Letícia Regina do Amaral Braga

Respostas comportamentais e metabólicas à ativação do sistema imune por injeção de lipopolissacarídeo (LPS) em *Scinax* gr. *perpusillus* (Anura: Hylidae)

Behavioral and metabolic responses to immune system activation by lipopolysaccharide (LPS) in *Scinax* gr. *perpusillus* (Anura: Hylidae)

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DEDICATÓRIA

Aos meus pais, que sempre estimularam
meu interesse pela Ciência mesmo sem perceber.

EPÍGRAFE

Eu não tenho filosofia: tenho sentidos...

Se falo na Natureza não é porque saiba o que ela é.

Mas porque a amo, e amo-a por isso,

Porque quem ama nunca sabe o que ama

Nem por que ama, nem o que é amar…

Alberto Caeiro

.

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SUMÁRIO

Esta dissertação está composta por um resumo inicial, seguido de uma introdução geral em português do tema abordado. Em seguida, encontra-se um artigo científico redigido em inglês e no formato da revista a que será submetido "*South American Journal of Herpethology*" contendo introdução, material e método, resultados, discussão e as figuras. Ao final, encontra-se uma conclusão geral redigida em português. O resumo do artigo não foi apresentado por ser equivalente ao já apresentado no início da dissertação. A dissertação é fechada com as referências bibliográficas, que também são comuns ao artigo e aos textos adicionais em português, obrigatórios da dissertação.

INTRODUÇÃO GERAL

Os organismos devem possuir um sistema imune capaz de reconhecer e destruir imediatamente patógenos e parasitas invasores, porém uma resposta imunológica demasiadamente forte pode acarretar custos elevados para o organismo. A montagem de uma resposta imune e a manutenção de um sistema imunológico competente é considerada por muitos autores como sendo uma atividade de elevada demanda energética e nutricional que pode vir a realocar recursos de processos biológicos diversos como crescimento, reprodução, cuidado parental, locomoção, regulação da temperatura corpórea e reparação tecidual (Sheldon e Verhults, 1996; Lochmiller e Deeremberg, 2000).

Animais com sistema imune ativado podem exibir uma série de comportamentos típicos, como atividade locomotora reduzida, anorexia, diminuição na ingestão de água, redução do comportamento reprodutivo em fêmeas, além de uma elevação da taxa metabólica de repouso (Hart, 1998; Martin et al., 2003). Este conjunto de alterações comportamentais e fisiológicas decorrentes de uma infecção tem sido investigado mais frequentemente em vertebrados endotérmicos, e tem sido interpretado como uma evidência de um ajuste fenotípico integrado, que favoreceria a resolução rápida e eficiente do processo infeccioso (Eraud et al., 2005; Viney et al., 2005)

O objetivo deste estudo foi verificar possíveis alterações de variáveis fisiológicas e comportamentais em machos adultos de *Scinax* gr. *perpusillus* em resposta a um desafio imunológico provocado por injeção de LPS, componente da membrana externa de bactérias gram-negativas que é capaz de ativar o sistema imune do hospedeiro. As hipóteses testadas foram as de que, ao ser comparados com

indivíduos controle, injetados com solução salina, os indivíduos injetados com LPS apresentariam: 1) Maiores taxas metabólicas de repouso; 2) Menores taxas de consumo alimentar; 3) Maior proporção de respostas passivas à simulação da predação; 4) Redução da atividade locomotora voluntária.

RESUMO

Parasitas e patógenos impõem uma importante pressão seletiva sobre a história de vida dos hospedeiros. Os desafios impostos aos hospedeiros resultam em respostas fisiológicas e comportamentais pouco conhecidas para a maioria das espécies de anfíbios anuros. O objetivo deste estudo foi quantificar os efeitos da ativação do sistema imune via injeção de LPS (lipopolissacarídeo) em um hospedeiro ectotermo modelo, como *Scinax* gr. *Perpusillus*. LPS foi administrado em duas concentrações, sobre o comportamento alimentar, taxa metabólica de repouso, atividade locomotora espontânea e comportamento anti-predatório de machos adultos da perereca das bromélias: *Scinax* gr. *perpusillus*. O tratamento com LPS provocou redução na quantidade de alimentos ingeridos, confirmando o efeito anorexigênico deste componente, e aumento na atividade locomotora espontânea, porém não apresentou efeito sobre a taxa metabólica de repouso ou sobre o comportamento anti-predatório das pererecas.

ABSTRACT

Infectious agents ~~should~~ are believed to play an important role modulating the evolution of key physiological and behavioral aspects of life history of the hosts. The challenges posed by pathogens to hosts result in well characterized changes in feeding, locomotion, reproduction and body temperature in mammals and birds but not clearly understood in most ecthoterm hosts species, such as anuran amphibians. The aim of this study was to quantify the effects of an LPS-induced inflammaroty response on the feeding behavior, resting metabolic rates, spontaneous locomotor activity, and antipredatory behavior of adult males of the bromeliad tree-frog: *Scinax* gr. *perpusillus.* Treatment with LPS caused a reduction of food intake confirming the anorexigenic effect of this component and an increase in spontaneous locomotor activity, but had no effect on the resting metabolic rate or in the the antipredator behavior of S. perpusillus.

INTRODUCTION

Within the last decade, the appreciation that parasites and infectious agents represent important selection pressures driving the evolution of life history of their hosts gave rise to the field of ecological immunology (Sheldon and Verhults, 1996; Lochmiller and Deerenberg, 2000). Several studies have been emphasizing that trade-offs between immunity and other functions are directly associated to the evolution and maintenance of secondary sexual characters (Hamilton and Zuk , 1982), generate reproductive costs (Møller,1997) and cycles of population regulation (Lochmiller, 1996). However, in order to be involved in life-history trade-offs, the immune function has to be costly to the organisms in some way (Sheldon and Verhulst 1996; Lochmiller and Deeremberg, 2000). The chronic maintenance of a competent immune system and the acute immune response to an infectious agent have been considered by many authors as activities of high energy and nutritional demand, associated to the relocation of resources diverted from important biological processes such as growth, reproduction, parental care, locomotion, regulation of body temperature and tissue repair (Sheldon and Verhults and 1996; Demas et al., 1997; Lochmiller and Deerenberg, 2000). The energy cost related to immune activation involves the production of immune cells, cytokines and antibodies and removing large amounts of antigen (Jin et al., 1995; Viney, 2005).

Simulated experimental infections have been frequently used to elucidate mechanisms and fitness implications of these energetic and nutritional trade-offs involving immune activation (Moret and Schimd-Hempel, 2000; Bonneaud et al., 2003; Martin et al., 2003). The injection of lypopolissacaride (LPS), a major component of the outer membrane of gram-negative bacteria, has been shown to increase metabolic rates

(Sherman and Stephens, 1998; Llewellyn et al, 2011), and decrease feeding rates (Webel et al., 1998; Llewellyn et al., 2011), reduce voluntary locomotor activity (Engeland et al., 2001; Lee et al., 2005), and lead to a poor reproductive outcome (Bonneaud et al., 2003). The physiological and behavioral components of sickness represent a highly organized strategy of endothermic organisms to fight infection (Hart, 1988). This strategy, generally referred as 'sickness behavior', is triggered by the pro-inflammatory cytokines produced by activated neutrophils and monocytes – macrophages in contact with invading microorganisms. These cytokines include mainly interleukin 1 (IL-1α and IL-1 β), IL-6 and tumour necrosis factor (TNF-α) (Konsman et al., 2002). The studies on components of sickness behavior have been conducted mainly using mammals and birds as models and, much less frequently, amphibians. The increased use of ectothermic vertebrates as models, however, would provide valuable insights on the diversity of physiological and behavioral responses followed an immune system activation, as well as its consequences to fitness.

In the present study, we used tree frogs (*Scinax* gr. *perpusilus*) inoculated with LPS at two different concentrations to test the following hypotheses:

1. Immune system activation reduces feeding rate. This prediction is sustained by the observation that, during immune challenge, the increased cellular production of the neurocytokine interleukin IL-1β results in decreased appetite in vertebrates (Bretdibat et al., 1995; Johnson, 2002).
2. Immune system activation increases resting metabolic rate. Such increased metabolic rates would be the result of increased cellular activity of the immune system (Jun et al., 1995; Lochmiller and Deerenber, 2000; Llewellyn et al., 2011).

3. Immune system activation should reduce voluntary locomotor activity. A reduced locomotion is a common sickness behavior and has been interpreted as an adaptive response to both illness and infection (Hart, 1988; Engeland et al., 2001). Previous experiments have found that animals injected with LPS show reduced locomotor activity and social interactions (Kozak et al., 1994; Llewellyn et al., 2011)
4. Immune system activation is expect to increase the frequency of passive antipredatory responses. Most of the studies analyzed only the effect of LPS on voluntary movements of individuals . However, it would be of great interest to investigate how an individual subjected to a physiological challenge responds to an aversive external stimulus of clear relevance to fitness, such as a predator attack. In this way, we also investigated the influence of LPS on the frequency of active and passive responses to a simulated predator attack.

Previous studies have shown that the response to simulated predation events can be highly varied, and depends on the physiological conditions of the individual, type of stimulus applied, temperature, and structural complexity of the environment (Ducey and Brodie, 1983; Brodie et al.., 1991; Aubert, 1999; Eilam et al., 1999; Gomes et al., 2002). Based on the inhibitory effect of LPS injection on the voluntary movements, we predicted that passive responses (freezing and death feigning) to a simulated predation would increase in frequency compared to active responses (jumping).

MATERIAL AND METHODS

SPECIMEN COLLECTION AND HUSBANDRY

Scinax gr. *perpusillus* are small bromeliad frogs (mean male mass 0,35 g) endemic from the Atlantic Forest, and can be found in large congregations from the South of the São Paulo State to Espírito Santo (Peixoto, 1986; Bell et al., 2012). Males are territorial and call from leaves of bromeliads and the females lay eggs in water that accumulates in the axils of these plants (Peixoto, 1986; Bell et al., 2012). We collected 29 adult males *of Scinax* gr. *perpusillus* at Ecological Station of Boracéia (Salesópolis/SP - 23°16´S, 48°24´W) from September to December 2011 (Figure 1). All animals were individually housed in perforated plastic containers 190 ml with access to free water, dry ground and a hiding place. Animals were transported to the laboratory at the University of São Paulo and placed at room temperature (20.99 °C ± 0.94; mean ± SD) and natural light cycle. Captive frog were fed three times a week wit*h Drosophila sp*. and newly hatched crickets prior the beginning of the study. Animals were fasted for 4 days before the beginning of any data collection

Immune challenge

Individuals were distributed in three groups ensuring a homogeneous distribution of individual body masses between the groups. Lipopolysaccharide (LPS), the major component of the outer membrane of gram-negative bacteria, was used to stimulate an immune response of the individuals of *Scinax* gr. *perpusillus*. Injections of LPS are able to activate both innate and humoral immune process, an effect mainly caused by the specific recognition of lipid A, one of the constituents of LPS (Kluger,

1991). LPS purified by phenol extraction (from the serotype of E. coli 0111: B4, Sigma Aldrich) was diluted in saline (PBS) and injected in animals. All subjects were injected into the inguinal portion of the right side of the body using a 30-gaugue needle attached via a cannula to a Hamilton syringe (10 µL). Individuals from the control group were injected with PBS; Group 1 individuals were injected with LPS at the dose of 2 µg g^{-1} body mass and Group 2 individuals were injected with LPS at the dose of 20 µg g^{-1} body mass. The solutions were injected at a volume of 0,002 mL g^{-1}These doses were established based on previous studies with other anuran species (Llewellyn et al., 2011).

MEASUREMENT OF METABOLIC RATES

The metabolic rates of the tree frogs were determined as the rates of oxygen consumption using intermittent-flow respirometry (Bartholomew and Lighton, 1986). The measurement routine began 24 h after PBS and LPS injections, and was repeated in the sixth and eleventh day after injections. Measurements were conducted during the period of inactivity (9h to 19h). Frogs were weighted before and after the BMR measurements and mean values were used for calculations.

Each frog was placed inside a small pexiglass metabolic chamber (142 mL) and kept in the dark in an incubator at 20 °C. Animals were maintained in these conditions for one hour before measurements to allow them to assume a resting posture and to equilibrate with the experimental temperature. During this period, humidified air was pumped through the chambers to prevent dehydration of the animals and changes in concentration of respiratory gases, and a piece of moistened cotton was added to the chamber to maintain humidity conditions.

At the start of data collection, a constant air flow (200ml/min) was pumped into the chambers for a period of 10 minutes in sequence, a period sufficient for a complete renew of air volume of each chamber. After this period, the chambers were sequentially closed for 240 minutes. Subsequently, the chambers were sequentially opened and air from outside was pumped for 10 minutes at 200 ml. min^{-1} by the use of the air pump (SS3- Sable Systems). The air leaving the chamber was first conducted to a filter containing the sequence Drierite/Ascarite/Drierite for complete absorption of water vapor and carbon dioxide and ultimately to an oxygen analyzer PA -1 (Sable Systems). The software Expedata (Sable Systems) was used for the data acquisition, storage and data analysis.

The rates of oxygen consumption were calculate as the area under the curve of oxygen concentration versus time, multiplied by the air flow used during the respirometry and divided by the time that chambers remained closed (Bartholomew and Lighton, 1986). The values were then converted to standard condition for temperature and pressure (Dejours, 1975) and expressed as the amount of oxygen consumed per gram of body mass per hour (mL $O_{2.}$ g^{-1} . h^{-1}). During the measurements, the metabolic chambers were monitored by video-camera (Vtek) connected to a recorder (H.264.Network.DVR). These videos were subsequently analyzed in Windows Movie Maker and data from individuals that have moved during the experiment were not considered for analyses.

EFFECT OF IMMUNE SYSTEM ACTIVATION ON FEEDING BEHAVIOR

After measurements of metabolic rates, animals were removed from metabolic chambers and returned to the individual containers. Tests of feeding performance were conducted during their period of activity. The hiding place and water were removed from the containers, and moistened cotton was added to prevent desiccation of the animals. Animals were exposed to fifteen newly hatched crickets in the plastic containers for 30 minutes. After this period, the remaining crickets were recorded and removed. Similar feeding trials were repeated in the sixth and eleventh days post-injection.

EFFECT OF IMMUNE CHALLENGE ON ANTIPREDATOR BEHAVIOR

To standardize and quantify the effect of immune system activation on the occurrence of antipredator behavior, we used an artificial mechanism to simulate a hostile stimulus in the laboratory. Frogs were placed individually into labeled 5 ml syringes with a hole was provided in its extremity. A rubber stopper was used to close this opening. The syringes were placed inside a temperature control chamber (Fitotron/Eletrolab) for 1h at the temperature of 20 ° C to reduce handling stress effects and to equilibrate animal body temperature with the environment.

For each trial, a syringe containing a resting frog was carefully placed on the floor of the climatic chamber. All manipulation was performed by the same experimenter to reduce variation in the stimulus applied, and care was taken to

minimize disturbance of the frogs. The rubber stopper was removed, and frogs were ejected by a sudden but gentle push from the plunger inside the syringe. If the frog jumped away, a landing spot on the floor was marked. If the frog jumped a second time, another landing spot was marked on the floor. If the animal exhibited a passive response to the initial stimulus, or if it stopped for more than 5 sec after a jump, we applied a second stimulus by gently touching the rear end of the frog with the experimenter's finger. If the frog jumped, we quantified the behavior by measuring the distance to the nearest 1 cm, jump distances were summed, and the total was referred to as fleeing distance; if frog did not move, we ended the trial. We also classified the animal according to its behavior during the trials: 0 = passive antipredator response and 1 = active antipredator response. Similar trials were repeated in the sixth and eleventh day postinjection.

EFFECT OF IMMUNE CHALLENGE ON SPONTANEOUS LOCOMOTOR ACTIVITY

During the 240 minutes period animals remained inside de metabolic chamber we quantified the spontaneous locomotor activity. We used the videos recorded during the respirometry to quantify the amount of time (seconds) the individuals spent in motion. We also quantified the frequency of movements during the same period. We classified the animal according to its behavior during the respirometry: 0 = didn't move and 1 = moved. Position adjustment was not classified as movement.

STATISTICAL ANALYSIS

To access the effects of LPS injection on patterns of feeding behavior on the three consecutive trials, we conducted Kruskal-Wallis non-parametric tests. To access the effects of LPS injection on metabolic rates, given that we excluded data from the individuals that moved inside the chamber, we conducted one-way ANOVA separately for each group. The antipredator behavior data was analyzed for the first and second stimuli independently, then as a pooled data. We performed the nonparametric Cochran's Q test, which is used for nominal data that can be coded as 0 or 1, to compare the proportion of active responses among the three-days repeated trials for each stimulus (first or second). The McNemar test, also used for dichotomous data, was performed to compare the proportion of active responses between the first and second stimulus within each trial day. The purpose of this test was to verify whether the animals responded more actively to the first or second stimulus.

To test whether there was a difference in the proportion of responses between the groups (Control and LPS), we performed chi-square tests for each stimulus, in each trial. The purpose of this test was to determine whether the proportion of active responses was modified by LPS injections. We conducted a Kruskal-Wallis test to verify if the distance jumped was modified by treatment with LPS on the trials days. To verify the total time LPS individuals spent engaged in ambulatory exploration inside the metabolic chambers we used non-parametric test of Kruskal-Wallis. We performed chi-square tests to verifiy if LPS injected individuals different from control in frequency of moviments inside metabolic chambers. We eliminated outliers from the analysis indentified by SPSS in a step of 1.5 × Interquartile Range.

RESULTS

EFFECT OF IMMUNE CHALLENGE ON FEEDING BEHAVIOR

LPS affected the amount of crickets consumed (Kruskal-Walli, $P \leq 0{,}002$). Particularly, treefrogs injected with LPS ate fewer crickets than tree-frogs injected with saline in the first day of trial (Pairwise Comparison, $P \leq 0{,}01$), and this effect was independent of the doses of LPS injected (Pairwise Comparison, $P = 1{,}00$). Although the proportion of prey consumed has increased in all groups after injection, individuals injected with LPS tended to eat less crickets than individuals injected with saline throughout the days of test (Figure 1).

EFFECT OF IMMUNE CHALLENGE ON METABOLIC RATE

The treatment with LPS did not affect resting metabolic rates in any of the trials (ANOVA, $P \geq 0{,}139$).

EFFECT OF THE IMMUNE CHALLENGE ON THE ANTIPREDATOR RESPONSE

The frequency of active responses against the predatory stimuli did not change among the three-day repeated test (Cochran Q, $P \geq 0.368$). Additionally, the proportion of active responses was not different to first and second predatory stimuli, within each trial (McNemar, $P \geq 0.219$). LPS injection had no effect on the proportion of active responses to first and second predatory simulation in any of the three days (X^2, $P \geq 0.160$). Additionally, treatment did not affect total distance traveled by the individuals

in response to the stimulus applied in any of the three days of trial (Kruskal-Wallis, $P \geq 0{,}176$; Figure 4)

EFFECT OF THE IMMUNE CHALLENGE ON VOLUNTARY LOCOMOTION

LPS affected voluntary locomotion inside the metabolic chambers (Kruskal-Wallis, $H = 8{,}751$, $P = 0{,}013$). Particularly, tree-frogs injected with the higher dose of LPS moved for longer periods than tree-frogs injected with lower doses of LPS or saline at the first day of trial, 24 hours after injection (Pairwise Comparison, $P = 0{,}014$; Figure 2). Although a higher proportion of tree-frogs injected with the higher doses of LPS tended to move more frequently inside the metabolic chambers throughout the trial days (Figure 3), differences among groups were not significant ($X^2 = 4{,}409$, $P \geq 0.11$).

DISCUSSION

Males of *Scinax* gr. *perpusillus* injected with LPS showed decreased food intake independently of the doses, a result that corroborate previous studies, including with anurans (Konsman et al, 2002; Hart, 1998; Llewellyn et al, 2010). Evidence of the involvement of cytokines released in response to LPS injection in the anorexic behavior comes from studies where they were injected both peripherally and in the central nervous system, thereby inhibiting food intake (Bluthé et al., 1992; Asarian and Langhans, 2005). Even though is well known that the pro-inflammatory cytokines play a key role in the expression of anorexic behavior by acting mainly in areas of the central

nervous system, such as the hindbrain, the forebrain and basal raphe in the midbrain (Nakamura et al., 2001; Langhans, 2007), the mechanisms of action of each cytokine is not entirely clear. Although these studies have been carried out on mammals, especially mice, it is probable that many of the molecular mechanism of cytokine action and consequently their behavioral and physiological responses are shared with other vertebrates (Hart, 1988; Dantzer and Kelley, 2007; Adelman and Martin, 2009).

Contrary to initial prediction, the resting metabolic rates of *Scinax* gr. *perpusillus* were not affected by LPS administration. Depending on the challenge posed by the pathogen to the host and on its health state, the immune system may require a relocation of energy and/or specific nutrients to assemble, maintain and activate the immune response (Klasing, 2004). Sheep erythrocytes (SRBC) and phytohemagglutinin (PHA) resulted in increased metabolic rates birds, revealing the energy cost involved in mounting of the immune response (Ots et al., 2001, Eraud et al., 2005, Lee et al., 2005). In contrast, resting metabolic rates did not change when guinea pigs were injected with sea slug hemocyanin (KLH) (Pilorz et al., 2005), or when blue tits were immunized with vaccines containing diphtheria and tetanus (Svensson et al., 1998). It is possible that these apparently conflicting results evidence that energy cost of the immune response may vary according to the type and intensity of infection, as well as to the components of the immune systems activated. Related species of birds show increased metabolic rates and decreased body mass when injected with sheep red blood cells (SRBC), but not when challenged by a diphtheria–tetanus vaccine (Svensson et al., 1998; Ots et al., 2001).

The difference between these two treatments may be related with the lower cost of producing the immunoglobulines in response to diphtheria-tetanus vaccine when

compared to the mobilization of T and B lymphocytes as an immune response against SRBC (Klasing and Leshchinsky, 1999). It must also be considered that energetic costs of immune system activation may be reflected in parameters other than resting metabolic rate. White-footed mice (*Peromyscus leucopus*) injected with SRBC, for example, did not show increased metabolic rates, but presented reduced masses of testes and small intestine (Derting and Compton, 2003). According to these authors, these results demonstrate energy relocation from small intestine and testes to mounting an immune response, with no detectable changes in resting metabolic rates. Unfortunately, our data do not allow testing the hypothesis of energetic costs relocation with parameters alternative to metabolic rates.

The influence of time post-treatment on the metabolic response to infection also deserves consideration. Llewellyn and collaborators (2011) detected increased metabolic rates in cane toads (*Rhinella marinus*) in response to LPS injection at both 25 and 32 ºC when respirometry measurements were taken immediately after injection. Sherman and Stephens (1998) studied the effects of LPS on the metabolic rate of cane toads 7h after injection and only detected elevated rates of oxygen consumption at higher temperatures (32 °C). Kakizaki et al. (1999), reported a temporal profile of IL-6, TNF-α after intraperitoneal administration of LPS in rats, and showed peak levels for these cytokines in the plasma 2.5 h and 1.5 h postadministration, respectively. It is possible that that metabolic changes in response to LPS are temporally associated to an early rise in cellular activity due to this cytokine peaks. Given that metabolic rates in *S.* gr. *perpusillus* were measured only at 20 ^{o}C, and measurements started only 24h post-administration, it is possible that we missed the peak of metabolic rates followed the immune challenge in these tree-frogs.

Although previous studies observed that LPS reduces voluntary locomotor activity in several groups of tetrapods, including anurans (Hart, 1988; Lee et al., 2005; Llewellyn et al., 2011), individuals of *Scinax* gr. *perpusillus* injected with the higher doses of LPS tended to move more frequently and moved for comparatively longer periods inside the metabolic chambers. However, it seems that the effect of LPS on voluntary locomotion interacts with novelty. Mice injected with LPS and put in a novel open field showed an attenuated response to LPS when compared to injected mice that were put back to the environments where they were habituated (Lacosta et al., 1999; Engeland et al., 2001). These results strongly suggest that the novelty of the environment reduced the inhibiting effects of LPS on voluntary activity. According to Llewellyn and collaborators (2011), individuals of *Bufo marinus* had their activity levels reduced by LPS injection, but the authors allowed these toads to explore enclosures for two nights before the LPS injection. Aubert (1999) suggested that cytokine-induced behavioral changes reflect motivational reorganization, so that the ability to respond to salient environmental stimuli remains intact, even though sick individual typically exhibits decrements in locomotor activity. In cases in which external cues become relevant enough, the sickness behavior will be interrupted so the individual can respond properly to environmental demands.

Exposure to a novel environment is stressful and typically causes a rise in adrenocorticotrophin (ACTH) and corticosterone plasma levels in rodents (Frederic el at., 1997; Bakshi and Kalin, 2000). Given that these substances inhibit cytokine release (including IL-1β and TNF-α), this mechanism might contribute to an attenuation of the effects of LPS on locomotor activity in a novel situation (Reichlin, 1993). Moreover, placing an animal in a novel environment increases IL-6 levels systemically, and this may reduce the inflammatory response by inhibiting, for instance, the synthesis of both

Il-1β and TNF-α (Barton, 1996; Willet et al., 2007). Previous studies reported that mice treated with IL-6 exhibited a significant and doses-dependent increase in ambulatory exploration of new environments (Zalcman et al., 1998) Thus, several putative mechanisms might inhibit the ability of LPS to reduce locomotor activity in novel environments, but additional studies are necessary to understand better their contribution to the interaction between novelty and infection on voluntary activity.

Individuals of *S.* gr. *perpusillus* can display a number of strategies of escape when threatened, including feeing death, immobility and jumps (Gomes et al., 2002). Previous studies have shown that the frequency of expression of different patterns of anti-predator responses depends on the type of stimuli to which individuals are subjected to as well as environmental and physiological conditions (Eilam et al., 1998; Gomes et al., 2002). Individuals of *Scinax* gr. *perpusillus* injected with LPS showed no significant reduction in the proportion of active responses (jumps) in response to simulated predation, both for the first and the second stimuli applied. The experiment was designed to observe the effect of LPS on the behavior forced by a hostile stimulus, rather than the more frequently studied pattern of spontaneous locomotion. Accordingly, the choice of standard behavioral response (active or passive) by the individual tested in response to the applied stimulus may determine its survival (Gomes et al., 2002). Thus, it is possible that individuals are able to, through changes in motivational state, momentarily breaking the behavior of immobility, giving precedence to the pattern of escape in response to the urgent need to escape from a predator (Aubert, 1999)

Conclusion

We were able to observe that LPS inhibited feeding behavior in males of *S.* gr. *perpusillus* and did not affect antipredator behavior. Additionally, our results showed that the higher doses of LPS increased voluntary locomotion, although this result may be influenced by interaction with environmental novelty in a non-controlled way. LPS did not affect resting metabolic rates, but we might have missed an acute and earlier effect. Additional experiments are necessary to explore the effects of LPS on voluntary locomotion and resting metabolic rates in *S.* gr. *perpusillus.* Although there are some studies that investigate the influence of the immune system activation via LPS injection in anurans on behavior and physiology (Sherman et al., 1991; Sherman and Stephens, 1998; Llewelyn et al., 201i; Llewelyn et al., 2011) the vast majority are still concentrated in mammals. We emphasize that more studies using other phylogenetic groups are necessary to increase our knowledge about the mechanisms and evolution of the expression of of sickness behavioral patterns in ecologically relevant contexts.

FIGURES

Figure 1: Male of *Scinax* gr. *perpusillus* collected at the Estação Biológica de Boracéia (Salesópolis/SP - 23°16´S, 48°24´W). Photo: Letícia Braga

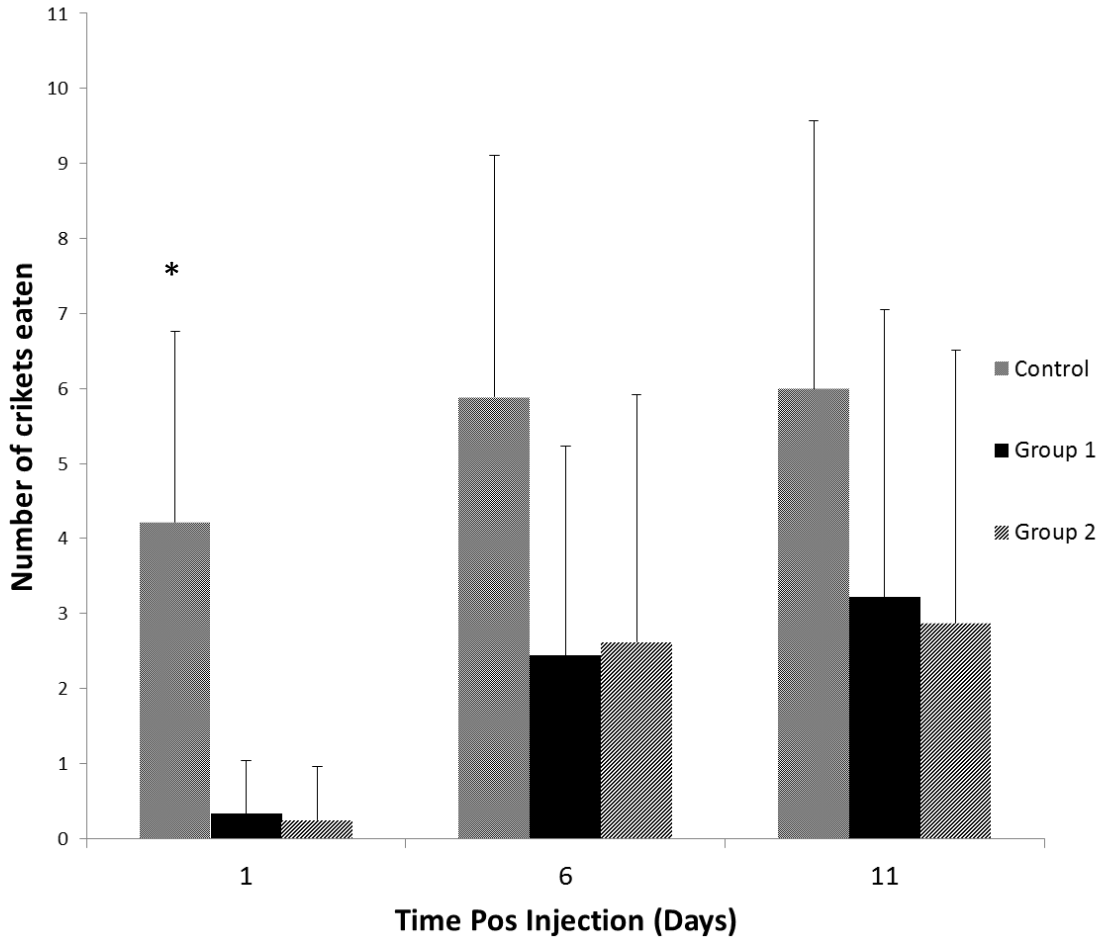

Figure 2: Number of crickets consumed (average and standard deviation) by males of *Scinax* gr. *perpusillus* treated with saline (Control, N= 9), 2µg LPS/ g (Group 1, N = 10), and 20µg LPS/g (Group 2, N = 10). Tests of feeding behavior were performed in the first, sixth and eleventh days after saline and LPS injections. The asterisks indicate statistically significant differences (at least P< 0,05).

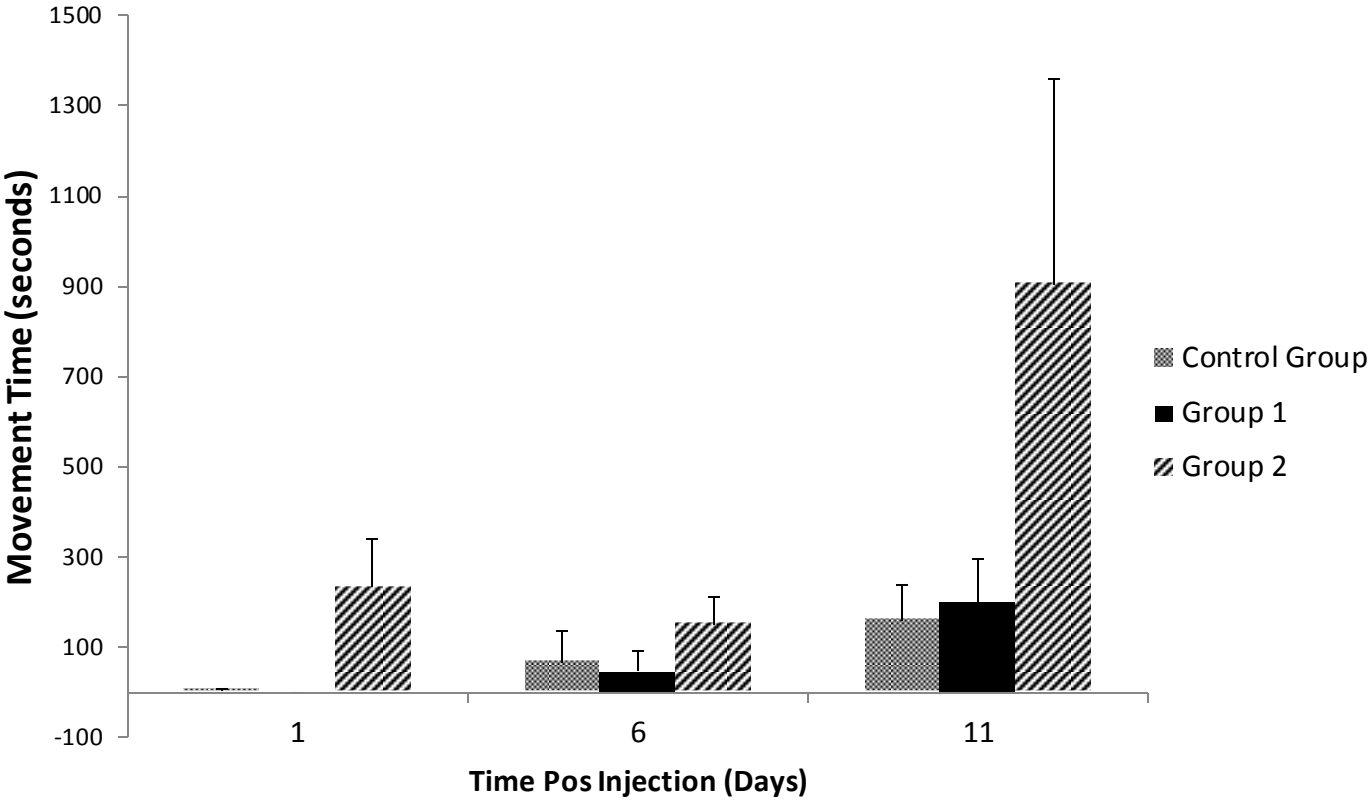

Figure 3: Movement time (average and standard deviation) for males of *Scinax gr. perpusillus* trated with saline (Control; LPS Group 1 = 2µg LPS/ g; Group 2 = 20 µg LPS / g). Tests were repeated in the first (Control: N = 8, Group 1: N = 8, Group 2: N = 9), sixth (Control: N = 4, Group 1: N = 6, Group 2: N = 7), and eleventh (Control: N = 7, Group 1: N = 8, Group 2: N = 10) days after saline and LPS injections. The asterisks indicate statistically significant differences (at least P< 0,05)

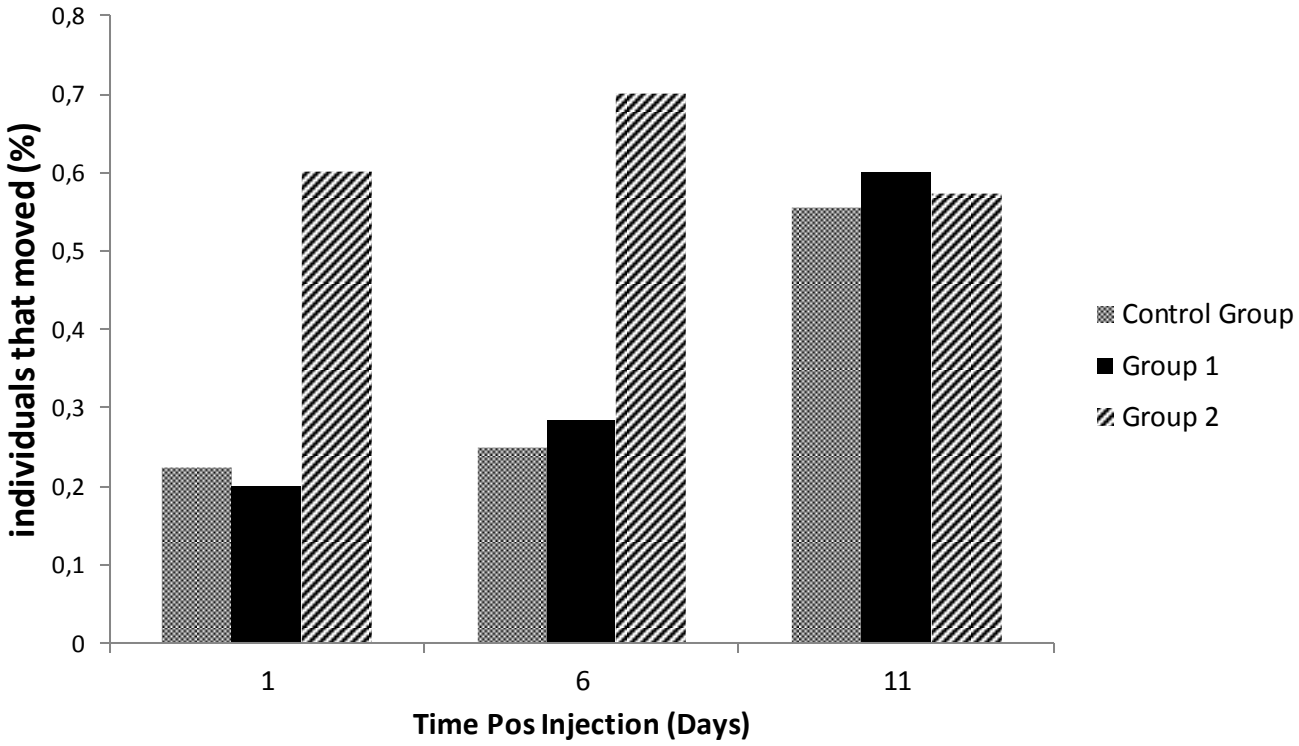

Figure 4: Frequency of males *of Scinax gr. perpusillus* that moved per group during the respirometry (Control = saline; LPS Group 1 = 2µg LPS/ g; Group 2 = 20 µg LPS / g). Tests were repeated in the first (Control: N = 9, Group 1: N = 10, Group 2: N = 10), sixth (Control: N = 4, Group 1: N = 7, Group 2: N = 7), and eleventh (Control: N = 9, Group 1: N = 9, Group 2: N = 10) days after saline and LPS injections.

CONCLUSÕES GERAIS

Neste trabalho concluímos que a injeção de LPS nas dosagens de 2 µg/g e 20 µg/g reduziu a ingestão de alimentos em machos de *Scinax* gr. *perpusillus*. Adicionalmente, o tratamento com LPS não afetou a proporção de respostas ativas e passivas frente à simulação predatória. Observamos que a injeção da dose mais elevada de LPS provocou um aumento da atividade locomotora voluntária dos indivíduos, embora este resultado possa ter sido influenciado pela interação do tratamento com uma novidade ambiental não controlada experimentalmente. Não detectamos alterações da taxa metabólica de repouso em resposta ao tratamento com LPS, porém é provável a ocorrência de um pico de elevação metabólica anterior ao período amostrado. Novos experimentos são necessários para se explorar os efeitos do LPS sobre a locomoção voluntária e sobre a taxa metabólica de repouso em *Scinax* gr. *perpusillus*.

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