where the prey was, was not altered between the treatment groups (ringer; LPS I; LPS II) (n=36; Kruskal-Wallis test $p=0.774$; Table 2). Regarding the number of preys ingested, it was reduced after the injection of LPS dose I (22%) and LPS dose II (38%), compared to animals injected with ringer (n= 36; Kruskal-Wallis test $p<0.001$; Table 2; Fig. 4). Total time to find access in the food box increased after LPS dose I (54%) and LPS dose II (96%) injection compared to animals injected with ringer (n=36; Kruskal-Wallis test $p<0.001$; Table 2. Fig. 4). The total time to find access to the food box also differed between the two LPS doses, increasing after LPS dose II injection (53%) compared to LPS dose I injection (n=36; Kruskal-Wallis test $p<0.001$; Table 2. Fig. 4). The feeding behavior of the animals did not change between test days (Table 2).

4.2 Locomotor Behavior

Intact Animals vs. injected with ringer

Most variables of locomotion remain unaffected by the ringer injection, including the total distance covered by the animal in the test arena, the number of jumps and the total time in the shelter (n=12; Wilcoxon test $p=0.119$; $p=0.367$; $p= 0.294$ respectively; Table 3). However, the total time with movement increased after the injection of ringer solution (12%) compared to intact animals (n=12; Wilcoxon test $p= 0.011$; Table 3. Fig. 5). The locomotor behavior of the animals did not change between the test days (Table 3).

Intact animals vs. injected with LPS I (2.0mg/Kg)

Locomotion, determined by the total distance covered and number of jumps, decreased after LPS dose I injection (40% and 62%, respectively), compared to control animals (n=12; Wilcoxon test $p<0.001$ and $p<0.001$, respectively; Table 3. Fig. 6). Voluntary movement, determined by the total time with continuous movement, was also reduced after LPS dose I injection (68%), compared to control animals (n=12; Wilcoxon test $p<0.001$; Table 3. Fig. 6). In relation to the total time in the shelter, there was an increase after LPS injection dose I (42%),

in relation to the control animals (n=12; Wilcoxon test p=0.017; Table 3. Fig. 6). The locomotor behavior of the animals did not change between the test days (Table 3).

Intact animals vs. injected with LPS II (3.0mg/Kg)

Locomotion, determined by total distance covered and number of jumps, decreased after LPS dose II injection (33% and 62%, respectively), compared to control animals (n=12; Wilcoxon test p=0.025 and p<0.001, respectively; Table 3. Fig. 7). In relation to control animals, voluntary movement, determined by the total time with continuous movement, also reduced after the injection of LPS dose II (57%) (n=12; Wilcoxon test p<0.001; Table 3; Fig. 7). Total time in the shelter increased after LPS dose II injection (86%) when compared to control animals (n=12; Wilcoxon test p=0.002; Table 3. Fig. 7). The locomotor behavior of the animals did not change between the test days (Table 3).

Comparison between treatment groups - anurans injected with ringer vs. anurans injected with LPS I vs. anurans injected with LPS II

Locomotion, determined by the total distance covered and the number of jumps, reduced after the injection of LPS dose I (55% and 68%) and LPS dose II (54% and 71%), in relation to animals injected with ringer solution (n=36; Kruskal-Wallis test p<0.001 and p<0.001, respectively; Table 4. Fig. 8). Voluntary movement, determined by the total time with continuous movement, was also reduced after LPS injection doses I and II (56% and 49%, respectively) compared to animals injected with ringer solution (n=36; Kruskal-Wallis test p<0.001; Table 4; Fig. 8). Total time in the shelter increased after LPS injection at dose I (21%) and dose II (74%), compared to animals injected with ringer solution (n=36; Kruskal-Wallis test p<0.001; Table 4. Fig. 8). Total time in the shelter also differed between the two doses of LPS, with a 67% increase in animals injected with dose II of LPS compared to animals injected with dose I of LPS (n=36; Kruskal-Wallis test p< 0.001; Table 2. Fig. 4). The locomotor behavior of the animals did not change between the test days (Table 3).

Principal Component Analysis - PCA

An overview of feeding and locomotor behavior was based on a principal component analysis (PCA), in which two components PC1 and PC2 explain most of the variation in the data (Table 6; Fig. 9). PC1 was heavily related by the variables associated with the feeding behavior of anurans (*eigenvalue* =4.31; variance=48%) whereas PC2 was more related by the

variables associated with the locomotor behavior of anurans (*eigenvalue*=1.22; variance=14%). The results of the principal components test showed that anurans injected with ringer (control group) are correlated with the variables of feeding and locomotor behavior, on the other hand, anurans injected with LPS are correlated with variables that demonstrate behavioral depression (total time to find access in the food box and total time spent in hiding) (Table 6; Fig. 8).

5. Discussion

According to the hypothesis of our research, after an immune challenge most sick animals exhibit changes in feeding and locomotor behavior, which was corroborated in the *A. catesbeiana* specie studied after simulated infection. Individuals injected with LPS showed a reduction in feeding and locomotor behavior under experimental conditions, apparently as a response to the activation of the immune system via a pro-inflammatory stimulus. Anurans treated with LPS spend more time inactive, corroborating the concept of behavioral depression postulated in the literature (Ader *et al*., 1987; Hart, 1988; Larson and Dunn, 2001). The reduction in feeding and locomotor behavior observed in *A. catesbeiana* compared to those reported for other tetrapods also after a simulated infectious process (Hart 1988; Bret-Dibat *et al*., 1995; Aubert, 1999; Dantzer, 2006; Adelman and Martin, 2009). Also, our results show that individuals of *A. catesbeiana* injected with LPS reduce prey ingestion, as seen in other studies with anurans under similar experimental protocols (Llewellyn *et al*., 2011; Braga, 2013).

Considering that feeding behavior involves metabolic and energy costs that may also be associated with the environmental landscape (Bennett & Licht, 1973; Emerson, 1976; Taigen and Pough, 1981; Taigen & Pough, 1983; Pough, 1984; Taigen & Pough, 1985; Randall *et al*., 1989; Pough *et al*., 1992; Pough *et al*., 2008), it is possible to infer that, in nature sick frogs in addition to presenting a reduction in feeding, may be easily deterred from obtaining prey. Individuals in the control group managed to feed even if facing some obstacles, but those injected with LPS drastically reduced the search for food, the attempt to capture and ingestion prey, in addition to spend more time to find the passage in the box with food (obstacle). It seems then, that how readily food is available may affect behavior, so that lower feeding rates may emerge often in this context (Taigen and Pough, 1983; Taigen and Pough, 1985; Davis *et al*., 2012). Regarding the ecological value of this pattern, decreased food intake may inhibit pathogenic multiplication, leading to low concentrations of nutrients (e.g., iron concentrations in blood plasma) important for the growth and proliferation of pathogens (Elin and Wolff, 1974;

Bullen, 1981; Hart 1988; Aubert, 1999; Dantzer, 2006; Adelman and Martin, 2009; Weinberg 2009; Llewellyn *et al*., 2011; Braga, 2013).

Given that frogs intact and injected with ringer showed similar behaviors, we assume that the ringer solution did not modify feeding and locomotor behavior. However, animals injected with ringer showed greater movement, which is related to the greater number of approaches and investments to the box with food. This increase may be related to the novelty of the experimental environment, although it was not observed in intact animals. However, we allowed for time for exploration, as has been recommended by some authors (Llewellyn *et al*. 2011; Braga, 2013), discarding the novelty effect. Most likely, the increase in movement and approximation of the box with food may be related to handling, especially considering that prey ingestion was not altered, so that the other variables related to feeding behavior were not affected by the control treatment with ringer.

The locomotion of anurans is relatively conserved and allows the animal to move around in the environment and perform crucial activities, often in a targeted manner, as in the case of searching for food (Nielsen- Schmidt, 1975; Taigen *et al.* 1982; Pough & Taigen, 1990; Pough *et al*., 2008; Katz, 2015). In ecological terms, these mechanisms are translated into a set of decisions associated with information from the environment, thus enabling guided or exploratory locomotion, which in the case of this research could be guided by the box with food or motivated by the exploration of the arena around the box with the preys. Given that LPS reduced the locomotion of anurans, despite the stimulus (food inside the box), these results corroborate that activation of the immune system by injections of LPS reduces locomotor activity, as demonstrated for several groups of tetrapods, including anurans (Hart, 1988; Lee *et al*., 2005; Llewellyn *et al*., 2011; Braga, 2013). Access to prey requires exploration of the environment, both in the laboratory, when food is not free, and in nature, and in the case of sick animals, exploration to obtain prey can be impaired due to reduced locomotion. In addition, the reduction of locomotor behavior may imply in the reduction or restriction of the performance of other activities dependent on locomotion, such as escape, reproduction and dispersion, for example (Taigen & Pough, 1983; Taigen & Pough, 1985; Lee *et al*., 2005; Llewellyn *et al*., 2011; Braga, 2013).

Furthermore, animals injected with LPS remained more time in the shelter and spent more time to find access in the box with the preys, due to less exploration of the experimental arena. This time increase was more visible when the LPS dose was increased. In anurans longer periods in shelter must relate to decreased feeding and locomotion in association with

behavioral depression. This state may cause animals to seek shelter, reduces appetite and decreases search for food, for example (Hart, 1988; Bret-Dibat *et al*., 1995; Llewellyn *et al*., 2011; Braga, 2013). In nature, staying in shelter during periods of illness can also reduce the risk of predation, due to less movement, and consequently, less exposure (Llewellyn *et al*., 2011), and may enhance hydration, granted access to humid shelters (Scwarzkopf, and Alford, 1996; Prates and Navas 2009).

The decrease in feeding and locomotor behavior in the face of an infectious process can lead to consequences, which will often be decisive for survival. However, by reducing these behaviors during immune challenges an individual's chances of survival increase (Hart, 1988; Aubert, 1999; Dantzer, 2006; Adelman and Martin, 2009; Llewellyn *et al*., 2011). Therefore, the depressed behavior, which in many cases characterizes sick individuals, is not a deleterious or undesirable effect of the disease, but an organized physiological strategy that can be ecologically critical (Ader *et al*., 1987; Hart, 1988; Lefcort and Eiger, 1993).

6. Conclusions

Aquarana catesbeiana showed reduced feeding and locomotor behavior after LPS injections in an environment where access to prey involves environmental exploration. The reduction of the feeding and locomotor response of individuals of *A. catesbeiana* with pro-inflammatory stimulus was expressed in the reduction of the search, attempt to capture and ingestion of prey, and in the reduction of the traveled distance and movement time. Consequently, downtime inside the shelter increased, characteristic of behavior depression. This response was dominant in all tested individuals. Our study corroborates that behavior depression, expressed as decreased activity performance, also occurs in tropical anurans, which may impact the survival of these animals in the wild when sick.

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Author contributions

T.O performed the experiments and data analysis. All authors contributed to the writing of the manuscript.

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Competitive interests

The authors declare no conflict of interest.

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Figures and Tables

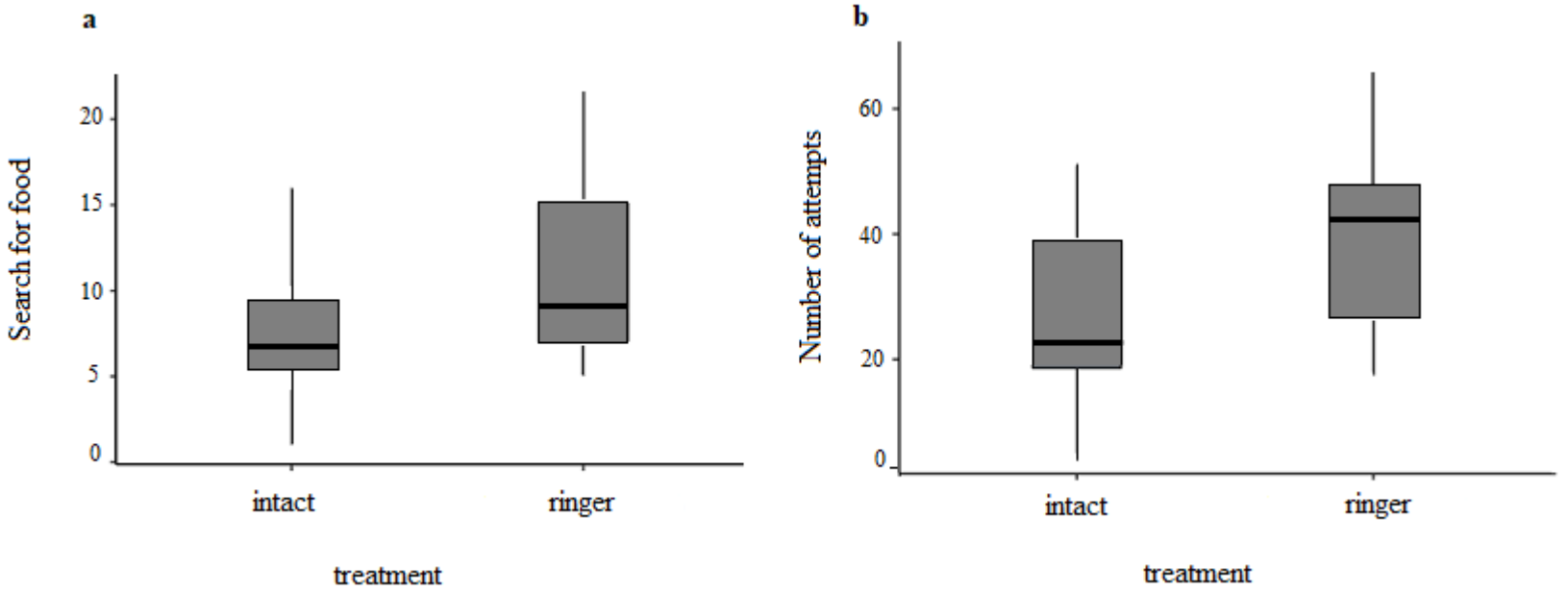

Fig. 1. Boxplot showing the distribution of data collected for each variable associated with feeding behavior of intact anurans vs. anurans injected with ringer solution in the experimental arena. **a.** Number of times animal goes to the food box (variable A). **b.** Number of attempts against to the food box (variable B). In this figure the X axis represents the treatment; the Y axis represents the analyzed variable; the bold line in the box represents the median of the samples.

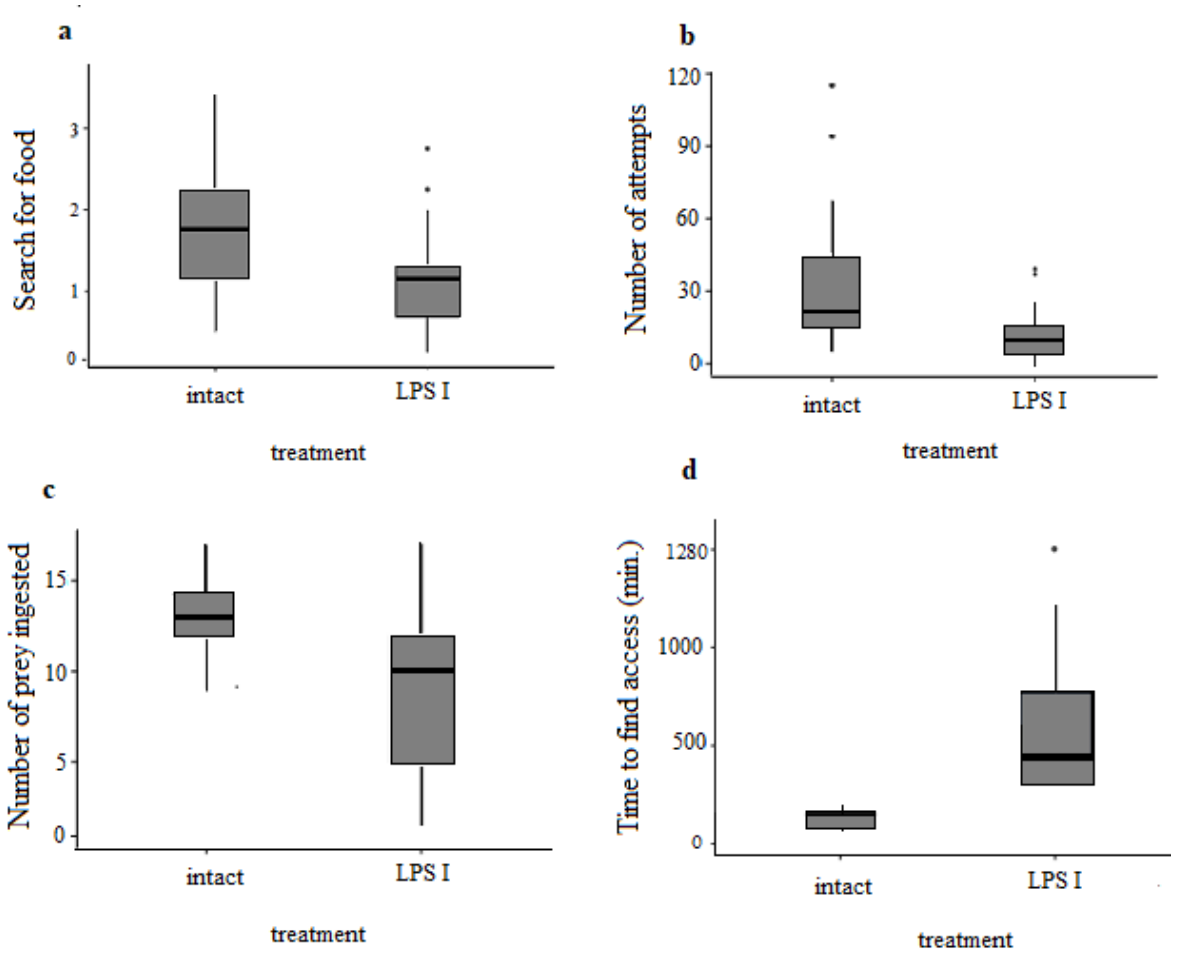

Fig. 2. Boxplot showing the distribution of data collected for each variable associated with the feeding behavior of intact anurans vs. anurans injected with LPS dose I (2.0mg/Kg) in the experimental arena. **a.** Number of times animal goes to the food box (variable A); **b.** Number of attempts against to the food box (variable B); **c.** Number of prey ingested (variable C); **d**. Total time to find access to the food box (variable D). In this figure the X axis represents the treatment; the Y axis represents the analyzed variable; the bold line in the box represents the median of the samples.

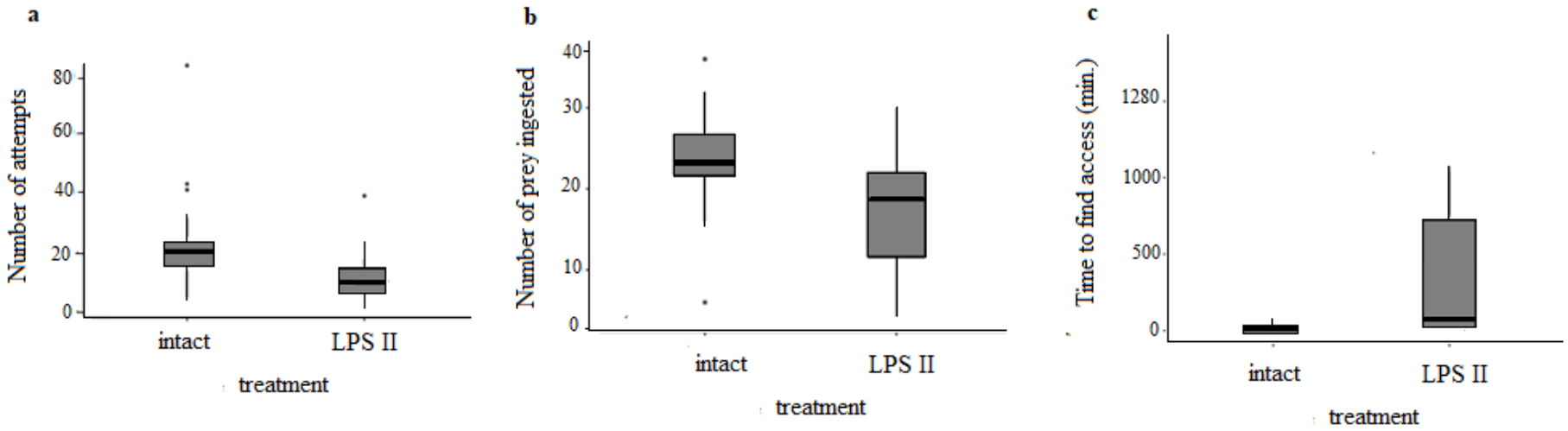

Fig. 3. Boxplot showing the distribution of data collected for each variable associated with the feeding behavior of intact anurans vs. anurans injected with LPS dose II (3.0 mg/kg) in the experimental arena. **a.** Number of attempts against to the food box (variable B); **b.** Number of prey ingested (variable C); **c**. Total time to find access in the food box (variable D). In this figure the X axis represents the treatment; the Y axis represents the analyzed variable; the bold line in the box represents the median of the samples.

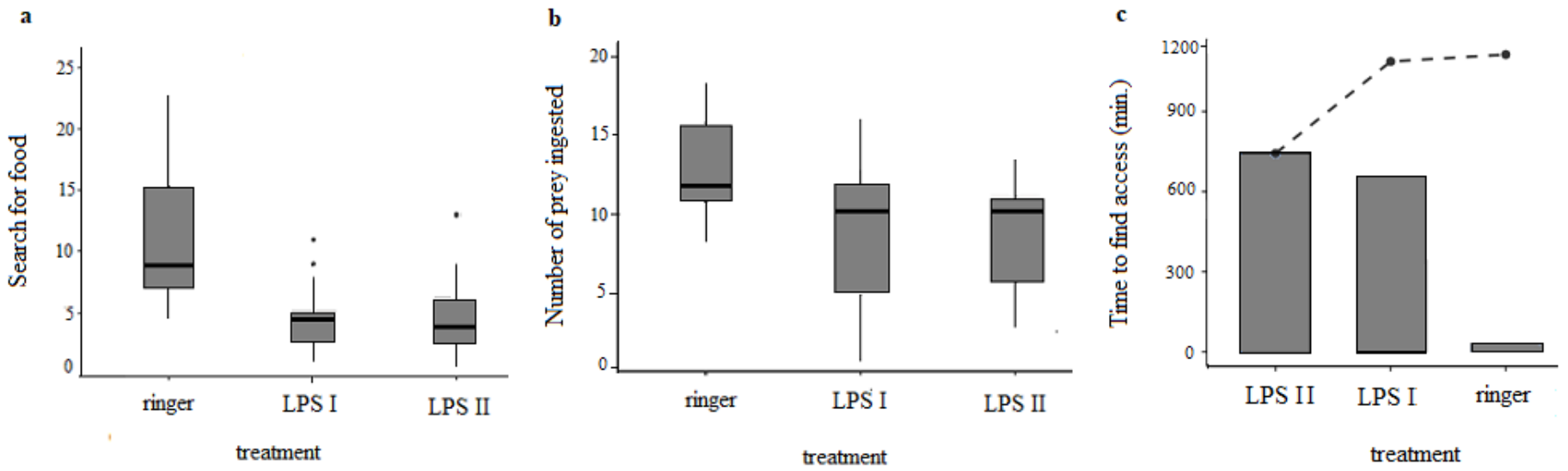

Fig. 4. Boxplot showing the distribution of data collected for each variable associated with feeding behavior between the experimental groups in the experimental arena: anurans injected with ringer (control) vs. anurans injected with LPS dose I (2.0 mg/kg) vs. anurans injected with LPS dose II (3.0 mg/kg). **a.** Number of time animal goes to the food box (variable A); **b**. Number of prey ingested (variable C); **c.** Total time to find access in the food box (variable D). In this figure the X axis represents the treatment; the Y axis represents the analyzed variable; the bold line in the box represents the median of the samples.

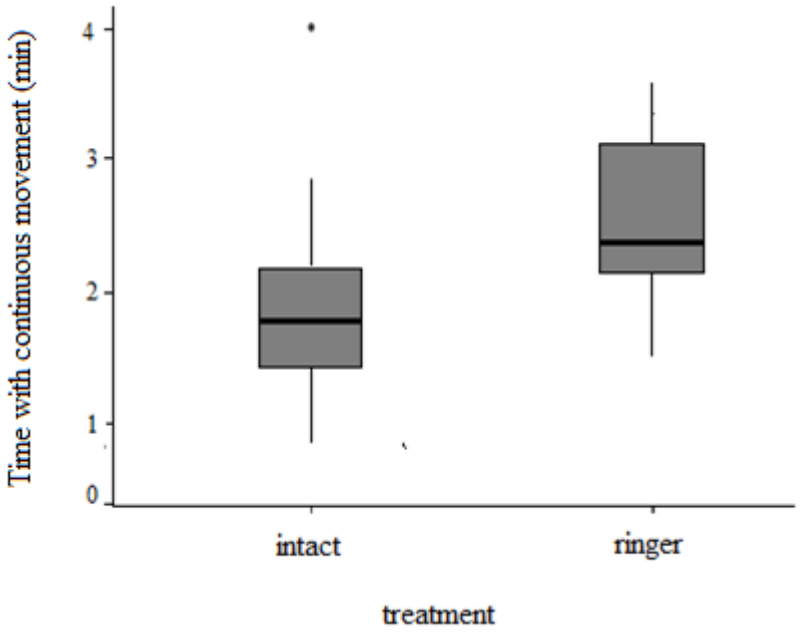

Fig. 5. Boxplot showing the distribution of data collected for the variable F, total time with continuous movement (min), associated with locomotor behavior of intact anurans vs. anurans injected with ringer solution in the experimental arena. In this figure the X axis represents the treatment; the Y axis represents the analyzed variable; the bold line in the box represents the median of the samples.

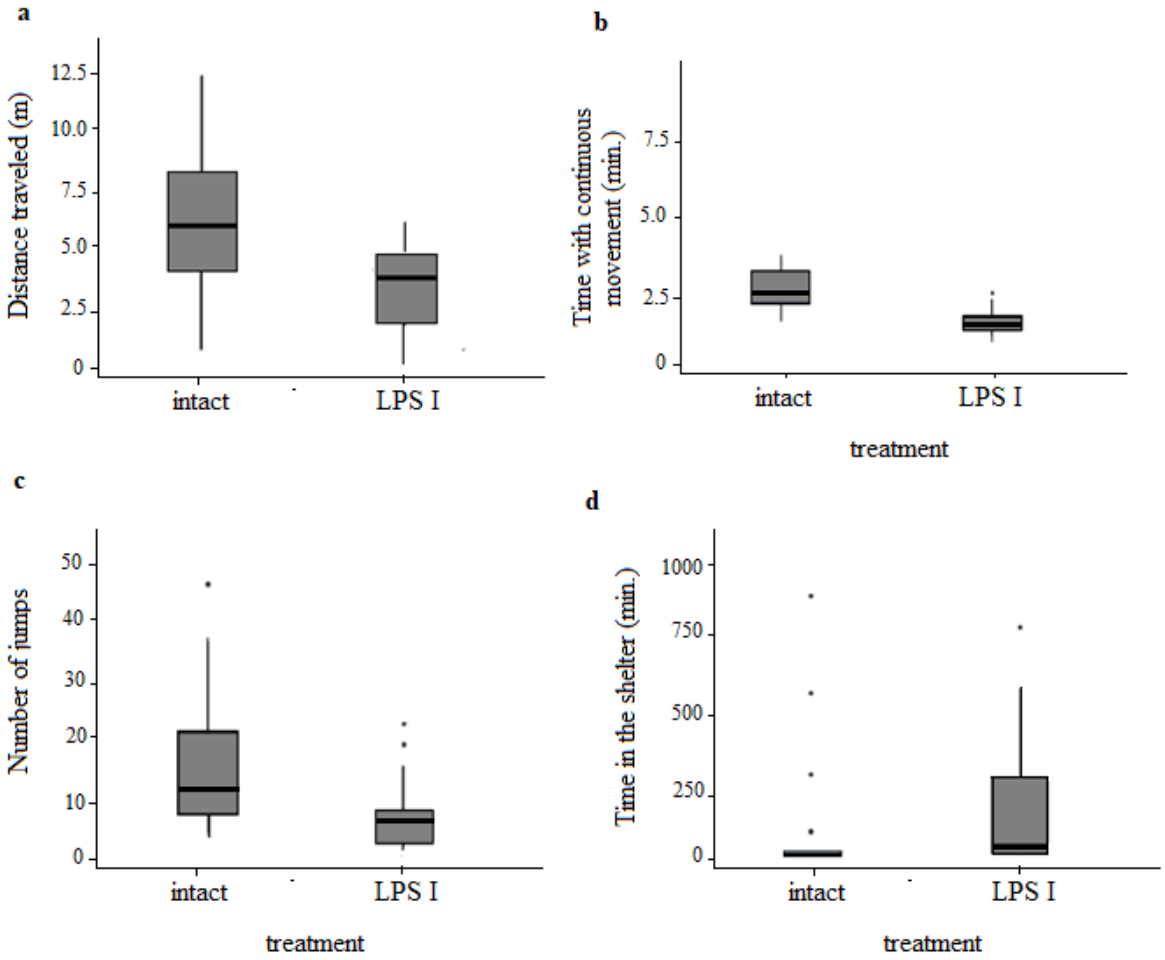

Fig. 6. Boxplot showing the distribution of data collected for each variable associated with locomotor behavior of intact anurans vs. anurans injected with LPS dose I (2.0 mg/kg) in the experimental arena. **a.** Total distance traveled (variable E); **b.** Total time with continuous movement (variable F); **c.** Number of jumps (variable G); **d.** Total time in the shelter (variable H). In this figure the X axis represents the treatment; the Y axis represents the analyzed variable; the bold line in the box represents the median of the samples.

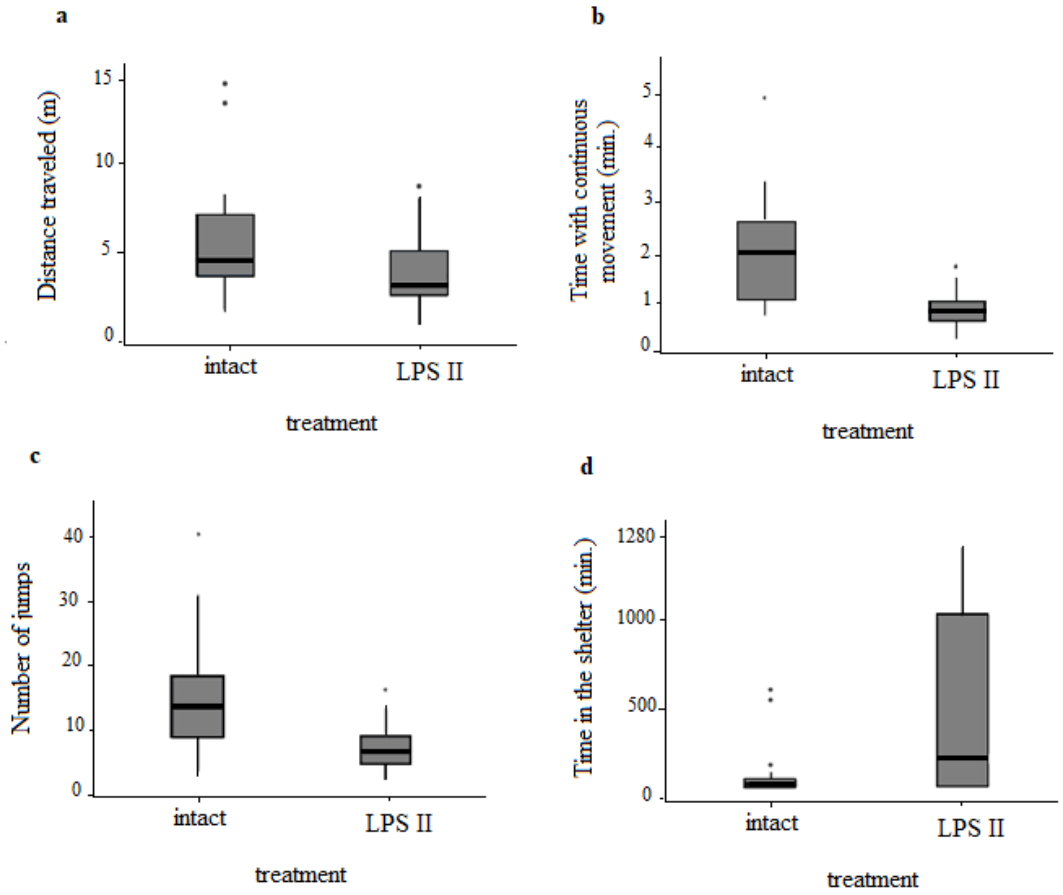

Fig. 7. Boxplot showing the distribution of data collected for each variable associated with locomotor behavior of intact anurans vs. anurans injected with LPS dose II (3.0 mg/kg) in the experimental arena. **a.** Total distance traveled (variable E); **b.** Total time with continuous movement (variable F); **c.** Number of jumps (variable G); **d.** Total time in the shelter (variable H). In this figure the X axis represents the treatment; the Y axis represents the analyzed variable; the bold line in the box represents the median of the samples.

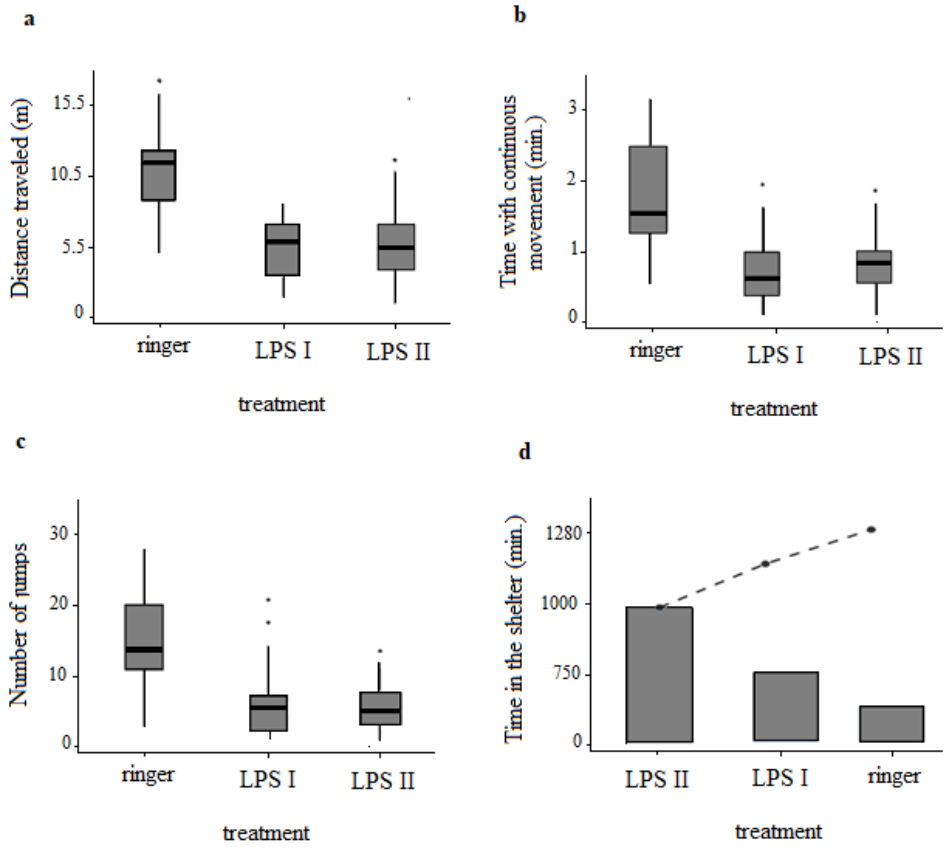

Fig. 8. Boxplot showing the distribution of data collected for each variable associated with locomotor behavior between the experimental groups in the experimental arena: anurans injected with ringer solution (control) vs. anurans injected with LPS dose I (2.0 mg/kg) vs. anurans injected with LPS dose II (3.0 mg/kg). **a.** Total distance traveled (variable E); **b**. Total time with continuous movement (variable F); **c**. Number of jumps (variable G); **d**. Total time in the shelter (variable H). In this figure the X axis represents the treatment; the Y axis represents the analyzed variable; the bold line in the box represents the median of the samples.

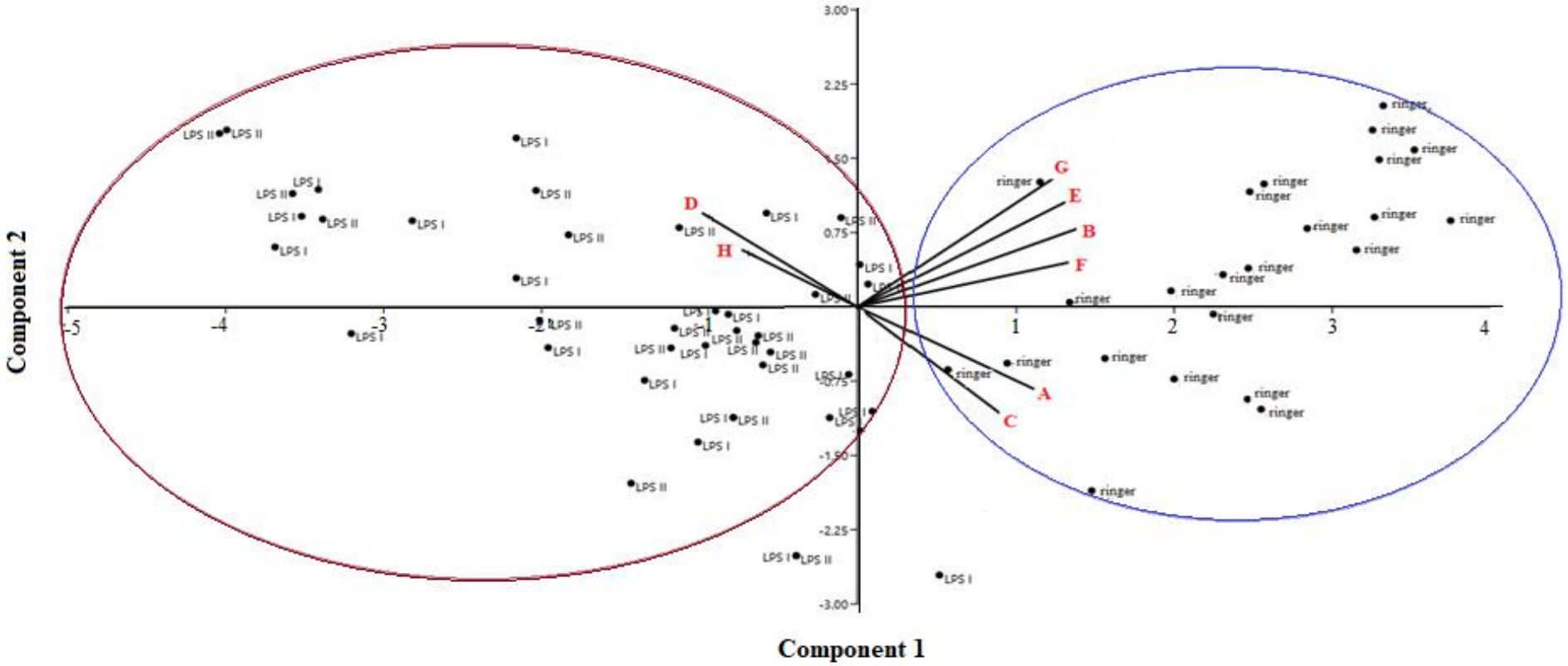

Fig. 9. Principal component analysis (PCA), demonstrating two components, PC1 and PC2, that explain most of the variation in data of feeding and locomotor behavior of anurans injected with ringer solution; anurans injected with LPS I (2.0 mg/kg); anurans injected with LPS II (3.0 mg/kg). Variables: A- number of times the animal goes to the food box; B- number of attempts against the food box; C- number of ingested preys; D- total time (min) that the animal spent to find the access in the box with food; F- total time with continuous movement (min); G- number of jumps; H- total time in the shelter.

Table 1. Wilcoxon nonparametric test, n=12, for each analyzed variable of the feeding behavior of intact anurans vs. with treatment (LPS I 2.0mg/Kg; LPS II 3.0mg/Kg). Variables: A- number of times the animal goes to the box with food; B- number of attempts against the food box; C – number of ingested preys; D – total time to find access in the box with food. The three columns with *p* values represent: *p* value between intact vs. after injections; the *p* value between the 1st and 2nd day analyzing only the intact animals (without treatment) of each experimental group, and *p* value between the 3rd and 4th day analyzing the anurans of each experimental group after treatment.

Treatment	Variable	Median	Wilcoxon Test	Wilcoxon Test Variation between 1st and 2nd day (intact)	Wilcoxon Teste Variation between 3rd and 4th day (injections)
Intact / ringer	A	7.50 / 9.00	$p = 0.002$	$p = 0.964$	$p = 0.533$
Intact / ringer	B	22.5 / 42.0	$p < 0.001$	$p = 0.326$	$p = 0.171$
Intact / ringer	C	12.5 / 12.0	$p = 0.903$	$p = 0.208$	$p = 0.124$
Intact / ringer	D	8.93 / 10.3	$p = 0.684$	$p = 0.204$	$p = 0.622$
Intact / LSP I	A	7.00 / 4.50	$p < 0.001$	$p = 0.386$	$p = 0.370$
Intact / LSP I	B	21.0 / 9.50	$p < 0.001$	$p = 1.000$	$p = 0.750$
Intact / LPS I	C	13.0 / 10.0	$p = 0.003$	$p = 0.623$	$p = 0.553$
Intact / LPS I	D	5.67 / 31.4	$p < 0.001$	$p = 0.204$	$p = 0.380$
Intact / LPS II	A	5.0 / 4.0	$p = 0.406$	$p = 0.357$	$p = 0.239$
Intact / LPS II	B	20.5 / 9.50	$p < 0.001$	$p = 0.289$	$p = 0.455$
Intact / LPS II	C	11.5 / 10.0	$p < 0.001$	$p = 0.438$	$p = 0.398$
Intact / LPS II	D	15.8 / 15.3	$p < 0.001$	$p = 0.970$	$p = 0.622$

Table 2. Nonparametric Kruskal-Wallis test, n= 12, of the variables associated with feeding behavior between the experimental groups (anurans injected with ringer vs. anurans injected with LPS I 2.0mg/Kg vs. anurans injected with LPS II 3 .0 mg/kg). Columns and *p*-values indicate comparisons between treatment groups by collected variable, and *p*-value between test days. Variables: A- number of times the animal goes to the box with food; B- number of attempts against the food box; C – number of ingested preys; D – total time to find access in the box with food.

Variable	Kruskal-Wallis Test for each variable	Pairwise comparisons (Ringer / LPS I)	Pairwise comparisons (Ringer / LPS II)	Pairwise comparisons (LPS I / LPS II)	Kruskal-Wallis Teste variance between day of test
A	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p = 0.981$	$p = 0.258$
B	$p = 0.774$	$p = 0.792$	$p = 0.858$	$p = 0.964$	$p = 0.071$
C	$p < 0.001$	$p = 0.011$	$p < 0.001$	$p = 0.631$	$p = 0.654$
***D**	*$p < 0.001$*	*$p < 0.001$*	*$p < 0.001$*	*$p < 0.001$*	*$p = 0.371$*

Note - The *D variable was analyzed using a test of Kolmogorov-Smirnov (bilateral).

Table 3. Wilcoxon nonparametric test, n=12, for each analyzed variable of locomotor behavior of intact anurans vs. with treatment (LPS I 2.0mg/Kg; LPS II 3.0mg/Kg). Variables: E – total distance covered; F – total time with continuous movement; G - number of jumps; H – total time in the shelter. The three columns with *p* values represent: *p*-value between intact vs. after injections; the *p* value between the 1st and 2nd day analyzing only the intact animals (without treatment) of each experimental group, and *p* value between the 3rd and 4th day analyzing the anurans of each experimental group after treatment.

Treatment	Variable	Median	Wilcoxon Test	Wilcoxon Test Variation between 1st and 2nd day (intact)	Wilcoxon Teste Variation between 3rd and 4th day (injections)
Intact / ringer	E	1.76 / 1.35	$p = 0.119$	$p = 0.151$	$p = 0.301$
Intact / ringer	F	22.5 / 42.0	$p = 0.011$	$p = 0.109$	$p = 0.176$
Intact / ringer	G	14.5 / 15.5	$p = 0.367$	$p = 0.224$	$p = 0.104$
Intact / ringer	H	11.2 / 8.91	$p = 0.294$	$p = 0.622$	$p = 0.850$
Intact / LSP I	E	6.30 / 3.84	$p < 0.001$	$p = 0.151$	$p = 0.131$
Intact / LSP I	F	1.65 / 0.575	$p < 0.001$	$p = 0.470$	$p = 0.450$
Intact / LSP I	G	11.0 / 5.00	$p < 0.001$	$p = 0.326$	$p = 0.305$
Intact / LSP I	H	1.89 / 31.3	$p = 0.017$	$p = 0.083$	$p = 0.677$
Intact / LPS II	E	4.44 / 3.49	$p = 0.025$	$p = 0.424$	$p = 0.850$
Intacto / LPS II	F	1.80 / 0.755	$p < 0.001$	$p = 0.119$	$p = 0.147$
Intacto / LPS II	G	12.0 / 4.50	$p < 0.001$	$p = 0.666$	$p = 0.064$
Intacto / LPS II	H	68.7 / 502	$p = 0.002$	$p = 0.233$	$p = 0.569$

Table 4. Nonparametric Kruskal-Wallis test, n= 12, of the variables associated with locomotion behavior between the experimental groups (anurans injected with ringer vs. anurans injected with LPS I 2.0mg/Kg vs. anurans injected with LPS II 3 .0 mg/kg). Columns and *p*-values indicate comparisons between treatment groups by collected variable, and *p*-value between test days. Variables: E – total distance covered; F – total time with continuous movement; G number of jumps; H – total time in the shelter.

Variable	Kruskal-Wallis Test for each variable	Pairwise comparisons (Ringer / LPS I)	Pairwise comparisons (Ringer / LPS II)	Pairwise comparisons (LPS I / LPS II)	Kruskal-Wallis Teste variance between day of test
E	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p = 0.967$	$p = 0.226$
F	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p = 0.437$	$p = 0.118$
G	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p = 0.952$
***H**	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	*$p = 1.000$*

Note - The *H variable was analyzed using a test of Kolmogorov-Smirnov (bilateral).

Table 5. T-test comparing body mass, n=12, of intact animals vs. injected with ringer; intact vs. injected with LPS I (2.0mg/Kg); intact vs. injected with LPS II (3.0mg/Kg).

Teste t – weight / Treatment	intact	Ringer	intact	LPS I	intact	LPS II
N	12	12	12	12	12	12
Mean	74,383	73,258	63,324	60,887	59,767	56,953
95% conf	(63,247)	(65,127)	(55,071)	(55,348)	(53,321)	(50,737)
Variance	307,23	163,78	168,73	75,987	102,92	95,734
Difference between means	1,125		2,4375		2,8133	
t	0,17957		0,53976		0,69146	
p	**0,85914**		**0,59478**		**0,49651**	
Critical t value (p=0.05)	2,0739		2,0739		2,0739	

Table 6. Principal component analysis (PCA) of feeding and locomotor behavior of anurans injected with ringer solution vs. anurans injected with LPS I (2.0mg/Kg) vs. anurans injected with LPS II (3.0mg/Kg). Variables: A- number of times the animal goes to the box with food; B- number of attempts against the food box; C – number of ingested preys; D- total time to find access in the food box; E – total distance covered; F – total time with continuous movement; G number of jumps; H – total time in the shelter.

PCA	PC 1	PC 2
A	0.33321	-0.24881
B	0.4225	0.24063
C	0.28048	-0.33645
D	0.20631	-0.61991
E	-0.29455	0.28413
F	0.3725	0.39157
G	0.39269	0.31719
H	-0.21869	0.17231

PC	eigenvalue	% variance
1	4.30962	47.885
2	1.22341	13.593

Capítulo 2

Impacts of immune system activation on locomotor behavior of male and female anurans of the species *Xenopus laevis.*

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Abstract

Locomotion is important for the survival and reproduction, however demand resources, such as energy and metabolites. Thus, locomotion can conflict with different aspects of physiology, like the immune responses derived of activation of the immune system due to an infection. Consequently, the allocation of energy and metabolites can impose limits on immune responses or activity patterns. When an animal is sick, immune responses can include depression of behavior, resulting in reduced activities such as locomotion. This reduction may be associated with energy and metabolites saving which can be destined to the activation of the immune system to fight an infection. This study investigated the effect of a simulated infection on locomotor performance and voluntary movement of male and female anurans of the species *Xenopus laevis*. The results show that a simulated infection resulted in a reduction in the locomotor endurance of individuals of both sexes when compared to the same individuals before injection. No differences between sexes in the treatment response were observed, however. Jump force also differed when compared to control animals, but also differed between sexes with females being more affected by the immune challenge. Voluntary movement, contrary to what was expected, was only marginally affected by the simulated infection. Our data suggest that a simulated infection leads to reduced locomotor performance in *X. laevis*, corroborating the data observed in the literature. However, there seem to be only few differences between sexes suggesting that similar mechanisms are likely responsible irrespective of differences in energy allocation strategies between the sexes.

Key words: Anurans; Behavioral Depression; Immune System; Locomotion.

1. Introduction

In animals the evolution of morphology and physiology is closely related to locomotor performance, which in turn is linked to the ecology of an animal (Taigen, Sharon and Pough, 1982; Taigen and Pough, 1985; Dickinson *et al.,* 2000; Irschick and Garland, 2000). The relationship between form and function has been suggested to be the result of adaptive processes because locomotion impacts functions such as foraging, reproduction and escape from predators (Taigen *et al.,* 1982; Taigen and Pough, 1985; Dickinson *et al.,* 2000; Gomes *et al*., 2009). Locomotor behavior requires energy and metabolic resources to occur and as such locomotion may conflict with other physiological processes that also demand these resources, such as immunological responses (Mayr, 1963; Barlow, 1964; Zug, 1972; Huey and Pianka, 1981; Taigen *et al.,* 1982; Andrews *et al.,* 1987; Arnold, 1992; Levington and Allen, 2005; Konuma and Chiba., 2007; Nathan *et al*., 2008; Herrel *et al*., 2009; Lowe, 2009; Pittman *et al*., 2014).

Activation of the immune system can occur due to pathogenic microorganisms that stimulate an immune response (Hart, 1988, Lefcort and Eiger, 1999; Dantzer *et al*., 2000, Inui, 2001; Larson and Dunn, 2001; Bicego, 20002; Dantzer, 2004; Dantzer, 2006; Pough *et al*., 2006; Adelman and Martin, 2009; Chown *et al*., 2010; Llewellyn *et al.,* 2011; Llewellyn *et al*., 2012; Braga, 2013; Moretti, 2016; Moretti, 2018). Immune responses may include an autonomic component and a complex group of physiological, hormonal, and behavioral responses (Hart, 1988; Sherman and Stephens, 1998; Lefcort and Eiger, 1999; Aubert, 1999; Inui, 2001) triggered by the production of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) that induce behavioral depression, both in endothermic animals (Hart, 1988, Lefcort and Eiger, 1999; Dantzer *et al*., 2000, Inui, 2001; Larson and Dunn, 2001; Dantzer, 2004; Dantzer, 2006; Adelman and Martin, 2009) and in ectotherms, such as anurans (Llewellyn *et al*., 2011; Braga, 2013).

Behavioral depression manifests itself by reduced activity in general, including feeding, locomotion, and reproduction (Hart, 1988; Lefcort and Eiger; 1993; Llewellyn *et al.,* 2011; Braga, 2013). In the case of reduced locomotion, this response can be seen as adaptive in an ecological context as this may lead to reduced exposure and, consequently, reduced predation risk. Moreover, energy and metabolic resources can be saved by the reduction in activity. Theoretically, these resources could then be used to activate the immune system and fight infections (Mhyre *et al.,* 1977; Hart, 1988; Sherman *et al.,* 1991; Lefcort and Eiger; 1993; Sherman and Stephens, 1998; Dantizer, 2006; Brow and Shine, 2014). In this way, behavioral

depression can be seen as part of a group of functionally important and ecologically critical responses to immune challenges (Lefcort and Eiger; 1993; Sherman and Stephens, 1998; Dantizer, 2006).

More generally, locomotion and immunological responses may be involved in physiological trade-offs (Arnold, 1992; Levinton and Allen, 2005; Konuma and Chiba., 2007). As reported in the literature, different species of anurans exhibit differences in the degree of basal activation of the immune system. Whereas some species may present a strong immune response, and with this, greater depression of behavior, and other species may present an immune system that is continually depressed allowing to maintain their ability to perform other activities, such as locomotion, even when sick (Llewellyn *et al*., 2012; Brow and Sine, 2014; Gardner, 2020). Consequently, no unique pattern of response to an immune challenge is present in anurans and the extent to which immune responses can affect the locomotor behavior of different species remains unclear.

The present study tested whether a simulated infection reduces locomotor behavior in male and female anurans. To do, so we used male and female individuals of the African claw frog *Xenopus laevis* (Daudin1802), a globally invasive species (Lillo *et al*., 2005, Lillo *et al*., 2011; Lobos and Jaksic, 2005; Eggert and Fouquet, 2006; Fouquet and Measey, 2006; Robert *et al.*, 2007; Faraone *et al*., 2008; Rebelo *et al*., 2010; Measey *et al*., 2012; De Busschere *et al*., 2016) that were injected with lipopolysaccharide (LPS) of the cell wall of gram-negative bacteria. We tested the impact thereof on locomotor performance (locomotor endurance and jump force) and voluntary movement. We hypothesize that a simulated infection will 1) reduce locomotor performance in *Xenopus laevis*; 2) reduce voluntary movement; 3) differentially impacts males and females due to the difference in basal metabolic rate (Louppe *et al*., 2018; Ducret *et al.*, 2020), the investment of resources in reproduction (higher in females compared to males), and the fact that females of many species are considered more immunocompetent (Ducret *et al*., 2020). Thus, we predicted that the decrease in locomotor performance would be more pronounced in females than in males (Kelly *et al*., 2018).

2. Materials and methods

2.1 Animals

The animals (N = 18 males and 18 females) were housed at the Function and Evolution laboratory at the Muséum National d'Histoire Naturelle in Paris, France. Individuals were taken from their home tanks, separated, and housed singly for the rest of the duration of the

experiments. Animals were fed beef heart twice weekly. Males and females were tested separately and sequentially before and after LPS treatment. All tests were done at 22°C, which is considered around the optimal temperature for the species (Miller, 1982).

2.2 Morphometry

All subjects were weighed on an electronic balance (Cgoldenwall model CNA-383H; precision ± 0.1g) before and after LPS treatment (see Table 1).

2.3 Administration and dosage of LPS

The simulation of an infection was performed by injecting LPS (gram-negative bacterial cell wall lipopolysaccharide) from the bacterium *Escherichia coli* (Serotype 0111: B4, purified by phenol extraction) at a dose of 2mg/Kg. LPS was injected into the dorsal lymph sac of the frog with an insulin syringe with 29-gauge needle attached similar to previous studies (Bicego and Branco, 2002; Llewellyn *et al*., 2011; Moretti, 2016; Olarte, 2017).

2.4 Experimental Groups

Six animals were used for each test (locomotor endurance; jump force; voluntary movement), totaling 18 animals of each sex. Animals were testes first without treatment (control group) and subsequently after LPS injection (treatment group). Therefore, each individual was a control of itself. Each animal was tested separately.

2.5 Locomotor endurance

Locomotor endurance was tested using a circular track measuring 3 meters in circumference. The track had a cork substrate and was covered with 5cm of water. Animals were placed into the track and forced to move until exhaustion, indicated by the lack of a response to the stimulus. Animals were tested three times for three days, with a rest interval of 48 hours between tests days. The animals were fed beef heart after each test day (Herrel *et al*.,2012; Herrel and Bonneaud, 2012a; Herrel and Bonneaud, 2012b; Herrel *et al*., 2014). We recorded the total time spent moving, the total distance covered, and the number of laps.

2.6 Jump force

The animals were tested, one at a time, on a piezoelectric Kistler force platform (20 x 10 cm), connected to a charge amplifier (see Herrel *et al*., 2014 for a detailed description of the

set-up). After being placed on the platform, were forced to jump by touching on the dorsal multiple times for 60 seconds. Each animal was teste for three times in three days, with 48-hour intervals between tests days. Forces were recorded at 500 Hz and the three best jumps (performed in 60 seconds) were analyzed using the Kistler Bioware software. To do so the peak X, Y- and Z-forces were extracted and the overall resultant force was calculated (Herrel *et al.*, 2014).

2.7 Voluntary movement

To quantify voluntary movement, the animals were placed in a tank (80 x 50 x 40 cm) with 20 cm of water and a shelter (Videlier *et al*., 2015). Next, animals were placed under the shelter and filmed for 12 hours (8:00 am - 8:00 pm) for three days, with 48 hours between subsequent days of recording. The videos were analyzed with a stopwatch and the total time spent moving, total time spent in the shelter, and the number of times the animal breached he surface for breathing, were recorded.

3. Statistical Analysis

Firstly, a normality test was performed following which the data were $\log_{10}$-transformed to comply with normality assumptions. The data were dependent (paired) when comparing control animals *vs.* the animals injected with LPS, where each animal is compared against itself (before *vs.* after). Therefore a 2-factor repeated measures ANOVA was performed. When the variables were compared between the sexes, the samples were independent (unpaired) because different experimental groups are compared (females *vs.* males). Consequently, an independent t-test was performed. First, the analysis was performed between control and LPS-injected animals of both sexes, then the sexes were compared.

The body mass of control animals versus LPS-injected animals was analyzed using a paired *t*-test, the comparison of body mass between sexes was performed using an unpaired *t*-test.

4. Results

4.1 Locomotor Endurance

Body mass and test days: Body mass showed no difference after treatment (male: p=0.587; female: p=0.910, respectively). And it was also no different between the sexes. (p=0.144) (Table 1 and 2). There was no difference between test days (Table 3).

Total time spent moving: There was significant differences between control males *vs.* males injected with LPS (n=6; *f*= 5.3; *p*=0.036), with a 17% reduction of time on the track after treatment with LPS. Control females were also different from females injected with LPS (n=6; *f*= 16; *p*=0.001), with a reduction of 21% after treatment with LPS (Table 3; fig 1). No differences between sexes were detected either in control or treatment animals (n=12; *p*=0.435) (Table 4; Fig. 1).

Number of laps until exhaustion: The results showed a significant difference between control males *vs.* males injected with LPS (n=6; *f*= 21; *p*< 0.001) and between control females *vs.* females injected with LPS (n=6; *f*= 12.7; *p*= 0.003). The decrease in performance was 31% in males and 30% in females after treatment with LPS (Table 3; Fig. 1). There was no difference between sexes, irrespective of treatment (n=12; *p*=0.052) (Table 4; Fig. 1).

Total distance covered: The results showed a significant difference between control males *vs.* males injected with LPS (n=6; *f*= 8.9; *p*=0.009) and between control females *vs.* females injected with LPS (n=6; *f*= 13; *p*= 0.002), with a decrease of 58% in males and 63% in females after treatment with LPS (Table 3; Fig. 1). There was no difference between the sexes irrespective of treatment (n=12; *p*=0.068) (Table 4; Fig. 1).

4.2 Jump Force

Body mass and test days: There was no difference in body mass after treatment (males: *p*=0.520; females *p*=0.057, respectively). Body mass did show a difference between the sexes (p=0.002) with males being heavier than females (Table 1 and 2). There was no difference between test days (Table 3).

Maximum jump force: The results showed a significant difference between control females *vs.* females injected with LPS (n=6; *f*= 6; *p*=0.025), with a 26% reduction in jump force after LPS treatment. For males the result was also significant (n=6; *f*= 6; *p*=0.036), with a reduction of 10% after treatment with LPS. There was a difference between the sexes (n= 12; *p*< 0.001), with jump forces being 27% lower in females compared to males. After LPS injections the difference was exacerbated, with jump force being 45% lower in females compared to males (Table 4; Fig. 2).

4.3 Voluntary movement

Body mass and test days: Body mass did not change after LPS injection in either males or females (males: p=0.356; females: p=0.810). Body mass was different between the sexes (p=0.002), with females being 41 % heavier than males (Table 1 and 2). There was no difference between test days (Table 3).

Total time spent moving: The results showed that there was no difference between control males *vs.* males injected with LPS (n=6; f= 1.16; p=0.111). Similarly, no difference was observed for females LPS (n=6; f= 2.15; p=0.163) (Table 3; fig.3). There was also no difference between the sexes (n=12; p=0.564) (Table 4; Fig. 3).

Total time in the shelter: The results showed that there was no treatment effect for either males (n=6; f= 1.56; p=0.230) or females (n=6; f= 0.16; p=0.688) (Table 3; fig.3). There was also no difference between the sexes (n=12; p=0.712) (Table 4; Fig. 3).

Number of breaths: There was a significant difference between control males *vs.* LPS-injected males (n=6; f= 10.5; p=0.005) with a 44% increase in breaths after LPS treatment. Similarly, there was also a difference between control females *vs.* LPS females (n=6; f= 2.46; p=0.038) with an increase of 31% after treatment with LPS (Table 3; fig.3). There was a significant difference between the sexes (n=12; p<0.001). Control females took 40% more breaths than males. After LPS injection the number of breaths became 47% higher in females compared to males (Table 4; Fig. 3).

5. Discussion

Previous studies have shown that immune responses triggered after activation of the immune system led to behavioral changes in anurans, including a reduction in locomotor behavior (Llewellyn, *et al*., 2011; Braga, 2013). These findings are also corroborated by our study. Our results showed that there were differences in locomotor behavior after a simulated infection. Treatment with LPS reduced locomotor endurance and jump force compared to the same individuals before injection. However, there were no differences between the sexes in endurance capacity contrary to previous results (Louppe *et al.*, 2017). Moreover, the impact of a simulated infection was similar in both sexes. For jump force, there was a difference between the sexes, with male frogs and animals with greater body mass exerting greater jump forces.

After injection the difference in performance between the sexes almost doubled, demonstrating a greater effect of LPS on jumping performance in females compared to males. For voluntary movement only the number of breaths increased after LPS treatment. There was also a difference between the sexes which was maintained after injection with LPS.

Locomotor behavior and activation of the immune system are expensive in terms of the use of energy and metabolic resources. Thus, the reduction of activities triggered by immune responses could lead to the savings of resources, which, theoretically, could be used for a stronger immunological response (Mhyre *et al*., 1977; Hart, 1988; Sherman *et al*., 1991; Lefcort and Eiger; 1993; Sherman and Stephens, 1998; Dantizer, 2006; Brow and Shine, 2014). However, few differences in voluntary behavioral were observed after injection suggesting little or no evidence for behavioral depression in the species. The only notable difference was an increase in the number of breaths suggesting an increased need for oxygen in animals after injection. In contrast to the behavioral results, trade-offs between locomotor performance and the immune system were clear. Indeed, LPS injection caused a reduction in two measures of locomotor performance relevant to fitness, endurance capacity and jump force. This provides indirect evidence for the role of resource allocation trade-offs in driving the observed decrease in performance of these animals.

Some species have a strong immune response, and with that, greater depression of behavior (Llewellyn, *et al*., 2011; Braga, 2013). Interestingly, some species maintain a depressed immune system, resulting in energy and metabolites being allocated for activities such as locomotion, which is commonly observed in invasive species (Llewellyn *et al*., 2012; Brow and Shine, 2014). This may explain why the anurans in this study, which are globally invasive, did not show changes in voluntary movements after LPS injection and maintained their activity contrary to our expectations. Only, the number of breaths increased after injection. A possible explanation for this may be that the increase in energy demands to fight infection is associated with a higher metabolic cost and oxygen demand (Choukèr *et al*., 2008; Hochgerner *et al.,* 2021). A previous study in mosquito fish showed that fish were able to increase their ATP production after an immune challenge, resulting in an elevated resting maximal metabolic rate (Bonneaud, *et al*., 2016). This has been suggested to allow fish to offset the energy requirements of mounting an immune response due to an immune challenge (Bonneaud *et al.*, 2016). The higher number of breaths observed in frogs after the immune challenge may reflect a similar strategy allowing these animals to increase the energy available to mount an immune response.

Contrary to our expectations, no interaction between sex and treatment was detected in the analysis of locomotor endurance and voluntary movements, suggesting that the effect of mounting an immune response after LPS injection was similar in males and females. Previous studies have suggested that sexual dimorphism in immune function is common in vertebrates, with females generally being more immunocompetent than males (see Nunn *et al*., 2009 for a review). Thus, females were expected to be more affected by the simulated infection. Furthermore, as female *Xenopus* have a higher basal metabolic rate (Louppe *et al.*, 2018; Ducret *et al.*, 2020) and allocate more energy for reproduction (Courant *et al.*, 2017), we would expect the impact in these variables would potentially be more pronounced in females. However, only the jump force differed between the sexes, with females showing lower jump force compared to males and a greater reduction in jump force after treatment. Although it is not yet clear why no effect was observed for endurance capacity, the fact that the animals were captive and thus not limited in resources such as energy and metabolites may have had an impact (Husak, *et al.*, 2016). Performing similar experiments with additional food restriction treatments would be insightful and could provide more information about potential differences in resource allocation between the sexes.

6. Conclusion

Our results highlight significant differences in locomotor performance of *Xenopus laevis* after LPS treatment. These results are consistent with studies that have shown that animals tend to show a decrease in their activity when faced with an infection, whether acquired in nature or through a simulated infection, such as LPS. Our results suggest that resource use trade-offs may underlie the observed decrease in locomotor performance. The spread of infectious diseases may thus not only directly impact an animals health, but also have indirect effects through its impact on locomotor performance, which can result in a reduced ability to escape predators, capture prey, reproduce and disperse.

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Competing interests

The authors declare no conflict of interest.

Author contributions

T.O. conducted experiments and analyzed the data. All authors conceived the study and contributed to the writing of the manuscript.

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Figures and Tables

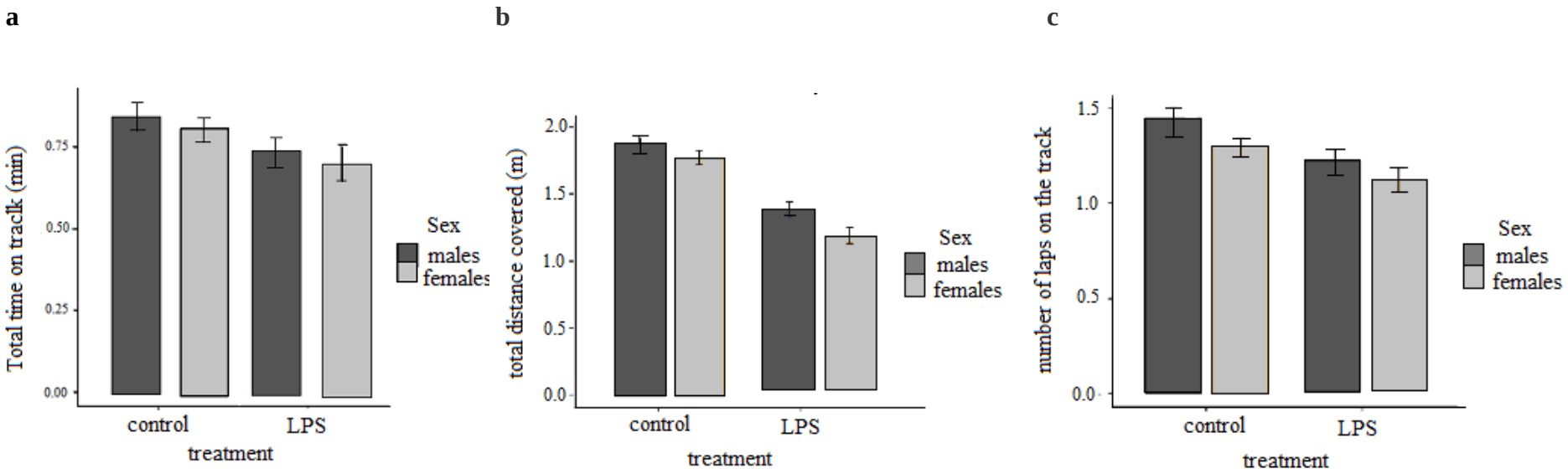

Fig. 1. Analysis of locomotor endurance of males control vs. males injected with LPS; control females vs. females injected with LPS; males and females control and injected with LPS. **a**. Total time on track. **b**. total distance covered. **c**. number of laps on track.

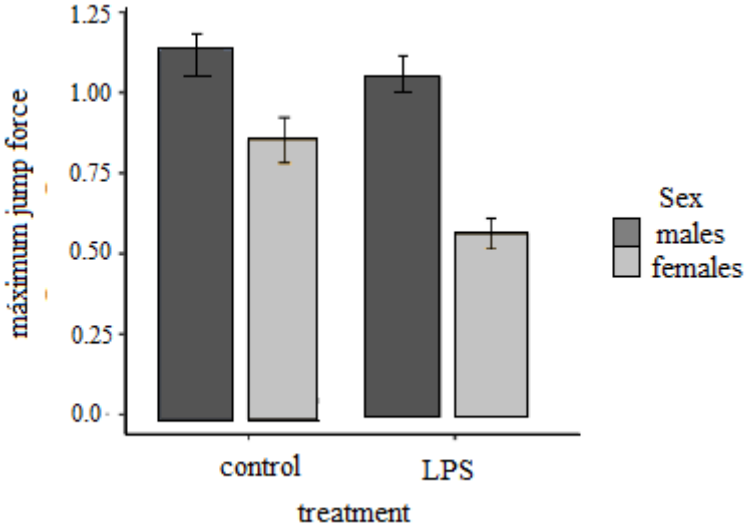

Fig. 2. Analysis of the maximum jumping force of control males vs. males injected with LPS; control females vs. females injected with LPS; males and females control and injected with LPS.

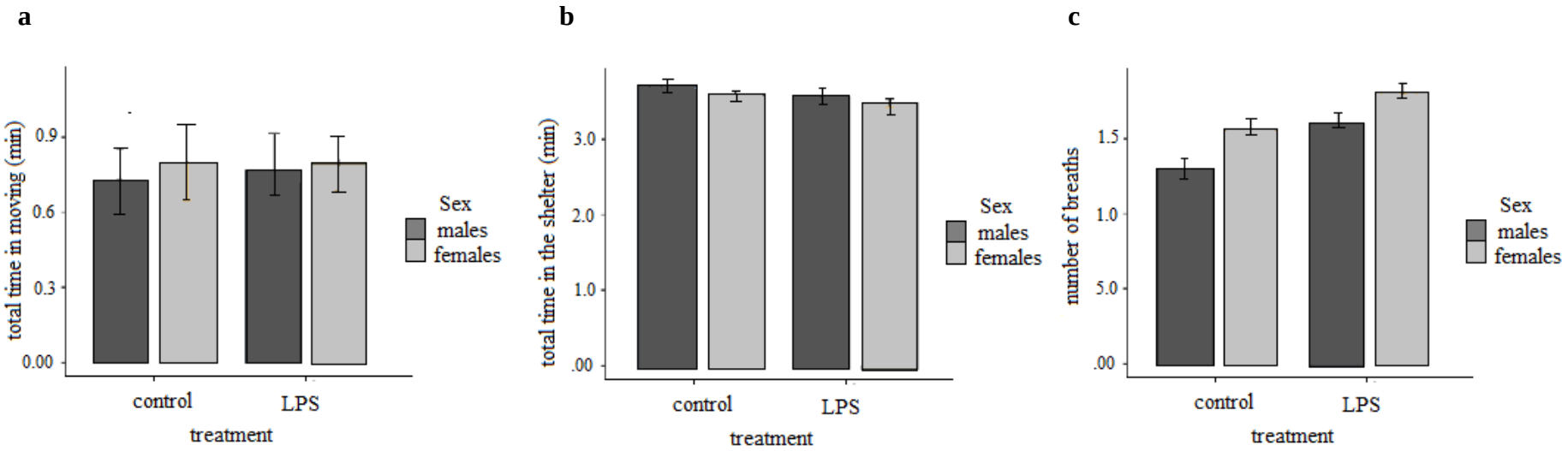

Fig. 3. Analysis of the voluntary movement of control males vs. males injected with LPS; control females vs. females injected with LPS; males and females control and injected with LPS. **a**. total time in moving; **b**. total time in the shelter; **c**. number of breaths during the test.

Table 1. Analysis of the body mass of the individuals used in the tests of each variable. Columns showing paired *t*-test: *p* of anurans body mass before LPS treatment (control) vs. anurans after LPS treatment. Unpaired *t*-test: *p* of body mass between sexes. Percentage (%) of body mass difference between sexes. (N=18 males; N=18 females).

Body mass analysis

	p body mass before vs. after injection LPS	*p body mass between sex*	*Percentage body mass difference between sexes*
		Locomotor Endurance	
Males *X. laevis*	0.587		
Females *X. laevis*	0.910		
Males vs. females		0.411	0
		Jump force	
Males *X. laevis*	0.520		
Females *X. laevis*	0.057		
Males vs. females		0.002	43% males > females
		Voluntary Movement	
Males *X. laevis*	0.356		
Females *X. laevis*	0.810		
Males vs. females		0.002	41.13 % females > males

Table 2. Relation of body mass of each individual used in the test of each variable before treatment with LPS (control) and after treatment with LPS. (N=18 males; N=18 females).

Individuals / sex		**Body mass of each individual - before and after LPS treatment in each variable**					
		Control animals			*LPS injected animals*		
		Locomotor endurance	**Jump force**	**Voluntary movement**	**Locomotor endurance**	**Jump force**	**Voluntary movement**
1	males	24.6	12.0	35.9	27.0	12.4	34.6
2	males	22.5	17.4	34.0	21.5	18.9	35.8
3	males	35.5	13.8	41.2	33.9	13.8	40.3
4	males	18.4	29.6	46.1	20.7	26.9	50.0
5	males	21.6	23.7	37.6	25.4	23.2	36.8
6	males	30.0	31.7	29.3	27.7	28.3	29.1
1	females	27.9	42.2	43.1	29.3	48.3	31.9
2	females	51.3	14.8	50.0	51.1	17.2	47.1
3	females	42.6	22.8	44.9	42.9	21.9	49.0
4	females	15.0	23.4	36.0	15.1	26.2	35.7
5	females	14.4	10.9	62.3	16.1	14.0	62.9
6	females	24.0	10.1	21.4	23.8	13.6	21.0

Table 3. ANOVA repeated measures (2-factor), demonstrating variables, difference between mean, standard error, *f* value, *t* value, *p* value, and *p* between days of the test, of control animals vs. animals injected with LPS in each variable analyzed in the tests for both sex. (N=18 males; N=18 females).

Comparison	**Repeated Measures ANOVA (two-factors)**							
Sex / treatment	*Variable*	*Mean Difference*	*SE*	*df*	*f*	*t*	*p*	*p between days of the test*
Locomotor Endurance								
Males control vs. males LPS	Total time spent moving	0.098	0.04	2	5.31	2.31	0.036	0.713
Females control vs. females LPS	Total time spent moving	0.121	0.02	2	16.3	4.04	0.001	0.433
Males control vs. males LPS	Total distance covered	0.176	0.05	2	8.95	2.99	0.009	0.298
Females control vs. females LPS	Total distance covered	0.177	0.08	2	13.6	3.69	0.002	0.119
Males control vs. males LPS	Number of laps until exhaustion	0.247	0.05	2	21.7	4.66	<0.001	0.322
Females control vs. females LPS	Number of laps until exhaustion	0.186	0.05	2	12.7	3.57	0.003	0.144
Jump Force								
Males control vs. males LPS	Jump force	0.029	0.07	2	6.16	0.40	0.036	0.215
Females control vs. females LPS	Jump force	0.202	0.08	2	6.17	2.48	0.025	0.708
Voluntary Movements								
Males control vs. males LPS	Total time in moving	0.251	0.14	2	1.16	1.69	0.111	0.340
Females control vs. females LPS	Total time in moving	0.212	0.14	2	2.15	1.47	0.163	0.966
Males control vs. males LPS	Total time in the shelter	0.035	0.02	2	1.56	1.25	0.230	0.852
Females control vs. females LPS	Total time in the shelter	0.022	0.05	2	0.16	0.41	0.688	0.935
Males control vs. males LPS	Number of breaths	0.247	0.07	2	10.5	3.24	0.005	0.267
Females control vs. females LPS	Number of breaths	0.068	0.06	2	2.46	1.57	0.038	0.606

Table 4. Independent test-*t* measure demonstrating variables, mean, standard valuation, standard error, statistic, *f* value, deviation *f* and *p* value between males vs. females in each variable analyzed in the tests.

Test t -comparisons males vs. females							
Comparison between sex	*Variable*	*Mean*	*SD*	*SE*	*Statistic*	*df*	*p*
Males vs. females	Total time on track	0.79 – 0.72	0.18 – 0.17	0.03 – 0.02	0.786	10	0.435
Males vs. females	Total distance covered	1.80 – 1.69	0.22 – 0.25	0.03 – 0.04	1.85	10	0.068
Males vs. females	Number of laps on the track	1.33 – 1.20	0.26 – 0.26	0.4 – 0.4	2.00	10	0.052
Males vs. females	Jump force	1.13 – 0.76	0.25 – 0.27	0.04 – 0.04	5.91	10	<0.001
Males vs. females	Total time in moving	0.85 – 0.93	0.58 – 0.55	0.09 – 0.10	0.58	10	0.564
Males vs. females	Total time in the shelter	2.78 – 2.77	0.10 – 0.16	0.01 – 0.01	0.37	10	0.712
Males vs. females	Number of breaths	1.38 – 1.49	0.27 – 0.28	0.04 – 0.05	1.56	10	<0.001

Capítulo 3

Impacts of immune system activation on locomotor behavior in invasive and non-invasive anurans.

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Abstract

Locomotion is essential for survival and demand resources such as energy and metabolites. Thus, locomotion can conflict with other aspects of physiology that also demand resources, such as the immune responses derived of activation of the immune system in the face of an infectious process. This conflict can impose limits on immune responses or activity patterns, as the allocation of energy and metabolites for locomotion reduces these resources for immune immunological responses. Conversely, a depressed immune system can free resources for activities such as locomotion. Previous studies have shown that invasive species may have a depressed immune response allowing them to maintain locomotor function and reproduction. The present study investigates the impact of a simulated infection on in locomotor performance (locomotor endurance and jump force) and voluntary movement of anurans of the species *X. laevis* (globally invasive species) and the congeneric *X. allofraseri* (non-invasive). The results showed that the simulated infection reduced the locomotor performance in both species. But, was more accentuated in anurans of the specie of *X. allofraseri*. Voluntary movement showed marginal differences between species. Our data suggest that a simulated infection leads to behavioral depression in anurans, and that this depression appears to be less pronounced in invasive species, which can maintain activities related to dispersal, such as locomotion, even when sick.

Key words: Anurans; Behavioral Depression; Dispersal; Immune System; Invasive Species;

Locomotion.

1. Introduction

Locomotion is an essential activity for survival, and is thus fitness-relevant. Indeed, locomotion is a key trait during foraging, escape from predators, and finding reproductive partners (Dickinson *et al*., 2000; Gomes *et al*., 2009). Locomotion is a costly activity that is sensitive to the energy or metabolite balance and reproductive status and health condition of an organism (Barlow 1964; Zug 1972; Levington and Allen 2005; Nathan *et al.*, 2008; Lowe 2009; Pittman *et al*., 2014). Locomotion can use a considerable part of the energy and metabolic budget but occurs in parallel with other activities that also demand them (Mayr 1963; Huey and Pianka 1981; Taigen *et al.*, 1982; Andrews *et al*., 1987). Thus, in certain contexts, locomotion may conflict with other physiological processes, such as the immunological responses resulting from an infectious process, what can generate a trade-off (e.g., Arnold 1992; Levington and Allen 2005; Konuma and Chiba 2007; Herrel *et al*., 2009; Llewelly *et al*., 2012).

Activation of the immune system can occur due to pathogenic microorganisms that stimulate an immune response, including infectious processes (Hart 1988, Lefcort and Eiger 1999; Dantzer *et al*., 2000, Inui 2001; Larson and Dunn 2001; Bicegeo 2000; Bicego 20002; Dantzer 2004; Dantzer 2006; Pough *et al.*, 2006; Adelman and Martin 2009; Chown *et al.*, 2010; Llewellyn *et al.*, 2011; Llewellyn *et al.*, 2012; Braga 2013). The immune response may include as an autonomic component resulting in a complex group of physiological, hormonal and behavioral responses (Hart 1988; Sherman and Stephens 1998; Lefcort and Eiger 1999; Aubert 1999; Inui 2001). These responses are typically triggered by the production of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) that induce behavioral depression in both endothermic (Hart 1988, Lefcort and Eiger 1999; Dantzer *et al*., 2000, Inui 2001; Larson and Dunn 2001; Dantzer 2004; Dantzer 2006; Adelman and Martin 2009) and ectothermic animals, such as anurans (Llewellyn *et al.*, 2011; Llewellyn *et al.*, 2012; Braga 2013) via the central nervous system. Depressed behavior is often reflected by reduced activity and locomotion (Hart 1988; Lefcort and Eiger 1993; Llewelly *et al*., 2011; Llewellyn *et al*., 2012; Braga 2013). Theoretically, resources freed up by reduced activity could be used to activate the immune system and fight an infection (Mhyre *et al.*, 1977; Hart 1988; Sherman *et al.*, 1991; Lefcort and Eiger 1993; Sherman and Stephens 1998; Dantizer 2006; Brow and Shine 2014). In this way, behavioral depression can be seen as part of a group of functionally important and ecologically critical responses to immune challenges (Lefcort and Eiger 1993; Sherman and Stephens 1998; Dantizer 2006).

Between anurans, different species can show substantial diversity in the degree of basal activation of the immune system. Whereas some species may exhibit a strong immune response, and with that, greater depression of behavior (Llewellyn *et al*., 2011; Braga 2013), others may exhibit a depressed immune system and maintain their ability to perform activities such as locomotion, even when sick (Llewellyn *et al*., 2012; Brow and Sine 2014). This premise arises from the energetic and metabolic cost, as maintaining an immune system in a permanent state of high responsiveness is expensive in terms of resource use (Brown and Shine 2014). Therefore, there seem to be two extremes along a continuum reflected on the one hand by a permanent and expensive immune state of alert, and on the other hand by immune depression as a basal state, but reversible in certain contexts (Brown and Shine 2014). The latter has been observed in different lineages, especially those with high dispersal capacity (Lee *et al.*, 2005; White and Perkins 2012; Llewellyn *et al*., 2012; Brown and Shine 2014; Ihlow *et al*., 2016; Gardner *et al.*, 2020).

Previous studies have shown that invasive anurans species are able to maintain their ability to move in contexts of infection (Brow and Shine 2014), thus being able to continue exploring new environments. In contrast, other studies, showed a reduction in activity in anurans in the face of an infection resulting in a lower rate of locomotion and feeding, both in the laboratory and in a semi-captive environment (Llewellyn *et al*., 2011; Braga 2013). Thus, depending on environmental conditions, a given immune response profile may confer an ecological advantage on invaders compared to non-invasive species. The latter would likely maintain a physiological state of increased immune activity (Brown and Shine 2014; Lee and Klasing 2004; Gardner *et al*., 2020). In expanding populations natural selection or spatial sorting can drive an increase in resource allocation to dispersal, growth, and reproduction, traits that can come at a cost to immune function (Brown and Shine 2014; Gardner *et al.*, 2020).

The present study focused on whether a simulated infection reduces the locomotor behavior of two congeneric species, one a globally invasive species and the other a non-invasive species. We predict that the reduction in activity would be more pronounced in non-invasive species. The study model used was the African claw toad *Xenopus laevis* (Daudin1802), a globally invasive species and the non-invasive *Xenopus allofraseri*. We tested if locomotion was affected by the immune response caused by a lipopolysaccharide (LPS) injection of the gram-negative bacterial cell wall. We analyzed two characteristics of locomotor performance (endurance and jump force) and voluntary movement before (control group) and after LPS injection (treatment group). We tested the following hypotheses: 1) the simulated

infection would reduce the locomotor behavior in both species; 2) the simulated infection will reduce voluntary movement; 3) the infection would differentially impact *X. laevis* and *X. allofraseri*; 4) the impact of infection would be lower for the invasive *X. laevis*.

2. Materials and methods

2.1 Individuals

The animals (*N* = 18 females of *X. laevis* and 18 females of *X. allofraseri*) were housed at the Function and Evolution laboratory at the Muséum National d'Histoire Naturelle in Paris, France. Individuals were taken from their home tanks, separated, and housed singly for the rest of the duration of the experiments. Animals were fed beef heart twice weekly. Both species were tested separately and sequentially before and after LPS treatment. All tests were done at 22°C, which is considered the optimal temperature for the species.

2.2 Morphometry

All subjects were weighed on an electronic balance (Cgoldenwall model CNA-383H; precision ± 0.1g) before and after LPS treatment (see Table 1).

2.3 Administration and dosage of LPS

The simulation an infection was performed by injecting LPS (gram-negative bacterial cell wall lipopolysaccharide) from the bacterium *Escherichia coli* (Serotype 0111: B4 purified by phenol extraction) at a dose of 2mg / Kg, injected into the dorsal lymph sac of the frog with syringe with 29-gauge needle attached (Bicego and Branco 2002; Llewellyn *et al*., 2011; Olarte 2017).

2.4 Experimental Groups

Six animals were used for each variable tested (locomotor endurance; jump force; voluntary movement), totaling 18 animals of each species. Animals were tested before (control group) and after LPS injection (treatment group). Therefore, each individual was a control of itself.

2.5 Locomotor endurance

Locomotor endurance was tested using a circular track measuring 3 meters in circumference. The track had a cork substrate and was covered with 5cm of water. Animals

were placed into the track and forced to move until exhaustion, indicated by the lack of a response to the stimulus. Animals were tested three times for three days, with a rest interval of 48 hours between tests days. The animals were fed beef heart after each test day (Herrel *et al*.,2012; Herrel and Bonneaud, 2012a; Herrel and Bonneaud, 2012b; Herrel *et al*., 2014). We recorded the total time spent moving, the total distance covered, and the number of laps.

2.6 Jump force

The animals were tested, one at a time, on a piezoelectric Kistler force platform (20 x 10 cm), connected to a charge amplifier (see Herrel *et al*., 2014 for a detailed description of the set-up). After being placed on the platform, were, were forced to jump by touching on the dorsal multiple times for 60 seconds. Each animal was teste for three times in three days, with 48-hour intervals between tests days. Forces were recorded at 500 Hz and the three best jumps (performed in 60 seconds) were analyzed using the Kistler Bioware software. To do so the peak X, Y- and Z-forces were extracted and the overall resultant force was calculated (Herrel *et al.*, 2014).

2.7 Voluntary movement

To quantify voluntary movement, the animals were placed in a tank (80 x 50 x 40 cm) with 20 cm of water and a shelter (Videlier *et al*., 2015). Next, animals were placed under the shelter and filmed for 12 hours (8:00 am - 8:00 pm) for three days, with 48 hours between subsequent days of recording. The videos were analyzed with a stopwatch and the total time spent moving, total time spent in the shelter, and the number of times the animal breached he surface for breathing, were recorded.

3. Statistical Analysis

First, a normality test was performed. Next, the data were $\log_{10}$-transformed to comply with normality assumptions. Parametric tests were performed according to the type of sample. The data were dependent (paired) in nature when comparing control animals *vs.* the animals injected with LPS (before vs. after). Therefore a 2-factor repeated measures ANOVA (rmANOVA) test was performed. When comparing between species, the data were independent (unpaired) because different experimental groups are compared (females of the species *X. laevis vs.* females of the species *X. allofraseri*). Consequently, an independent t-test was performed. First, we compared control *vs.* LPS-injected animals of both species, then the

species were compared. The difference in body mass of control *vs.* LPS-injected animals was analyzed using a paired *t*-test, the comparison of animal body mass between species was performed using an unpaired *t*-test.

4. Results

Body mass between species utilized and test days

Body mass was significantly different between species (Table 3) with females of *X. laevis* being larger than females of *X. allofraseri* (Tables 1; 2). There was no difference between test days (Table 3).

4.1 Locomotor endurance

Body mass before and after treatment: Body mass did not differ between control and LPS-injected animals of either species (*X. laevis*: p=0.910; *X. allofraseri*: p=0.374).

Total time spent moving: The results showed that there was a significant difference with a reduction of 23% of the total time spent moving in females injected with LPS in comparison to control females in *X. allofraseri* (n=6; f= 13.2; p= 0.002). For *X. laevis* females there was also a statistical difference with a decrease of 21% in the total time spent moving in females injected with LPS (n=6; f=13.6; p=0.001) (Table 3; fig. 1) compared to the same individuals before injection. There was a significant difference between species before injection (n=12; p<0.001); control females of *X. allofraseri* spent 23% less time moving until exhaustion compared to control females of *X. laevis*. After LPS injection the difference between species nearly doubled with *X. allofraseri* showing a 47 % less time compared to *X. laevis* (Table 4; Fig. 1).

Number of laps on the track: The results showed that there was a significant difference between control females *vs.* females injected with LPS (n=6; f= 12.7; p= 0.036) in *X. laevis*, with a decrease of 21% after the treatment with LPS. *Xenopus allofraseri* also showed a significant effect (n=6; f= 12; p=0.003) with a reduction of 41% after treatment with LPS (Table 3; Fig. 1). There was a significant difference between the species (n=12; p<0.001), with control females of *X. allofraseri* performing 32% fewer laps on the track compared to females of *X. laevis*. After treatment with LPS, females of *X. allofraseri* showed a reduction of 47% in relation to the females of *X. laevis* showing that the impact of LPS injection was greater in *X. allofraseri* (Table 4; Fig. 1).

Total distance covered on the track: The results showed that there was a significant difference between control females *vs.* females injected with LPS (n=6; *f*= 6; *p*= 0.025) in *X. laevis* with a decrease of 19% after treatment with LPS. The results for *X. allofraseri* were also significant (n=6; *f*=12; *p*=0.002), with a reduction of 31% after treatment with LPS (Table 3; fig. 1). There was a significant difference between the species (n=12; *p*<0.001) and females of the species *X. allofraseri* traveled 37% less distance on the track compared to females of the species *X. laevis*. After the injection of LPS *X. allofraseri* covered 46% less distance in relation to *X. laevis* showing that the impact of LPS injection was greater in *X. allofraseri* (Table 4; Fig. 1).

4.2 Jump force

Body mass before and after treatment: The body mass of female *X. laevis* and female *X. allofraseri* did not change after the injection with LPS (*p*=0.057; *p*=0.059, respectively).

Jump test: The results showed that there was a difference between control *X. laevis vs.* females injected with LPS (n=6; *f*=6.1; *p*=0.025), with a reduction in force of 26% after treatment with LPS. Females of the species *X. allofraseri* also showed a difference in jumping force (n=6; *f*= 4.7; *p*=0.027), with a reduction of 28% in females injected with LPS compared to control females (Table 3; Fig. 2). There was a difference in the jumping force between the species (n=12; *p*<0.001), both in control animals as well as after LPS injections. The results showed a reduction of 28% in females of *X. allofraseri* controls compared to the *X. laevis* controls. After LPS injections the difference was similar (30% - Table 4; Fig. 2).

4.3 Voluntary Movement

Body mass before and after treatment: The body mass did not differ before and after LPS injection for either species (*X. laevis*: *p*=0.134; *X. allofraseri*: *p*=0.119).

Total time spent moving: The results showed that there was a significant difference between control females vs. females injected with LPS (n=6; *f*= 8; *p*=0.013) in *X. allofraseri*, with a reduction of 24% of the time in movement after the treatment with LPS. No significant difference (n=6; *f*= 2.1; *p*=0.163) was observed for *X. laevis* (Table 3; fig.3). There was a difference between the species (n=12; *p*=0.017), with *X. allofraseri* spending 30% less time moving compared to *X. laevis* controls. The difference remained at 31% after treatment with LPS (Table 4; Fig. 3).

Total time spent in the shelter: There was no difference between control females *vs.* females injected with LPS for either *X. laevis* (n=6; *f*= 0.16; *p*=0.688) or *X. allofraseri* (n=6; *f*= 1.9; *p*=0.538) (Table 3; fig. 3). There was a difference between the species (n=12; *p*=0.028) with *X. allofraseri* spending 12% less time in the shelter compared to *X. laevis*. After LPS injection *X. allofraseri* spent 16% less time in the shelter compared to *X. laevis* (Table 4; fig. 3).

Number of breaths: There were no differences between control females *vs.* females injected with LPS in either species (*X. laevis:* n=6; *f*= 2.4; *p*=0.138; *X. allofraser*i: n=6; *f*= 1.9; *p*= 0.186) (Table 3; fig.3). There was a difference between species (n=12; *p*=0.002), with the number of breaths being 24% greater in *X. allofraseri.* After LPS injections the difference between species was similar (22%, Table 4; fig. 3).

5. Discussion

Our results showed that LPS treatment altered the locomotor behavior of the frogs included in our study. Moreover, there were significant differences between females of the species *X. laevis* and *X. allofraseri,* with *X. laevis* being less affected by the simulated infection as predicted. Indeed, previous studies have shown that invasive species tend to maintain a depressed immune system, resulting in energy and metabolites being allocated to activities such as locomotion (Lee *et al*., 2005; Llewellyn, *et al*., 2012; White and Perkins 2012; Brown and Shine 2014). The immune responses triggered after activation of the immune system resulted in a reduced locomotor performance but few or no differences in behavior, as demonstrated in this study. The reduction in endurance after injection appears to be a general pattern also observed in other anurans (Llewellyn *et al*., 2011; Braga 2013). Interestingly, locomotor endurance (time, distance, and the number of laps) showed a greater reduction in *X. allofraseri.*

Our results further showed that jumping force was also reduced after LPS injection in both species (Llewellyn *et al*., 2011; Braga 2013). However, the reduction in force was not exacerbated in the non-invasive species contrary to what was observed for endurance. This may be due to the fact that jumping is an explosive behavior, mainly relying on energy stores present in the muscles (Zug, 1972; Taigen *et al*., 1982; Dickinson *et al*., 2000; Gomes *et al*., 2009). Interestingly, few or no differences in voluntary movement were observed after LPS injection suggesting that animals showed little behavioral depression after mounting an immune response. Yet, differences between species were apparent with the *X. allofraseri* taking more breaths, spending less time in the shelter but also moving less. These interspecific differences

may simply reflect the differences in habitat use between species with *X. allofraseri* being a forest-dwelling species in contrast to *X. laevis* which lives in more open habitats and may thus spend more time in its shelter (Lillo *et al*., 2005; Faraone *et al.,* 2008). Only females of *X. allofraseri* showed a reduction in total time with movement after LPS injections compared to control animals suggesting that this species may use partial behavioral depression to free up energy for mounting an immune response.

Overall, our results suggest that locomotor performance is reduced in anurans, corroborating studies from the literature (Llewellyn *et al.*, 2011; Braga 2013). In addition, the invasive species *X. laevis* was less affected similar to other invasive anurans species (Llewellyn, *et al*., 2012; Brow and Shine 2014). Finally, *X allofraseri* showed a partial behavioral depression possibly because it allocated more energy to the immune response. Therefore, this study corroborates previous studies in showing that invasive frogs have a depressed immune system allowing them to maintain relatively higher levels of locomotor capacity. This is likely related to the importance of dispersal in novel environments allowing animals to gain access to novel resources and competitor-free environments.

6. Conclusion

X. laevis and *X. allofraseri* showed reduced locomotor performance after a simulated infection. These results are consistent with other studies that have shown that animals tend show reduced locomotor performance after an immune challenge. The globally invasive *X. laevis* was less affected by the simulated infection than *X. allofraseri*, a non-invasive species. This provides evidence that invasive species may maintain a depressed immune system, allocating more energy to activity and locomotion.

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Competing interests

The authors declare no conflict of interest.

Author contributions

T.O. conducted experiments and analyzed the data. All authors conceived the study and contributed to the writing of the manuscript.

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Figure and Tables

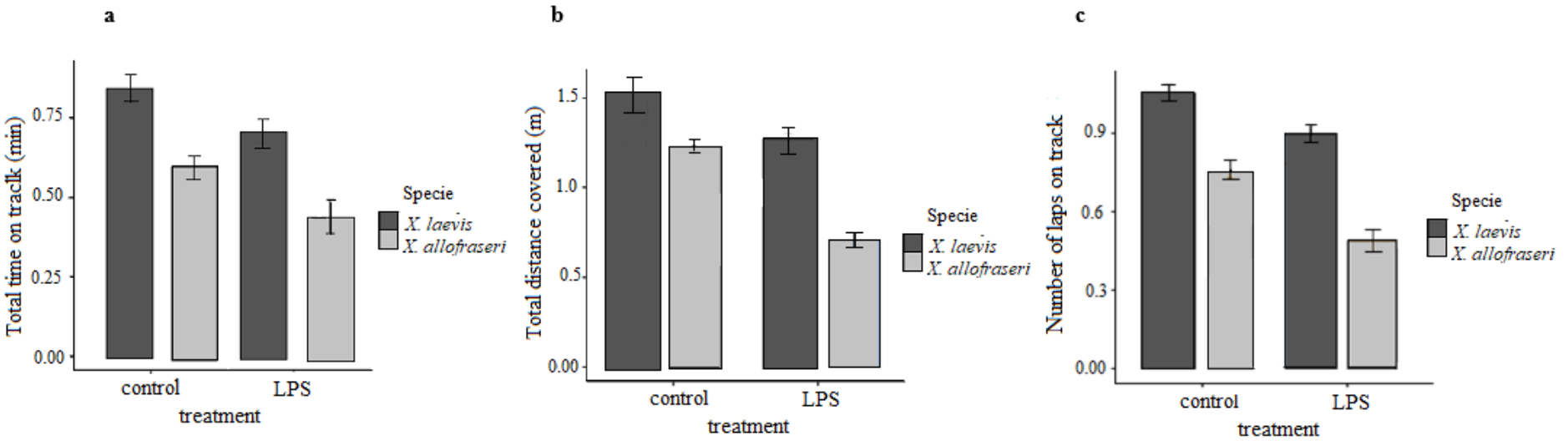

Fig. 1. Behavior analysis of control females vs. females injected with LPS of the species *X. laevis* and *X. allofraseri*; comparison between species. **a**. Total time on track. **b**. total distance covered. **c**. number of laps on the track.

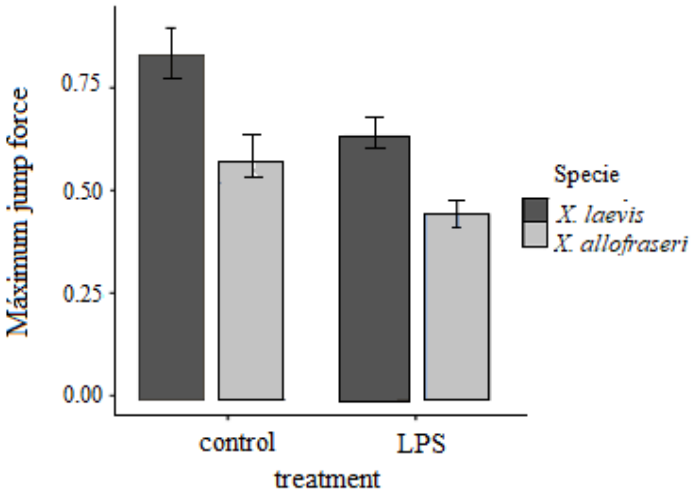

Figure 2. Analysis of the maximum jumping force of control females vs. females injected with LPS of the species *X. laevis* and *X. allofraseri;* comparison between species.

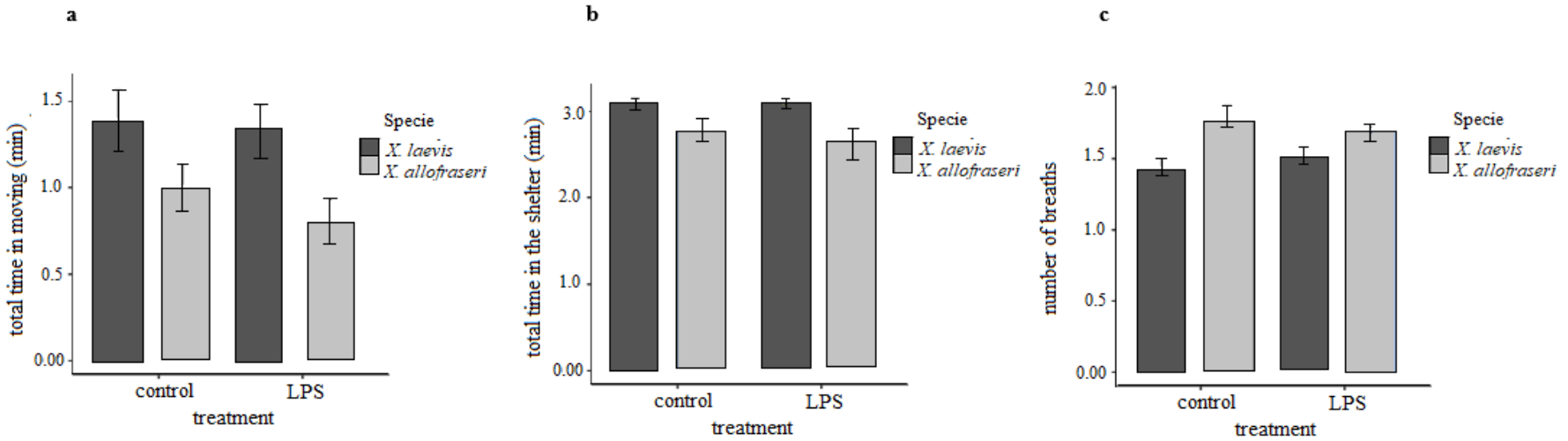

Figure 3. Analysis of voluntary movements of control females vs. females injected with LPS of the species *X. laevis* and *X. allofraseri*; comparison between species. **a**. Total in moving. **b**. total time in the shelter. **c**. number of breaths.

Table 1. Analysis of the body mass of the individuals used in the tests of each variable. Paired *t*-test: *p* of anurans body mass before LPS treatment (control) vs. after LPS treatment. Unpaired *t*-test: *p* of body mass between species. Percentage (%) of body mass difference between species.

Species	***p* body mass before vs. after injection LPS**	***p* body mass between species**	**Body mass difference between species (%)**
Locomotor Endurance			
Females *X. laevis*	0.910		
Females *X. allofraseri*	0.374		
X. laevis* vs. *X. allofraseri		0.003	56.18 % *X. laevis* > *X. allofraseri*
Jump force			
Females *X. laevis*	0.057		
Females *X. allofraseri*	0.059		
X. laevis* vs. *X. allofraseri		0.018	38.45 % *X. laevis* > *X. allofraseri*
Voluntary Movement			
Females *X. laevis*	0.134		
Females *X. allofraseri*	0.119		
X. laevis* vs. *X. allofraseri		<0.001	97.11 % *X. laevis* > *X. allofraseri*

Table 2. Relation of body mass of each individual used in the test of each variable before treatment with LPS (control) vs. after LPS treatment.

Individuals/sex		Body mass					
		Control animals			**LPS injected animals**		
		Loc. endurance	**Jump force**	**Vol. movement**	**Loc. endurance**	**Jump force**	**Vol. movement**
1	***X. laevis***	27.9	42.2	43.1	29.3	48.3	31.9
2	***X. laevis***	51.3	14.8	50.0	51.1	17.2	47.1
3	***X. laevis***	42.6	22.8	44.9	42.9	21.9	49.0
4	***X. laevis***	15.0	23.4	36.0	15.1	26.2	35.7
5	***X. laevis***	14.4	10.9	62.3	16.1	14	62.9
6	***X. laevis***	24.0	10.1	21.4	23.8	13.6	21.0
1	***X. allofraseri***	13.8	17.5	10.1	13.3	17.8	8.7
2	***X. allofraseri***	15.3	12.8	10.4	16.8	11.0	9.5
3	***X. allofraseri***	5.8	20.2	12.2	6.4	18.4	10.4
4	***X. allofraseri***	13.0	10.7	9.7	14.4	10.5	9.2
5	***X. allofraseri***	14.0	9.7	15.0	14.0	8.7	13.3
6	***X. allofraseri***	14.0	21.9	16.3	13.4	20.5	16.1

Table 3. The ANOVA test repeated measures (2-factor) of the comparison between control and LPS-injected animals of each species. Columns demonstrating variables, difference between mean, standard error, *f* value, *t* value and *p* value the differences between the 3 days of testing, of the anurans females of the species *X. laevis* and *X. allofraseri* control and injected with LPS. (N= 18 individuals of each species).

Species / treatment	**Variable**	**Mean Difference**	**SE**	**d*f***	***f***	***t***	***p***	***p* between days of test**
Locomotion Endurance								
***X. allofraseri* control vs. *X allofraseri* LPS**	Total time on track	0.113	0.031	2	13.2	3.6	0.002	0.903
***X. laevis* control vs. *X. laevis* LPS**	Total time on track	0.121	0.029	2	13.6	4.0	0.001	0.433
***X. allofraseri* control vs. *X allofraseri* LPS**	Total dist. covered	0.0561	0.037	2	12.3	1.5	0.002	0.898
***X. laevis* control vs. *X. laevis* LPS**	Total dist. covered	0.177	0.084	2	6.35	3.7	0.025	0.778
***X. allofraseri* control vs. *X allofraseri* LPS**	Num. of laps	0.0517	0.037	2	11.9	1.2	0.003	0.853
***X. laevis* control vs. *X. laevis* LPS**	Num. of laps	0.186	0.051	2	12.7	3.6	0.036	0.758
Jump Force								
***X. allofraseri* control vs. *X allofraseri* LPS**	Jump force	0.131	0.060	2	4.7	2.2	0.027	0.986
***X. laevis* control vs. *X. laevis* LPS**	Jump force	0.202	0.081	2	6.17	2.5	0.025	0.708
Voluntary Movements								
***X. allofraseri* control vs. *X allofraseri* LPS**	Total time in moving	0.287	0.102	2	7.9	2.9	0.013	0.599
***X. laevis* control vs. *X. laevis* LPS**	Total time in moving	0.212	0.144	2	2.15	1.5	0.163	0.966
***X. allofraseri* control vs. *X allofraseri* LPS**	Total time in shelter	0.0917	0.145	2	0.40	0.6	0.538	0.592
***X. laevis* control vs. *X. laevis* LPS**	Total time in shelter	0.0222	0.054	2	0.17	0.4	0.688	0.935
***X. allofraseri* control vs. *X allofraseri* LPS**	Number of breaths	0.0967	0.070	2	1.92	1.2	0.186	0.527
***X. laevis* control vs. *X. laevis* LPS**	Number of breaths	0.0681	0.061	2	2.46	1.6	0.138	0.606

Table 4. Independent *t*-test measure of the comparison between the specie. Columns demonstrating variables, mean, standard valuation, standard error, statistic, *f* value, deviation *f*, and *p* value of the anuran's females of the species *X. laevis* vs. *X. allofraseri* control and injected with LPS. (N= 18 individuals of each species).

Comparison Between Species							
Species	**Variable**	**Mean**	**SD**	**SE**	**Stat.**	**d*f***	***p***
X. allofraseri* vs. *X laevis	Total time on track	0.5–0.8	0.1– 0.2	0.02– 0.03	6.71	10	<0.001
X. allofraseri* vs. *X laevis	Total dist. covered	1.5– 0.8	0.2– 0.2	0.02– 0.03	18.7	10	<0.001
X. allofraseri* vs. *X laevis	Num. of laps	1.0– 0.8	0.2– 0.3	0.03– 0.3	6.26	10	<0.001
X. allofraseri* vs. *X laevis	Jump force	1.1– 0.8	0.2– 0.3	0.04 – 0.04	5.91	10	<0.001
X. allofraseri* vs. *X laevis	Total time in moving	0.5– 0.7	0.2– 0.3	0.03– 0.04	3.99	10	0.017
X. allofraseri* vs. *X laevis	Total time in shelter	1.0– 0.7	0.6– 0.5	0.09 – 0.08	2.45	10	0.028
X. allofraseri* vs. *X laevis	Number of breaths	2.4– 2.7	0.5– 0.2	0.09 – 0.02	3.61	10	0.002

Discussão Geral e Conclusões

A ativação do sistema imune estimulada por pirógenos exógenos como o LPS, desencadeia respostas imunológicas que envolvem diferentes tipos celulares e agem mediados por citocinas pró-inflamatórias, como IL-1, IL-6 e TNF-α (Hart, 1988; Aubert, 1999; Sherman *et al*., 1991; Sherman & Stephens, 1998; Bicego, 2000; Dantizer, 2000; Larson & Dunn, 2001; Bicego, 2002; Dantizer, 2004; Dantizer, 2006; Adelman & Martim, 2009; Braga, 2013; Abbas, *et al*., 2014; Madigan, *et al.,* 2016; Gardner, 2020; Ferreira *et al*., 2021; Bastos *et al*., 2022). A ação dessas citocinas pode desencadear a depressão comportamental que leva a redução da alimentação, locomoção, reprodução, dentre outros (Hart, 1988; Lefcort & Eiger, 1993; Aubert, 1999; Larson & Dunn, 2001; Adelman & Martin, 2009). A depressão comportamental já foi estudada em diferentes linhagens de mamíferos, aves, peixes, insetos, e répteis em diferentes contextos (Reynolds *et al.*, 1976; Hart 1988; Reynolds, 1976; Hatalski & Lipkin, 1997; Aubert 1999; Inui, 2001; Asarian & Langhans, 2005; Lee *et al*., 2005).

Embora em anfíbios anuros o número de trabalhos considerando a depressão comportamental ainda seja incipiente, é demonstrada em indivíduos de diferentes espécies quando doentes em condições experimentais (e.g *Rhinella marina*, *Scinax perpusillus*) (Llewellyn *et al*., 2011; Braga, 2013). Estes estudos mostram que após um processo infeccioso os indivíduos apresentam redução de atividades como alimentação, locomoção, hidratação, interação social e reprodução (Llewellyn *et al*., 2011; Braga, 2013). A redução da alimentação e da locomoção decorrente da ação de citocinas após estímulo inflamatório por injeções de LPS também foi observada em indivíduos da espécie *A. catesbeiana* analisados nesta pesquisa. Os indivíduos reduziram a busca e interesse pelo alimento e a ingestão de presas, permanecendo mais tempo no esconderijo. Anuros das espécies *X. laevis* e *X. allofraseri* estudados também reduziram o comportamento locomotor, especificamente a resistência locomotora e a força de salto, após o tratamento com LPS. Portanto, estes resultados mostram que as três espécies estudadas demonstram a ocorrência da depressão comportamental como uma das respostas imunológicas diante de um processo infeccioso.

A ocorrência da depressão comportamental pode estar relacionada ao custo das respostas imunes, relativo ao uso de recursos, como energia e metabólitos (Hart, 1988; Aubert, 1999). Pois, no contexto da história natural se as respostas imunes levam a custos pela utilização de recursos, então devem competir com outros aspectos da fisiologia e ecologia que também precisam de energia e metabólitos, como a alimentação e a locomoção por exemplo. Portanto, após a ativação do sistema imune, a realização do comportamento alimentar e

locomotor pode levar a um *trade-off*. Este trade-off seria gerado pois, o indivíduo precisa alocar recursos (energia, metabolitos e tempo) entre a realização de diferentes atividades e as respostas imunes (Hart, 1988; Aubert, 1999; Llewellyn *et al.*, 2012). Assim, a depressão comportamental pode permitir a economia de energia e metabólitos que podem ser utilizados no sistema imune (Hart, 1988; Aubert, 1999; Dantzer, 2006; Adelman and Martin, 2009). Além disso, reduzindo a alimentação e a locomoção o indivíduo consegue diminuir a ingestão de nutrientes importantes para o crescimento e multiplicação patogênica, e tem um menor o risco de predação devido a menor exposição em contextos de doença (Hart, 1988; Aubert, 1999; Dantzer, 2006; Adelman & Martin, 2009; Llewellyn *et al*., 2011).

Apesar da depressão do comportamento ser observada e caracterizar a maioria dos indivíduos doentes, já foi observado que algumas espécies investem menos recursos na função imune e com isso conseguem manter sua capacidade para realizar atividades como alimentação, locomoção e reprodução. Este caso já foi observado principalmente em diferentes linhagens de espécies invasoras, especialmente aquelas com alta motilidade e capacidade de dispersão (Lee *et al*., 2004; Lee *et al*., 2005; White e Perkins, 2012; Llewellyn *et al.*, 2012; Brown e Shine, 2014; Ihlow *et al*., 2016, Gardner *et al*., 2020). Llewellyn e colaboradores (2012), Brow e Shine (2014) e Gardner, (2020) mostraram que o anuro invasor *Rhinella marina* parece demonstrar um sistema imune deprimido e mantem sua capacidade de locomoção, percorrendo longas distâncias, principalmente na faixa de invasão. Portanto, conseguem se dispersar em novos ambientes mesmo doentes, mantendo sua capacidade de exploração, busca por alimento e água e reprodução, em relação aos indivíduos não invasores. Este contexto foi corroborado nesta pesquisa, quando o comportamento locomotor de indivíduos de *X. laevis* (espécie invasora) e de indivíduos de *X. allofraseri* (espécie não invasora) foi analisado após o tratamento com LPS. Ambas as espécies apresentaram redução da resistência locomotora (distância, tempo, e n° de voltas na pista) componente do desempenho locomotor, no entanto a espécie não invasora *X. allofraseri* foi mais afetada em relação a *X. laevis*, que mesmo com o processo infeccioso conseguiu manter maiores taxas de locomoção.

Portanto, entre as espécies de anuros parece haver diferentes respostas comportamentais após um desafio imune. Algumas espécies podem apresentar uma forte resposta imune, e com isso, maior depressão do comportamento (Llewellyn *et al.*, 2011; Braga, 2013), e ao contrário, outras espécies podem apresentar um sistema imunológico deprimido mantendo sua capacidade de realizar atividades mesmo quando doentes (Llewellyn *et al.*, 2012; Brow e Sine, 2014; Gardner *et al*., 2020). Assim, dependendo das condições ambientais, um determinado perfil de

resposta imune pode conferir uma vantagem ecológica a algumas espécies, como os invasores em relação às espécies não invasoras. Em um contexto evolutivo, este cenário pode levar as espécies invasoras a expandir ainda mais sua área colonizada, aumentando a alocação de recursos para dispersão, crescimento e reprodução, características que podem ter um custo para a função imunológica (Brown e Shine, 2014; Gardner *et al.,* 2020).

Além das respostas imunes, o comportamento alimentar também pode ser influenciado pelo ambiente, como detalhado no capítulo 1. Particularmente a obtenção de alimento geralmente envolve a busca por presas, principalmente em predadores mais ativos, e o tipo de habitat influencia diretamente devido as características da paisagem (e.g. distâncias até fontes de alimento, solos mais difíceis de percorrer) (Bennett & Licht, 1973; Emerson, 1976; Taigen & Pough, 1981; Taigen & Pough, 1983; Pough, 1984; Taigen & Pough, 1985; Pough *et al*., 1992; Deban *et al.,* 2001; Pough *et al*., 2008). Assim, a abordagem que utilizamos nesta pesquisa para estudar o impacto da infecção no comportamento alimentar de *A. catesbeiana* levou em consideração os possíveis custos ambientais que estes animais poderiam encontrar na natureza para se alimentar, inclusive antes da manipulação invasiva dos indivíduos. Os resultados mostraram que os indivíduos do grupo controle conseguem manter o comportamento alimentar mesmo com o obstáculo para a obtenção das presas, mas aqueles injetados com LPS reduziram drasticamente a alimentação. Essa abordagem amplia o panorama de análise levando em consideração respostas variadas, diferente do que é apresentado tradicionalmente na literatura, onde alimentação geralmente é oferecida ad libitum (Llewellyn *et al*., 2011).

Diante do contexto apresentando, concluímos que se torna importante investigar quais os impactos das respostas imunes no comportamento de anfíbios em laboratório, levando em consideração a história natural das diferentes espécies, para que seja possível fazer inferências sobre estes animais na natureza. Estes estudos permitem manter o vínculo entre a fisiologia, ecologia e conservação e podem demonstrar as diferenças entres as espécies no contexto das repostas dos anfíbios a doenças emergentes, demonstrar as implicações da ativação do sistema imune no comportamento e, com isso, contribuir ainda para evidenciar fatores que levam ao sucesso de espécies invasoras.

Finalmente, nossa pesquisa conclui que *A. catesbeiana* apresenta redução do comportamento alimentar e locomotor e que anuros das espécies *X. laevis* e *X. allofraseri* apresentam redução do comportamento locomotor após infecção simulada por injeções de LPS. Além disso, concluímos que o ambientem também tem influência na realização do comportamento alimentar, portanto é possível pensar que no campo os custos relacionados as

características da paisagem podem diminuir ou inibir a alimentação em algumas espécies de anuros quando doentes. Adicionalmente, esta pesquisa é compatível com a ideia de que espécies invasoras apresentam menor influência da ativação do sistema imune na locomoção em relação às espécies não invasoras (Llewellyn *et al*., 2012; Brow e Sine, 2014; Gardner *et al*., 2020). Esta pesquisa também corrobora resultados observados em outras espécies de anuros, as quais indicam a ocorrência da depressão comportamental neste grupo (Llewellyn *et al*.,2011; Braga, 2013), e acrescenta evidências sobre o efeito de uma doença (e em consequência a ativação da resposta imune) nas respostas comportamentais de anuros.

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Anexos e Apêndices

Capítulo 1.

Imagem 1: Indivíduo da espécie *Aquarana catesbeiana* usado como modelo de estudo (animal juvenil, 60g).

Imagem 2: Arenas onde os animais eram testados.

Imagem 3: Arena e cubo de acrílico onde as presas eram colocadas.

Imagem 4: Animal dentro do cubo de acrílico se alimentado.

Capítulo 2 e 3.

Imagem 1: Indivíduo da espécie *Xenopus laevis* usado como modelo de estudo (animal macho adulto, 25g).

Imagem 2: Indivíduo da espécie *Xenopus allofraseri* usado como modelo de estudo (animal fêmea adulto, 15g).

Imagem3: Pista usada para medir a resistência locomotora.

Imagem 4: Plataforma piezoelétrica utilizada para medir a força de salto.

Imagem 5: Tanques utilizado para medir os movimentos voluntários.