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MAGNITUDE DO CUSTO ENERGÉTICO DA ATIVAÇÃO DO SISTEMA IMUNE EM MORCEGOS: EFEITOS SAZONAIS E DA RESTRIÇÃO ALIMENTAR

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Dissertação apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Zoologia.

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TÍTULO DA DISSERTAÇÃO: Magnitude do custo energético da ativação do sistema imune em morcegos: efeitos sazonais e da restrição alimentar

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“My mind seems to have become a kind of machine for grinding general laws out of large collections of facts…”

(Charles R. Darwin)

RESUMO

Um dos componentes da resposta imune inata é a resposta de fase aguda (RFA), a qual tem como objetivo reestabelecer a homeostase e promover a cura de um organismo através de uma serie de alterações fisiológicas que podem acarretar num alto gasto energético. Algumas das alterações são aumento da taxa metabólica, temperatura corpórea (febre) e granulócitos no sangue (eosinófilos, basófilos e neutrófilos). Os animais podem também apresentar anorexia, diminuição da atividade e perda de massa corpórea, minimizando o gasto de energia durante a ativação da RFA. Em situações de aumento da demanda de energia e diminuição da ingestão calórica, os organismos estão sujeitos a uma deficiência nas reservas energéticas, a qual poderia dificultar o combate efetivo contra patógenos. Nos induzimos a ativação da RFA em morcegos frugívoros da espécie *Carollia perspicillata* por meio de injeção de lipopolissacarídeo (LPS). Quantificamos o custo energético da RFA e como o mesmo é afetado pela sazonalidade (inverno e verão) e disponibilidade de alimento (restrição alimentar e alimentados *ad libitum*). A perda de massa corpórea durante a RFA foi significativa em ambas as estações, porém apenas em morcegos em restrição alimentar, provavelmente devido a que nos morcegos em restrição alimentar foi necessária a mobilização de reservas energéticas, enquanto que morcegos alimentados *ad libitum* cobriram parte do custo da RFA através da mobilização de carboidratos ingeridos horas antes do experimento. Não observamos febre nem leucocitose, contudo, a razão neutrófilo/linfócito (N/L) teve aumento significativo unicamente em morcegos em restrição alimentar e durante o inverno. Este último parâmetro é também um indicador de estresse, o que sugere que à combinação de baixa disponibilidade de alimento e aumento na demanda energética para termoregulação durante o inverno pode ser uma situação estressante para estes morcegos. Aparentemente os morcegos deste grupo conseguiram ativar uma RFA, porém não foi possível mensurar se a efetividade da mesma foi comprometida. O custo energético da RFA não diferiu entre grupos experimentais. Nossos dados mostram que o custo da ativação da RFA em *C. perspicillata* é baixo (1% do orçamento diário de energia). Portanto, mesmo em situações onde existe maior demanda de energia e baixa disponibilidade de alimento, *C. perspicillata* ainda é capaz de ativar uma RFA, e consequentemente, manter a imunocompetência sem comprometer o seu orçamento de energia.

Palavras-chave: Custo energético, Resposta imune, Taxa Metabólica, Sazonalidade, Restrição alimentar, Resposta de fase aguda

ABSTRACT

One of the innate immune response components is the APR, which can be activated by trauma, infection, stress and inflammation. The APR activation aims to restore homeostasis and health of an organism exposed to the before mentioned events, through several physiological modifications that may result in high energetic cost. Some modifications are increase in metabolic rate, fever and granulocytes in the blood (eosinophils, basophils and neutrophils). Animals may also show anorexia and body mass loss, reducing the energy expenditure during the APR activation. In situations where animals are exposed to increase energy demand and decrease caloric intake, they could experience a deficiency in energy reserves, which may hamper the effectiveness of an immune response. We induced the APR activation in the Short-tailed fruit bats (*Carollia perspicillata*) by LPS injection. We quantified the energetic cost of the APR and how it is affected by seasonality (winter and summer) and food availability (food restriction and fed *ad libitum*). Body mass loss during the APR activation was observed in both seasons, but only in bats in food restriction, probably due to the mobilization of energy reserves, while bats fed *ad libitum* covered part of the APR cost by the mobilization of carbohydrates from the recent ingested food. Bats did not show fever or leukocytosis, nevertheless, we found increase in N/L ratio when bats were in food restriction and during winter. This parameter is also a stress indicator, so probably the combination of limited food availability and increase in the energy demand for thermoregulation during winter, may be a stressful situation for this bat. It seems that bats from this group were able to activate the APR, nevertheless we could not measure if the effectiveness of the response was jeopardized. The energetic cost of the APR activation did not vary between experimental groups. Our results showed that for *C. perspicillata*, activating the APR entails low energy cost (1% of the daily energy budget). Thus, they seem to be capable of activating an immune response even in situations that demand more energy and when food availability is limited, and consequently preserve immunocompetence without compromising their energy budget.

Key-words: Energetic cost, Immune response, Metabolic rate, Seasonality, Food restriction, Acute phase response.

SUMÁRIO

INTRODUÇÃO GERAL

A imunocompetência reflete a capacidade dos organismos de ativar e manter uma cascata de respostas imunológicas apropriadas para combater patógenos aos quais são expostos ao longo de seus ciclos de vida, e assim, assegurar a manutenção da homeostase frente aos potencias efeitos deletérios causados por doenças (Costantini and Møller, 2009; Demas et al., 2011; Råberg et al., 1998). A primeira linha de defesa contra patógenos é a resposta inata, durante a qual é induzida inflamação local, através da produção de citocinas inflamatórias. Caso uma leve inflamação não seja suficiente para restaurar a saúde do organismo, inicia-se uma inflamação sistêmica, acarretando um alto gasto de energia (Lee, 2006). Durante a inflamação sistêmica aumenta a produção de proteínas de fase aguda pelo fígado, ocorrem mudanças no metabolismo de energia e nutrientes, diminuem as atividades sociais e de locomoção, e é apresentado comportamento de anorexia (diminuição na ingestão de alimento) e febre (Hart, 1988; Klasing and Leshchinsky, 1999). Estas alterações comportamentais e fisiológicas caracterizam a resposta de fase aguda (RFA) que tem como objetivo acelerar o processo de cura, mas que requerem um alto gasto de energia e nutrientes para ser ativada (Klasing and Leshchinsky, 1999; Lee, 2006; Sheldon and Verhulst, 1996). Por esta razão, tem sido postulado que a ativação do sistema imune inato, o menos em aves e mamíferos e especificamente da RFA, poderia levar a um conflito entre a ativação do sistema imune e outras funções fundamentais para a sobrevivência dos organismos que também dependem de recursos energéticos, tais como reprodução e termoregulação (Ardia, 2005; Sheldon and Verhulst, 1996). Por exemplo, Deerenberg e col. (1997) manipularam o tamanho da ninhada e carga de trabalho parental em indivíduos da espécie *Taeniopygia guttata* e observaram que o aumento no esforço reprodutivo acarretou redução na produção de anticorpos. Whitaker e Fair (2002) observaram que após um desafio imune, filhotes de *Poecile gambeli* apresentaram flutuação assimétrica no comprimento das penas das asas, indicando que a ativação da resposta imune durante a fase de crescimento, teve um custo energético que comprometeu o desenvolvimento dos filhotes. Sköld-Chiriac e col. (2015) desafiaram o sistema imune de indivíduos de *Taeniopygia guttata* e observaram variações de temperatura corpórea. Quando os indivíduos eram desafiados durante a noite, eles ativavam uma resposta febril, porém, ao serem desafiados durante o dia eles apresentavam hipotermia. A conclusão dos autores foi que a hipotermia deve minimizar o risco de superaquecimento e evitar um custo metabólico excessivo durante o dia, período no qual a temperatura corpórea

tende a ser alta. Porém durante a noite, quando a temperatura corpórea é naturalmente mais baixa nessas aves, a resposta febril é mais benéfica.

A perda de massa corpórea durante a RFA já foi observada em muitos organismos (Burness et al., 2010; Otálora-Ardila et al., 2016.; Owen-Ashley et al., 2006; Owen-Ashley and Wingfield, 2006; Schneeberger et al., 2013a). Como a RFA induz anorexia (Kyriazakis et al., 1998), esta perda de massa é comumente associada à mobilização de reserva de reservas energéticas que seriam utilizadas para cobrir, em parte, os custos de ativar a RFA (Moreno-Rueda, 2011; Ricklefs and WIlkelski, 2002; Zera and Harshman, 2001). Outro evento observado durante a RFA é a febre (Evans et al., 2015; Marais et al., 2011; Otálora-Ardila et al., 2016).Altas temperaturas corpóreas reduzem a replicação de vírus e bactérias (Lwoff, 1971; Osawa and Iel, 1964), provocam a proliferação e tráfego de linfócitos para os órgãos linfáticos secundários (linfonodos e baço), migração de neutrófilos e células dendríticas e produção de citocinas inflamatórias (Appenheimer et al., 2005). Portanto, em principio, certos parâmetros hematológicos que variam em decorrência da resposta febril, tais como a razão N/L e a contagem total de leucócitos, também podem ser indicadores da ativação da resposta imunológica (Feldman et al., 2000). Por fim, diversos estudos mostram que a ativação do sistema imune, induzida por diferentes desafios produz aumento na taxa metabólica (Cutrera et al., 2010; King and Swanson, 2013; Marais et al., 2011; Martin et al., 2003; Otálora-Ardila et al., 2016; Ots et al., 2001). No caso da RFA, este aumento pode ocorrer devido à resposta febril, já que para cada 1 ° C de aumento na temperatura corpórea, há incremento de 10 – 25% na taxa metabólica (Kluger, 1979). Todavia, este aumento também pode ocorrer devido à modulação da síntese de proteínas no fígado, como observado num trabalho realizado por Ksiazek et al., (2003). Estes autores detectaram aumento no tamanho do fígado e rim durante desafio imune em ratos de laboratório (Swiss Webster), e atribuíram tal variação de tamanho ao incremento da síntese de proteínas, associada com o aumento da taxa metabólica durante a ativação da RFA.

Os padrões das respostas imunológicas podem variar em decorrência de diversos fatores, como a história de vida do organismo, o seu estado nutricional e até mesmo o período do ano no qual ocorre o processo imunológico de defesa (Lee, 2006; Martin et al., 2008). Conforme mencionado anteriormente, a suposição de que a ativação da resposta imune requer alto gasto de energia, postula-se que muito desta variação deve-se as possíveis soluções de compromisso entre o custo de ativar o sistema imune e a alocação de energia para outra funções. No caso especifico da RFA, este compromisso seria mais evidente em situações de de baixa disponibilidade de alimento no ambiente ou alta demanda energética como, por

exemplo, durante o inverno (Bronson, 1987; Kunz et al., 1998; Moreno-Rueda, 2011). Em ambas as situações, seria esperado que os organismos priorizassem as respostas menos custosas em termos de energia, isto é as respostas do sistema imune adaptativo (respostas específicas não inflamatórias – Lee, 2006). Por exemplo, foi observado que em aves da espécie *Passer montanus* com altas taxas reprodutivas possuem respostas de memoria imunológica fracas, porém fortes respostas inflamatórias não específicas em comparação com indivíduos da espécie *Passer domesticus,* os quais possuem taxas reprodutivas menores (Lee et al., 2006). Assim também, durante o período reprodutivo, foi observado que aves da espécie *Anas platyrhynchos* produzem uma versão truncada de anticorpo durante a resposta imune, a qual minimiza a resposta inflamatória contra a invasão de bactérias (Humphrey et al., 2004), diminuindo assim o custo da mesma. Em um estudo com primatas (*Macaca mulata*) foi observado uma diminuição da produção de citocinas (as quais requerem alto custo energético, porém, aumento na proliferação de linfócitos (que requerem menor custo energético) durante a ativação de uma resposta imune no inverno, que coincide com a estação reprodutiva desta espécie (Mann et al., 2000). Tal resultado sugere que esta espécie prioriza a ativação do sistema imune adquirido durante o período reprodutivo (Mann et al., 2000). Assim também, durante a resposta imune indivíduos da espécie *Passer domesticus* submetidos a uma dieta de baixa qualidade foram capazes de produzir uma alta quantidade de anticorpos (Buchanan et al., 2003), enquanto que quando submetidos a uma dieta de alto teor proteico, isto é, indivíduos em bom estado nutricional, produziram uma baixa quantidade de anticorpos, porém apresentaram forte resposta inflamatória (Gonzalez et al., 1999). As variações nos padrões da resposta imune observadas nos trabalhos citados anteriormente confirmam a importância de considerar diversos parâmetros imunológicos em diferentes situações às quais os organismos são expostos durante o seu ciclo de vida (como períodos com alta demanda de energia e baixa disponibilidade de alimento) quando se tem como objetivo a compreensão dos processos relacionados à ativação da resposta imune.

Variações sazonais como diminuição da temperatura ambiental, redução da disponibilidade de alimento, migração, confinamento, falta de refugio e aumento na taxa de predação podem causar alterações na função imunológica de aves e mamíferos, comprometendo a sua sobrevivência (John, 1994; Lochmiller et al., 1994; Nelson and Demas, 1996). Por exemplo, num estudo realizado com roedores da espécie *Peromyscus maniculatus,* os autores observaram que indivíduos mantidos a 8°C apresentavam níveis menores de anticorpos do que indivíduos mantidos a 20°C (Demas and Nelson, 1998), e em aves da espécie *Saxicola torquata,* os autores observaram que quando mantidas em temperaturas

baixas (~10°C), as aves apresentavam menor capacidade bactericida contra *Escherichia coli* do que aves mantidas a temperaturas mais altas (~20°) (Versteegh et al., 2014). Estudos também tem observado que a redução na disponibilidade de alimento pode afetar a imunocompetência dos organismos. Por exemplo, no estudo realizado por Nakamura e col (1990) com ratos de laboratório, os autores observaram que ratos submetidos à restrição alimentar apresentavam perda da massa dos órgãos linfáticos (timo 25% e baço 27%) e diminuição da atividade de células exterminadoras naturais em comparação com o grupo controle (alimentado *ad libitum*), assim como aumento de cortisol e catecolaminas no plasma sanguíneo os quais acredita-se, atuam como supressores das respostas mitogênicas dos linfócitos (Syvalahti, 1987). Assim também, Alonso-alvarez e Tella (2001) realizaram um estudo com aves da espécie *Larus cachinnans*, as quais eram submeteram a três regimes alimentares: jejum, restrição (um terço da ingestão média diária) e aves alimentadas *ad libitum*. Os autores detectaram correlação entre a condição corpórea dos indivíduos e a sua capacidade de ativar uma resposta imune inflamatória.

No presente trabalho nos analisamos a presença e magnitude da RFA em morcegos. Morcegos são os mamíferos de maior diversidade e distribuição geográfica, podendo ser encontrados em todos os continentes com exceção da Antártida (Schipper et al., 2008). A sua capacidade de voar e cobrir longas distâncias, habilidade para habitar diversos refúgios e ocupar nichos ecológicos variados, torna os morcegos um dos organismos mais sucedidos do planeta, porém também os faz eficientes vetores na transmissão de doenças (Bernard et al., 2012; Calisher et al., 2006; Kuzmin et al., 2011; Wong et al., 2007; Woo et al., 2009). Por exemplo, o seu comportamento de agregação nos refúgios facilita a transmissão de doenças entre indivíduos, e o deslocamento entre refúgios ou mesmo o comportamento migratório pode facilitar a transmissão entre colônias da mesma espécie ou ainda de espécies distintas. Morcegos são hospedeiros de diversos microrganismos como bactérias, fungos, vírus e parasitas (Bausch and Schwarz, 2014; Brook and Dobson, 2015; Calisher et al., 2006; Mühldorfer, 2013). Existem diversos estudos sobre doenças emergentes em morcegos, porém a maioria se concentra no estudo de vírus e parasitas (Bausch and Schwarz, 2014; Calisher et al., 2006; Plowright et al., 2015; Saéz et al., 2014). De fato, morcegos são vulneráveis a diversas doenças como vírus Nipah, Hendra, Ebola, Marburg, ou de coronavirus do tipo SARS (Food and Agriculture Organisation of the United Nations, 2011; Kuzmin et al., 2011), mas também a bactérias como *Pasteurella*, *Salmonella*, *Escherichia* e *Yersinia* spp.

(Mühldorfer, 2013) que podem causar doenças nos próprios morcegos, mas que também podem ser transmitidas para seres humanos e animais domésticos.

Baker e col. (2013) fizeram uma revisão detalhada sobre as respostas do sistema imune inato e adaptativo em morcegos, e observaram que embora compartilhem diversos componentes do sistema imune com outros vertebrados, a sua imunocompetência é diferenciada em termos quantitativos e qualitativos. Porém os padrões das respostas imunes podem variar ainda entre espécies de morcegos. Diversos estudos tem avaliado a imunocompetência de morcegos e os resultados tem sido controversos. Por exemplo, Schneeberger e col. (2013a) desafiaram o sistema imune de morcegos da espécie *C. perspicillata* com injeção de LPS e observaram leucocitose e aumento do estresse oxidativo após a resposta inflamatória. Assim também, Stockmaier e col. (2015) induziram uma RFA através de injeção de LPS em morcegos da espécie *Molossus molossus,* os quais apresentaram perda de massa corpórea mas não desenvolveram febre nem leucocitose. Os autores concluíram que a resposta febril pode não ser uma característica conservativa entre mamíferos e que o fato de não terem observado leucocitose pode estar associado às variações de temperatura corpórea da espécie, já que existem evidências de baixas temperaturas corpóreas reduzirem o número de leucócitos circulando na corrente sanguínea em outros mamíferos. Pelo contrário, Otálora-Ardila e col. (2016) observaram febre e aumento da taxa metabólica de morcegos da espécie *Myotis vivesi* após induzir uma resposta imune através de injeção de LPS. Estes trabalhos sugerem que os padrões de resposta do sistema imune podem variar mesmo entre espécies taxonomicamente próximas. Isso torna de suma importância o estudo da ativação da RFA em diversas espécies de morcegos, assim como em diversas situações às quais os mesmos estão expostos durante o ciclo de vida. Somente dessa forma será possível aumentar a nossa compreensão sobre a imunocompetência destes organismos, podendo contribuir para planos que visem a sua conservação.

Dentro deste contexto, nos analisamos se determinados componentes da RFA (perda de massa corpórea, febre, aumento na razão neutrófilo/linfócito, na contagem total de leucócitos e na taxa metabólica) estariam presentes no morcego frugívoro *C. perspicillata*, apos seu sistema imune ter sido desafiado com a inoculação de LPS. Estes parâmetros foram avaliados durante duas estações do ano (verão e inverno) e em morcegos submetidos a dois regimes alimentares (alimentados *ad libitum* e restrição alimentar). Por fim, o custo energético de ativação da RFA |foi estimado com base em medidas da taxa metabólica *C. perspicillata* possui hábito frugívoro, e tem preferência por frutos de *Piper* sp. (Mello et al., 2004), os quais são escassos durante o inverno. Sendo assim, durante essa estação, os

morcegos são susceptíveis à diminuição na disponibilidade de recursos energéticos, e ao aumento da demanda de energia para outras funções fisiológicas tais como termoregulação (Lochmiller and Deerenberg, 2000; Nelson and Demas, 1996; Schetter et al., 1998). Porém, é importante considerar que os organismos podem desenvolver adaptações que lhes permitiram ter sucesso durante períodos de condições mais severas (Nelson and Demas, 1996). Se *C. perspicillata* tiver desenvolvido adaptações para se antecipar a possíveis variações ambientais durante o ciclo anual, nós esperamos que em ambas as estações e ambos os regimes alimentares, os indivíduos consigam ativar uma RFA, e que o custo da mesma seja semelhante entre os grupos. Porém, se a espécie não for adaptada para lidar com tais variações, esperamos que morcegos no verão e alimentados *ad libitum* consigam ativar a RFA com menor custo energético, e morcegos no inverno e em restrição alimentar apresentem o maior custo de ativação da RFA. Esperamos detectar perda de massa corpórea em todos os grupos experimentais, porém devido a contradições nos resultados de estudos anteriores, realizados com morcegos, as nossas hipóteses sobre febre e aumento nos parâmetros hematológicos não são concretas. Entender o efeito de tais variações na imunocompetência dos morcegos e de outros organismos é fundamental para entender os fatores que determinam a sua sobrevivência na natureza.

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The energetic cost of the acute phase response in the Short-tailed fruit bat (*Carollia perspicillata*): Effects of seasonality and food restriction

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List of symbols and abbreviations

ANOVA	Analysis of variance	**N/L**	Neutrophil/lymphocyte
ANCOVA	Analysis of covariance	**PBS**	Phosphate buffer saline
APR	Acute phase response	**RM ANOVA**	Repeated measure analysis of variance
ALS	Ad libitum during summer		
ALW	Ad libitum during winter	$\mathbf{T_b}$	Body temperature
FRS	Food restriction during summer	$\mathbf{T_{bi}}$	Initial body temperature
FRW	Food restriction during winter	$\mathbf{T_{bp}}$	Peak body temperature
LPS	Lipopolysaccharide	$\mathbf{T_{bf}}$	Final body temperature
$\mathbf{M_b}$	Body mass	$\mathbf{\Delta T_b}$	Increase in body temperature
$\mathbf{M_{bi}}$	Initial body mass	**TEC**	Total energetic cost
$\mathbf{M_{bf}}$	Final body mass	$\mathbf{VO_2}$	Rates of oxygen consumption
$\mathbf{\Delta M_b}$	Body mass loss	**WBC**	White blood cell
MR	Metabolic rate		

ABSTRACT

One of the innate immune response components is the APR, which can be activated by trauma, infection, stress and inflammation. The APR activation aims to restore homeostasis and health of an organism exposed to pathogens, through several physiological modifications that may result in high energetic cost. In situations where animals are exposed to increase in energy demand and decrease in caloric intake, they could experience a deficiency in energy reserves, which may hamper the effectiveness of an immune response. We induced the APR activation in the Short-tailed fruit bats (*Carollia perspicillata*) by LPS injection. We quantified the energetic cost of the APR and how it is affected by seasonality and food availability. Bats injected with LPS show increase in MR when compared to control (120–180%). ΔM_b during the APR was observed in both seasons, but only in bats in food restriction. Bats did not show fever or leukocytosis; nevertheless, we found increase in N/L ratio when bats were in food restriction and during winter, probably as a stress indicator. Furthermore, the energetic cost of the APR activation did not vary between seasons or food availability. Our results showed that for *C. perspicillata*, activating the APR entails low energy cost (1% of the daily energy budget). Thus, they seem to be capable of activate an immune response even in situations that demand more energy and when food availability is limited, consequently preserving immunocompetence without compromising their energy budget.

Key-words: Energetic cost, Immune response, Acute phase response, Metabolic rate, Seasonality, Food restriction.

INTRODUCTION

The immune response can be generally classified in innate or adaptive. An innate response is initiated relatively fast after contact with pathogens, acting as first line of defense. During this response, blood vessels contract in order to inhibit the propagation of pathogens through the bloodstream and phagocytic cells and molecules of the innate immune system are recruited (Murphy, 2012) inducing local inflammation through cytokine production. If a simple inflammation is not enough to fight the invasion, a highly energy demanding systemic inflammation is activated (Lee, 2006), with the increase of acute phase proteins (APP) production by the liver, changes in energy and nutrient metabolism, decrease in social and locomotor activities as well as anorexia and fever (Hart, 1988; Klasing and Leshchinsky, 1999). These behavioral and physiological modifications characterized the APR, which

accelerates the healing process, but results in a high energy cost (Cai et al., 2009; Klasing and Leshchinsky, 1999; Lee, 2006; Martin et al., 2003; Sheldon and Verhulst, 1996). When infection exceeds the fight capacity of the innate arm, an adaptive immune response is induced. This arm consists in highly specialized cells, capable of create an immunological memory to pathogens, allowing the organism to fight faster and efficiently any known future invasion (Klurfeld, 1993).

The APR is usually induced by LPS inoculation, which is an antigen present in most gram-negative bacteria that mimics an infection without actually getting the animal sick, inducing an inflammatory response by promoting the release of cytokines few hours after being injected (Burness et al., 2010; Lopes et al., 2012; MacDonald et al., 2012; Sköld-Chiriac et al., 2014).

The immune responses may vary due to several factors, as life-history, nutritional status and season of the year (Lee, 2006; Martin et al., 2008), in order to prioritize those processes which are more advantageous for the organism survival. Animals may suppress the activation of an inflammatory response during specific time of the year, for example during reproductive season or when food availability decreases. In a study made with *Passer montanus*, which have high reproductive rates, authors found week antibody responses but higher inflammatory responses than those observed in individuals of *Passer domesticus*, which have lower reproductive rates (Lee et al., 2006). These findings suggest that birds with lower reproductive rates prioritize the allocation of resources for reproduction over immunocompetence. In other study with *Passer domesticus*, individuals where separated in two groups, one received a low quality diet and the other a high-protein diet (Gonzalez et al., 1999). Birds in high protein diet had higher inflammatory responses than birds in low quality diet, on the other hand birds in low quality diet showed higher antibody responses, suggesting that inflammatory immune defense may actually be costly to activate. Therefore, in situations where animals are exposed to severe conditions of increase in energy demand and decrease of food availability, we could expect a trade-off between immune function and other energetically costly activities.

Seasonal variations as decrease in ambient temperature, reduction of food availability, migration, confinement, lack of refuge and increase in risk of predation may cause some alterations in the immune function of populations, compromising their survival (John, 1994; Lochmiller et al., 1994; Nelson and Demas, 1996). For example, Demas and Nelson (1998) observed in mice of the species *Peromyscus maniculatus*, that individuals kept at 8°C

produced lower levels of antibodies than individuals kept at 20°C, and Versteegh et al. (2014) found that birds of the species *Saxicola torquata* kept at low ambient temperatures (~10°C) showed lower bactericidal capacity against *Escherichia coli* than birds kept at higher ambient temperatures (~20°C). Other studies showed that the reduction of food availability may affect immunocompetence. For example, Nakamura et al. (1990) found that laboratory mice in food restriction showed mass loss of the lymphatic organs (thymus 25% and spleen 27%) and reduction of the natural killer cells activity when compared with the control group (fed *ad libitum*), as well as increase in plasma levels of cortisol and catecholamines, which are thought to suppress the mitogen responses of lymphocytes (Syvalahti, 1987). Furthermore, in a study with *Larus cachinnans,* the birds where divided in three experimental groups: fasted, restricted (fed with one third of the average daily intake) and birds fed *ad libitum* (Alonso-alvarez and Tella, 2001). The authors detect a correlation between body condition and the capacity of individuals to activate an inflammatory response.

We quantified the cost of the APR activation by measuring the MR of C. perspicillata challenged with LPS, and searched for variations between seasons (summer and winter) and availability of food (*ad libitum* and food restriction). We measured T_b, M_b and hematological parameters (N/L ratio and WBC count), which are indicators of the APR activation. Then we used the MR measurements to calculate the TEC of the response. During winter, animals experience an increase in energy demand for thermoregulation, to cope with low ambient temperatures (Lochmiller and Deerenberg, 2000; Schetter et al., 1998) and *C. perspicillata* is also susceptible to a decrease in caloric intake, because most *Piper* species do not produce fruits during this season (Mello et al., 2004). Some studies had also showed that energy restriction in diet can lead to suppression of the immune system (Klurfeld, 1993; Lochmiller and Dabbert, 1993), so both situations increase their vulnerability to disease and may hamper their immune response effectiveness (Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996). If *C. perspicillata* have developed adaptations to anticipate to any possible environmental variation during the annual cycle, we expect that in both seasons and both diets, the individuals will be capable of activating an APR, and that the cost of such activation will be similar between groups. On the other hand, if the species is not adapted to such changes, we expect that bats during summer and fed *ad libitum* will activate an APR with lower energy cost, and bats in winter and food restriction will show higher energy cost during the APR activation. We also expect to detect ΔM_b in every experimental group, yet because of the controversial results found in previous studies with bats, our hypothesis about the fever

response and variations in the hematological parameters are not established.

MATERIAL AND METHODS

Animal capture and housing

Adult individuals of *C. perspicillata* were mist-netted at the entrance of an arenitic cave located at the municipality of Corumbatai, Southeastern Brazil (22°14'54" S, 47°42'24" W), during summer (2015 and 2016) and winter (2015). In summer we only captured adult male individuals (n=13), because virtually all females were pregnant or lactating. In winter, we tried to prioritize the capture of male bats, but most of those that were mist netted were juvenile, so we include some non-reproductive, adult females (males: n=7, females: n=5). Because of the reduced sample size, it was not possible to test for variations between sex. Upon capture, bats were kept in an outdoor 3 x 3 x 3 m flight cage, at Universidade Estadual Paulista at Rio Claro, exposed to natural conditions of photoperiod and temperature (Summer: 18.8 - 30.4 °C; Winter: 12.0 – 26.5 °C - CEAPLA/IGCE/UNESP). During this period, bats were fed with banana, papaya and apple, and maintained M_b. Before the experiments, during each season, bats were separated in two groups: fed *ad libitum* and with food restriction. Thus, we had four experimental groups: 1) fed *ad libitum* in summer (ALS, n=6 males), 2) food restriction in summer (FRS: n=7 males), 3) fed *ad libitum* in winter (ALW: males: n=3, females: n=3) and 4) food restriction in winter (FRW: males: n= 4, females: n=2). Bats in food restriction group did not received food during a 24 hour period before the experiment, but they received about 0.5 ml of saline to avoid dehydration. We opt for a 24 hour period because this situation mimics a possible natural food deprivation that bats may experience in the wild and also because more than 24 hour food restriction could lead *C. perspicillata* bats to starvation, dehydration and death. Once the experiments were finished, bats were marked and released at the site of capture. Ethical permits for this study were issued by the Comissão de Ética no uso de Animal, of the Universidade Estadual Paulista at Rio Claro (Authorization: 20/2014).

Immune challenge

We challenged the immune system of bats by injecting 50µL of a 1mg mL^{-1} solution of LPS (L2630, Sigma-Aldrich, USA) diluted in PBS (P4417, Sigma-Aldrich, USA). Because immune response varies between individuals (Krams et al., 2012), each bat participate in both the challenge (LPS) and control (PBS) experiment, and the order was assigned randomly. Bats were injected subdermally in the back and the skin surrounding the injection site was

sterilized with ethanol prior and after the injection. The injection procedure took less than five minutes. Pilot experiments showed that this dose was high enough to elicit a sustained and significant response in MR; lower doses did not cause a measurable response and higher doses elicited a blunted response.

We calculated the mass-specific dose of LPS injected per individual ([1000 * 0,05 mg LPS] / M_{bi}), and tested for variation in those values between experimental groups using a one-way ANOVA. We found that the mass-specific dose differed statistically between experimental groups ($F_{3,24} = 7.23$, $p = 0.002$ – table 1). Such variations occurred because bats in food restriction had higher ΔM_b during captivity than bats fed *ad libitum*, so we used mass-specific dose as covariate when we analyzed the variations in TEC.

Measuring the APR components

Body mass – The bats were weighted to the nearest 0.1 grams (Ohaus Precision Balance, USA) at the beginning (M_{bi}) and end (M_{bf}) of the experiments. ΔM_b during the experiments was calculated in relative terms ($[M_{bf} – M_{bi}] / M_{bi}$).

Body temperature - Rectal temperature was assessed to the nearest 0.1 °C with a type K thermocouple (Cole-Parmer model 91100-20, USA) immediately before the injection (T_{bi}), three hours after the injection (T_{bp}) and at the end of the experiments (T_{bf}). During pilot experiments, it was possible to determine that the peak of the APR, in terms of maximum increase in MR, occurred approximately three hours after the injection. Thus, we assumed that the T_b measured after this time would represent the T_b that would be associated with a fever response and then calculate ΔT_b in relative terms ($[T_{bp} - T_{bi}] / T_{bi}$).

Hematological parameters - At the beginning and end of each experiment, blood was collected from the propatagial vein for blood smears. The blood was fixed with methanol and stain with Giemsa (20% dilution). N/L ratio and WBC count were made similar to protocols followed by Krams et al. (2012) and Schneeberger et al. (2013a) respectively.

Metabolic rate – MB was indirectly assessed by measuring the rates of oxygen consumption (VO_2) in bats prior and after LPS or PBS injection using an open-flow system (Voigt and Cruz-Neto, 2009). Bats were placed in 165ml cylindrical metabolic chambers, fitted with inlet and outlet ports. Respirometric chambers were then placed in a BOD incubator set at 27 ºC, which lies within the species thermoneutral zone (Cruz-Neto and Jones 2006). A same-sized, but empty, respirometric chamber was used for baseline measurements. A tube containing Drierite™ (W. A. Hammond Drierite, Xenia, Ohio, USA) absorbed the water from the incurrent and excurrent air. The air passed through a flow meter (FB8 – Sable

System, USA) sending 300-400 ml air per minute. A Multiplexer (Multiplexer V2.0 – Sable System, USA) at the outlet port of the chambers allowed us to make automatic baseline recordings, receiving air from both respirometric chambers (empty and with the bat) but sending the air of only one of them at a time to the rest of the system. Out from Multiplexer, the air passed through Drierite^{TM} again, and through another flow meter (Sierra Side-Track) connected to a mass flow reader (MFC2 – Sable System, USA). The air was sub-sampled (200 ml/min) and send to an O_2 analyzer (Foxbox – Sable System, USA). All the routine was made through an A/D converter (UI2 – Sable System, USA).

Experimental protocol: Experiments started between 06:00-7:00 am, measuring one bat at a time, and starting by taking a 30 minutes baseline reading from the empty chamber and then a continuous two hours reading of the excurrent air form the experimental chamber with the bat. Pilot experiments showed that bats usually settled down in the chamber after one hour; thus, this period was enough for MR to achieve steady-state that could be used as standard for comparing the incremental responses associated with treatments. After the first two hours, the bat was injected either with PBS or LPS, and during the injection procedure, excurrent air from the empty chamber was monitored. After that, the bat was placed back in the chamber and we recommenced the recording for the next three hours, followed by a 20 minutes baseline, another six hours of bat recording and a final 30 minutes baseline. Data was recorded at a rate of one point each five seconds, and analyzed by the software Expedata 1.4.9 (Sable Systems International), where VO_2 was calculated using the equations 11.2 from Lighton (2008).

Data Analysis

Variations in the parameters that indicate the activation of the APR (M_b, T_b, N/L ratio, WBC count and MR) were analyzed within each experimental groups, between LPS and PBS experiments. The TEC obtain from the MR measurements was the only variable analyzed between experimental groups, since our objective in this study is to determine the energetic cost of the APR activation and the effects of seasonality and food restriction.

Body mass and Body Temperature - We used a one-way ANOVA, considering the treatment (LPS and PBS) as variable, to detect variations in M_{bi}, $T_{bi,}$ and relative changes (ΔM_b and ΔT_b) within each of the experimental groups.

Hematological parameters – We used a two-way ANOVA, considering the time (at the beginning and end of the experiments), to test for differences, within each experimental group, in N/L ratio and WBC count.

Metabolic rate - VO_2 fluctuated during the experiments due to random movements of the bats inside the chamber. To minimize such fluctuations, we used the analytical tools of the Expedata software to divide the whole trace of VO_2 into segments of 60 minutes. From these segments, we used the *nadir* function to select the lowest and most constant 15 minutes trace, and an average of these values was used to characterize the VO_2 for each time bin. These values of VO_2 were then transformed to their MR equivalents [(VO_2*60) *0.0198] in order to obtain values in kJ h^{-1}, which were used in all subsequent analysis (Burton, 2000).

Differences in pre-injection MR within each experimental group were tested using a one-way RM ANOVA, considering the treatment as variable. Variations in post-injection values were tested by using a two-way RM ANOVA, with individuals used as the RM and the time (1 to 9 hours after injection) and treatment as variables. We also calculated the mean value of post-injection RMR for both treatments (LPS and PBS) during the time of response for each experimental group. With these means we calculated the percentage of increase of pre-injection MR after LPS injection with respect to the increase after PBS injection.

Total energetic cost of the immune response – We calculated the total energetic cost (TEC) associated with mounting an immune response, following a protocol somewhat similar to that described by Marais et al. (2011). After detecting variations in post-injection MR within each experimental group, using the two-way RM ANOVA, we used a Holm-Sidak test, which allowed us to identify until what time MR differed between LPS and PBS experiments, that is, the duration of response. In order to compare the TEC between experimental groups, it must be calculated during the same period of time. For that, we standardize the duration of response as being the mean time of response between experimental groups. Once we established the duration of response, we subtract for each individual, the MR of each post-injection time bin when the bat was injected with PBS, from that when injected with LPS, eliminating any cost of handling. Following the trapezoid method (Tai, 1994), we used these values to calculate the TEC associated with the APR activation per individual, integrating the area under the curve that describes the variation of MR in time. The TEC analysis between experimental groups was made using an analysis of covariance (ANCOVA), with mass-specific dose as covariate and experimental group as variables.

All values are expressed as mean ± s.d. and statistical significance was considered at $p \leq 0.05$.

RESULTS

Body mass – There was no statistical difference in M_{bi} between treatments for any of the experimental groups, ΔM_b was significantly higher after LPS injection only in FRS and FRW groups (table 2).

Body temperature – We did not found variations in T_{bi} between treatments for any of the experimental groups. Also, there was no significant difference in ΔT_b between treatments, for any of the experimental groups (table 3).

Hematological parameters – The hematological parameters varied considerable within groups (figures 1 and 2). For the ALS and FRS groups, N/L ratio varied as function of time (ALS: $F_{1,23} = 15.54$, $p < 0.001$; FRS: $F_{1,27} = 6.19$, $p = 0.02$), but not as a function of the treatment (ALS: $F_{1,23} = 0.07$, $p = 0.8$; FRS: $F_{1,27} = 4.18$, $p = 0.05$) or as a function of treatment-time interaction (ALS: $F_{1,23} = 2.81$, $p = 0.11$; FRS: $F_{1,27} = 0.78$, $p = 0.39$). For the ALW group we found that N/L ratio did not varied as function of time ($F_{1,23} = 2.97$, $p = 0.1$), treatment ($F_{1,23} = 0.89$, $p = 0.36$), or by the interaction between these variables ($F_{1,23} = 0.08$, $p = 0.78$). In the FRW group we found effect of time ($F_{1,23} = 7.28$, $p = 0.02$), no effect of treatment ($F_{1,23} = 1.11$, $p = 0.31$) but a significant time-treatment interaction ($F_{1,23} = 12.22$, $p < 0.01$) (figure 1). In this group, the N/L ratio at the end of the experiments was 1.54 times higher when bats were injected with LPS than when they were injected with PBS.

We found no evidence for leukocytosis. In fact, for all experimental groups, WBC counts did not varied as a function of time (ALS: $F_{1,23} = 2.13$, $p = 0.16$; FRS: $F_{1,27} = 1.1$, $p = 0.31$; ALW: $F_{1,23} = 3.61$, $p = 0.07$; FRW: $F_{1,23} = 1.71$, $p = 0.21$), treatment (ALS: $F_{1,23} = 0.53$, $p = 0.48$; FRS: $F_{1,27} = 0.07$, $p = 0.79$; ALW: $F_{1,23} = 0.5$, $p = 0.49$; FRW: $F_{1,23} = 4.07$, $p = 0.06$), or by the interaction between these variables (ALS: $F_{1,23} = 0.13$, $p = 0.73$; FRS: $F_{1,27} = 0.01$, $p = 0.92$; ALW: $F_{1,23} = 0.11$, $p = 0.75$; FRW: $F_{1,23} = 0.11$, $p = 0.74$) (figure 2).

Metabolic rate – We found no difference in pre-injection MR between PBS and LPS experiments for any of the experimental groups. (ALS: $F_{5,11} = 5.57$, $p = 0.07$; FRS: $F_{6,13} = 0.01$, $p = 0.94$; ALW: $F_{5,11} = 2.35$, $p = 0.19$; FRW: $F_{5,11} = 6.39$, $p = 0.05$) (figure 3). When testing, within each experimental group, for variations in post-injection MR, we found effect of time in ALS ($F_{8,107} = 11.47$, $p < 0.001$), FRS ($F_{8,125} = 4.01$, $p < 0.01$) and ALW groups ($F_{8,107} = 2.75$, $p = 0.02$). No effects of time was observed for in the FRW group ($F_{8,107} = 1.8$, $p = 0.11$). A treatment effect was detected in the ALS ($F_{1,107} = 25.84$, $p < 0.01$), FRS ($F_{1,125} = 13.35$, $p = 0.01$) and FRW experimental groups ($F_{1,107} = 45.09$, $p < 0.01$) but not in ALW experimental group ($F_{1,107} = 3,48$, $p = 0,12$). Nevertheless, all experimental groups showed time-treatment interaction (ALS: $F_{8,107} = 3.63$, $p < 0.01$; FRS: $F_{8,125} = 2.49$, $p = 0.02$; ALW:

$F_{8,107}$ = 8.20, p < 0.001; FRW: $F_{8,107}$ = 8.67, p < 0.001). This interaction between variables indicates that bats MR was different between treatments, depending on time. In fact, the mean duration of the response (time when MR of LPS injected bats was different from MR of PBS injected bats – i.e the duration of the APR response) varied from 3 hours for ALW, to 5 hours in FRS and FRW to 6 hours in ALS. (figure 3) The increase in MR after LPS injection was of 125.0% in ALS, 151.3% in FRS, 120.8% in ALW and 180.8% in FRW.

Total energetic cost of the immune response – We found no variation in the TEC between experimental groups ($F_{4,24}$ = 0.97, p = 0.45) suggesting that the TEC was similar in bats in all experimental groups (ALS: 0,33±0,07 kJ; FRS: 0,38±0,1 kJ; ALW: 0,25±0,05 kJ; FRW: 0,47±0,08 kJ) (figure 4).

DISCUSSION

The APR, a suite of physiological and behavioral changes, is thought to be the first line of defense of vertebrates when exposed to pathogens (Burness et al., 2010; Lee, 2006; Owen-Ashley and Wingfield, 2007; Sköld-Chiriac et al., 2014). Although the APR is triggered by different stimulus, the dynamics of the responses associated with it are thought to be universally presented in all vertebrates, and results in a complex systemic reaction that would reestablish homeostasis (Owen-Ashley and Wingfield, 2007). ΔM_b, a fever response, leukocytosis and an increase in MR have been suggested as being central aspects associated with the APR in vertebrates (Copeland et al., 2005; Gabay and Kushner, 1999). Usually studies that measured these parameters challenged the immune system by administration of LPS (Burness et al., 2010; Demas et al., 2011; MacDonald et al., 2012; Marais et al., 2011; Otálora-Ardila et al., 2016; Owen-Ashley and Wingfield, 2007; Sköld-Chiriac et al., 2015; Stockmaier et al., 2015). However, one worth aspect that should be considered when analyzing the results from these studies is that the responses to LPS are highly variable, within and between species, even when the same LPS batch is used (Demas et al., 2011). This is specially the case in our study, where the dose administrated varied between groups, being smaller for the food-restricted group (table 1). Even though we analyzed most of the effects within each experimental group (which should minimize the problem of differences in the mass-specific dose used), our interspecific comparisons and interpretations between group differences should be viewed with some caution.

ΔM_b during the APR might be due to anorexia, but also can be indicative of energy reserve mobilization to cover the costs associated with the APR (Evans et al., 2015; Stockmaier et al., 2015). In bats, significant ΔM_b after LPS challenge has been documented in

C. perspicillata (Schneeberger et al., 2013b), *M. molossus* (7% - Stockmaier et al., 2015), *M. vivesi* (8% - Otálora-Ardila et al., 2016) and the Palla's long-tongued bat (*Glossophaga soricina*) (9% - Cabrera-Martinez et al., *Unpublished results*). In our experiments, we observed that ΔM_b of *C. perspicillata* varied between 9 and 12% after LPS challenge. These high rates of ΔM_b observed in our study, as compared to those observed for other bats, cannot be attributed only to differences in dose used. (2.7 – 3.3 mg LPS kg^{-1}, as compared the reported mass-specific doses of 2.84 mg LPS kg^{-1} for *G. soricina*, 1.75 mg LPS kg^{-1} for *M. vivesi* and 4.5 mg LPS kg^{-1} for *M. molossus*). However, bats in food restriction received a higher dose, and they were the only ones with significantly ΔM_b when compared to bats injected with PBS, irrespectively of season. Since bats fed *ad libitum* had access to the food until the start of the experiments, we suggest that the lack of significance in ΔM_b for these bats might be because part of the APR cost was covered by the mobilization of carbohydrates from the recent ingested food. In fact, nectar and fruit eating bats are capable of channeling the substrates of recently eaten food to meet their energetic requirements (Amitai et al., 2010; Voigt et al., 2012). Thus, these bats might rely on exogenous sources of energy to cover, at least partially, the costs of the APR, a possibility that was not available for bats in food restriction, which have to rely on fat reserves to cover this cost.

Fever is considered an essential component of the APR in vertebrates, and its adaptive advantage relies on the fact that it creates a hostile environment to the pathogens and, at the same time, enhances the host's immune system (Blatteis, 2003). It is interesting, thus, that our study did not find any evidence of fever in any of the experimental groups. In bats, fever after LPS administration was also not observed for the *M. molossus* (Stockmaier et al., 2015) but was present in *M. viviesi* (Otálora-Ardila et al., 2016), and is an integral part of the APR in birds (Marais et al., 2011; Sköld-Chiriac et al., 2015) and small non-flying mammals (Bilbo et al., 2002; Rudaya et al., 2005). For the food-restricted group, especially in winter, the lack of fever might be an expected result. In fact, Romanovsky and Székely (1998) suggested that, since fever might be energetically expensive, the absence of a fever response would be expected when food is scarce. However, it is difficult at the present moment, to reconcile the absence of fever for bats with access to food with those of the literature, especially with the conflicting results observed for bats.

Leukocytosis is another phenomenon that is thought to be an integral part of the APR (Copeland et al., 2005; Gabay and Kushner, 1999). As with fever, however, we also did not find any evidence for changes in WBC count of bats in any of the experimental groups. This result contradicts the findings of Schneeberger et al. (2013a) which reproted leukocitosis in *C.*

perspicilatta after LPS admininstration, but is in tandem with those reported for the *M. molossus* (Stockmaier et al., 2015). Furthermore, WBC count baselines observed in our study were low (three cells on average per visual field at 200X) when compared with WBC count made for other bat species and even with *C. perspicillata* (Schneeberger et al., 2013a, 2013b; Stockmaier et al., 2015). The reduction in the numer of circulating leukocytes have been observed in other mammals at low T_b (Bouma et al., 2010), but this is not the case in our study, since we did not have evidence of hipothermia during the experiments in any of the experimental groups. Therefore, the inconsistence between results may be explained by the time between blood samples, since we collected the blood nine hours after the injection, while Schneeberger et al. (2013a) collected only 24 hours after the injection. If the increase of circulating leukocyte started after the nine hour post-injection period in our study, it was not possible for us to detect it.

We only observed significant increase in N/L ratio in bats injected with LPS in the FRW group. This group showed increase of 1.54 times above the PBS values. Besides the lack of food the night before experiments, this group experienced also low ambient temperatures in contrast with summer groups. The increase in N/L ratio is commonly used as a stress indicator (Davis et al., 2008), since the release of glucocorticoids due to stress caused neutrophilia and lymphopenia. Glucocorticoids are known for causing immunosuppression (Ader and Cohen, 1993), but they may also enhance the immune function, and that is why they have an important role during the APR. The production of APP in the liver is induced by a synergistic action of cytokines and glucocorticoids (Wilckens and De Rijk, 1997), so we could expect that in the FRW group specifically, the increase in glucocorticoids will be beneficial since it will boost the immune response of these bats. Considering this, we believe that the increase in N/L ratio is a consequence of the increase of glucocorticoids in the bloodstream, which may be a mechanism used by the bats to enhance the effectiveness of the APR during extremely challenging situations (since we did not detect increase in N/L ratio after the PBS injection).

We observed a significant increase in the MR of *C. perspicillata* in all experimental groups, with an increase of 120-180% after LPS injection when compared to bats injected with PBS. Other studies have also reported the increase in MR after an LPS challenge. For example, Marais et al. (2011) observed an increase of 33% in Pekin ducks (*Anas platyrhynchos*) injected with 0.1 mg LPS kg^{-1} and King and Swanson (2013) observed increase of 40% in house sparrows (*Passer domesticus*) injected with 5 mg LPS kg^{-1}. In mammals, MacDonald et al. (2012) found a 10% increase in lab rats (*Rattus norvegicus*)

injected with 0.05 mg LPS kg^{-1}, while the administration of a dose of 0.5 mg LPS kg^{-1} did not cause increase in MR of *Mus musculus* (Baze et al., 2011). Nevertheless, when we tried to compare our results with those obtained in other studies, we noticed that, besides the mass-specific doses used differed between studies, every author calculated the percentage of increase in a total different way, which hinders any further comparison.

Because of these considerations, in our study we calculated the percentage of increase of the post-injection MR in a similar way it was calculated by Otálora-Ardila et al. (2016). These authors found an increase of 140-185% in MR in *M. viveisi* injected with 1.75 mg LPS kg^{-1}, which is similar to that observed by Cabrera-Martinez et al. (*Unpublished results*) in *G. soricina* (180% increase) after receive a dose of 2.84 mg LPS kg^{-1}, and also similar to what we found in *C. perspicillata* (120-180%) injected with 2.7-3.3 mg LPS kg^{-1}.

Some studies observed variations in the APR with respect to seasonality. There is evidence that shows that short-day lengths reduce the duration of LPS response in contrast to long-day lengths, as male Siberiam Hamsters (*Phodophorus sugorus*) and White-crowned Sparrows (*Zonotrichia leucophrys gambelii*) (Bilbo et al., 2002; Owen-Ashley et al., 2006). Owen-Ashley and Wingfield (2007) mentioned that such reduce may be correlated to seasonal changes in body fat composition and energy allocation, and that the APR is tightly regulated by energy reserves at the time of infection. At proximate levels, the differential allocation of limited internal energy resources is thought to drive trade-offs. In support of this, increased quality and quantity of food can result in improved immune responses (Brzek and Konarzewski, 2007), as it was observed in northwestern song sparrows (*Melospiza melodia morphna*), with seasonal variation in the magnitude of an individual's response to LPS, probably in part due to variation in energy stores (Owen-Ashley and Wingfield, 2006). However, we did not find any seasonal variation in the TEC of bats between the four experimental groups. Nelson and Demas (1996) suggested that immune function may not vary between seasons when environmental changes are not severe enough or even when the animals develop adaptations to cope with such changes. We hypothesized that, if the energetic cost of the APR did not vary between experimental groups, it will probably reflect an adaptation of *C. perspicillata* to environmental changes, specifically to variations in food availability and seasonality, which were the variables tested in our study. It may also reflect that for these bats, activating an APR may not have a substantial energetic cost, in such a way that even when they are susceptible to higher energy demands and lower resources availability, they are capable to maintain immunocompetence.

To fully interpret the magnitude of the increase in MR in this context, it is necessary to calculate the TEC associated with the response and then compare it in the context of the total energy budget of the species. Our calculations for the TEC during the APR return values that ranged between 0.25 to 0.47 kJ. Using an allometric equation with data on field MR for bats compiled by Speakman and Król (2010), we calculated that the average daily energy expenditure of a 16 grams *C. perpsicillata* would be about 50 kJ day^{-1}. Thus, the energy invested in mounting an innate immune response is only, at most, 1% of the daily energy budget of this species. Herbst (1986) measured the energy content of *Piper amalago*, which is one of the most common fruits eaten by *C. perspicillata*, and found that the energy reward obtain by ingesting one infructescens (~1.25 g) is about 6.1 kJ for the bats. Considering that only 70% of the infructescens is assimilated due to incomplete fruit uptake, and that the absorption of nutrients is limited, the bats would need to harvest on average 12 fruits of *P. amalago* to fulfill their daily energy requirement of 50 kJ day^{-1} (Voigt et al., 2006). Thus, the costs associated with the APR in *C. perspicillata* could be fully covered if these bats eat an additional single fruit per night. Such trivial costs might explain why we did not find any seasonal effects on the TEC. If just one fruit is what takes to pay the cost of the APR activation, we could expect that even during winter, when food availability is lower and bats need to expend more energy with thermoregulation, it would be feasible to increase the food intake in such little amount to over both costs. Studies on bats that measured the total costs in a way similar as we did here, found that the costs of the APR comprises between 2 and 12% of the energy budget of, respectively, *G. soricina* (Cabrera-Velarde et al, *Unpublished results*) and *M. vivesi* (Otálora-Ardila et al., 2016). Although the increase in MR seems to be substantial in bats after LPS injection, the total cost is tirival and do not jeopardize their energy budget. In this context, we found no suport for the suggestion that the activating the innate immune system, and the APR in particular, entails a substantial energetic expenditure.

In summary, our results suggest that the magnitude of activating an immune response in *C. perspicillata* is energetically costly (120-180% increase in TEC), but the extra energy needed to pay the APR activation do not compromise the bats daily energy budget (1% of the daily energy budget). Bats are long-lived species which need to survive through several reproductive seasons in order to achieve fitness. Some author suggest that for long-lived species it would be more advantageous to rely in the adaptive immune system in contrast to short-lived species that would depend on a fast and effective response as those of the innate arm, in order to fight infection and survive (Cutrera et al., 2010; Lee, 2006; Lochmiller and

Deerenberg, 2000; Martin et al., 2006a, 2006b). To understand in what extent bats rely more in the innate or adaptive arm of the immune system requires additional study.

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Table 1. Mass-specific doses of LPS inoculated in each experimental group. Values are presented as mean ± s.d. Numbers in parenthesis denote range of observations.

	ALS	FRS	ALW	FRW
Mass-specific dose (mg kg-1)	**2.7 ± 0.3** (2.4 - 3.2)	**3.3 ± 0.2** (3.1 – 3.7)	**2.8 ± 0.2** (2.6 - 3.0)	**3.2 ± 0.3** (2.8 - 3.7)

Table 2. Initial body mass (M_{bi}), final body mass (M_{bf}) and body mass loss (ΔM_b) of *C. perspicillata* challenged with either LPS or PBS. Values are presented as mean ± s.d. Numbers in parenthesis denote range of observation.

	Treatment	M_{bi}(g)	M_{bf} (g)	Body mass loss (%)
ALS	LPS	**18.43 ± 1.92**	**16.08±1.9**	**12.8± 1.7**
		(15.4 – 20.8)	(13.3-18.6)	(-0.154 to - 0.106)
	PBS	**17.87±1.51**	**16.05±1.6**	**10.2±2.9**
		(15.2-19.5)	(13.4-18.3)	(-0.134 to - 0.062)
		$F_{1,11} = 0.323$; $p = 0.582$		$F_{1,11} = 3.676$; $p = 0.084$
FRS	LPS	**15.22 ± 1.03**	**13.82 ± 0.99**	**9.2 ±1.5**
		(13.6-16.2)	(12.3-14.8)	(-0.111 to -0.069)
	PBS	**15.35 ±0.99**	**14.18 ±0.8**	**7.5 ±1.2**
		(14.0-16.3)	(13.0-14.7)	(-0.093 to -0.057)
		$F_{1,13} = 0.101$; $p = 0.756$		$F_{1,13} = 6.314.$; $p = 0.027$
ALW	LPS	**17.75 ±1.06**	**15.78 ±0.87**	**11.0 ±2.3**
		(16.4-18.9)	(14.4-16.5)	(-0.143 to -0.078)
	PBS	**17.58 ±1.6**	**15.68 ±1.39**	**10.8 ±0.7**
		(15.5-19.2)	(14.0-17.0)	(-0.115 to -0.097)
		$F_{1,11} = 0.0452$; $p = 0.836$		$F_{1,11} = 0.0645$; $p = 0.805$
FRW	LPS	**15.82±1.6**	**14.23±1.21**	**9.9±2.0**
		(13.5-18.1)	(12.6-15.9)	(-0.122 to -0.067)
	PBS	**15.02±1.37**	**14.10±1.38**	**6.1±2.9**
		(13.1-16.9)	(11.8-15.9)	(-0.099 to -0.014)
		$F_{1,11} = 0.867$; $p = 0.374$		$F_{1,11} = 6.880$; $p = 0.025$

Table 3. Initial (T_{bi}), peak (T_{bp}), final (T_{bf}) body temperature and body temperature increase (ΔT_b) of *C. perspicillata* after injection of either LPS or PBS. Values are presented as mean ± s.d. Numbers in parenthesis denote range of observations.

	Treatment	T_{bi} (°C)	T_{bp} (°C)	T_{bf} (°C)	Body temperature increase (%)
ALS	LPS	**33,8±2,1** (30,1-35,9)	**35,1±1,8** (32,8-37,2)	**34,6±1,8** (31,9-36,6)	**03.69±03.85** (0,0028-0,0887)
	PBS	**33,8±1,8** (31,1-35,7)	**34,1±1,9** (31,0-36,2)	**34,6±1,5** (32,5-36,8)	**0.71±0.96** (-0,0059-0,0182)
		$F_{1,11}$ =0.002; p = 0.965			$F_{1,11}$ = 3.345; p = 0.097
FRS	LPS	**31,7±1,7** (31,1-34,2)	**33,7±1,8** (30,0-35,4)	**33,6±0,7** (32,7-34,4)	**5.87±4.67** (-0,0148-0,1114)
	PBS	**31,5±1,8** (28,7-33,9)	**32,2±1,2** (30,7-33,8)	**33,2±1,3** (31,0-34,5)	**1.96±3.93** (-0,0211-0,1031)
		$F_{1,13}$ = 0.0327; p = 0.86			$F_{1,13}$ = 2.813; p = 0.119
ALW	LPS	**33,7±1,0** (32,1-34,8)	**35,1±0,9** (34,2-36,7)	**34,0±1,4** (32,3-36,2)	**3.81±3.23** (-0,0087-0,0708)
	PBS	**33,0±1,8** (29,9-34,6)	**33,5±1,1** (32,0-34,8)	**34,6±0,9** (33,4-35,7)	**1.51±3.97** (-0,0269-0,08)
		$F_{1,11}$ = 0.819; p = 0.387			$F_{1,11}$ = 1.161; p = 0.307
FRW	LPS	**32,2±1,7** (30,0-34,0)	**34,4±2,1** (31,1-36,4)	**33,6±2,9** (29,6-36,7)	**6.23±6.09** (-0,0245-0,1566)
	PBS	**30,1±2,5** (27,8-33,8)	**31,7±2,4** (29,0-34,5)	**32,9±2,4** (30,1-35,8)	**4.77±7.87** (-0,07-0,1716)
		$F_{1,11}$ = 3.3039; p = 0.112			$F_{1,11}$ = 0.092; p = 0.768

FIGURE CAPTIONS

Figure 1. N/L ratio in *C. perspicillata* before and after injection of either LPS or PBS and for each experimental group: fed *ad libitum* during summer (ALS), in food restriction during summer (FRS), fed *ad libitum* during winter (ALW) and in food restriction during winter (FRW). Asterisks denote statistical significance between pre and post injection, observed only in the FRW group when bats were injected with LPS.

Figure 2. WBC in *C. perspicillata* before and after injection of either LPS or PBS. No statistically differences were found between pre and post-injection values in any of the experimental groups. Details of the experimental groups are the same as in Figure 1.

Figure 3. Metabolic rate of bats following an injection of either LPS (black) or PBS (white). Details of the experimental groups are the same as in Figure 1. Asterisks indicate statistical difference between treatments ($p \leq 0.05$).

Figure 4. Total energetic cost (TEC) of the APR in *C. perspicillata* after immune challenge with LPS, with each curve representing one experimental group. The TEC was not statistically different between groups. Details of the experimental groups are the same as in Figure 1.

Figure 1.

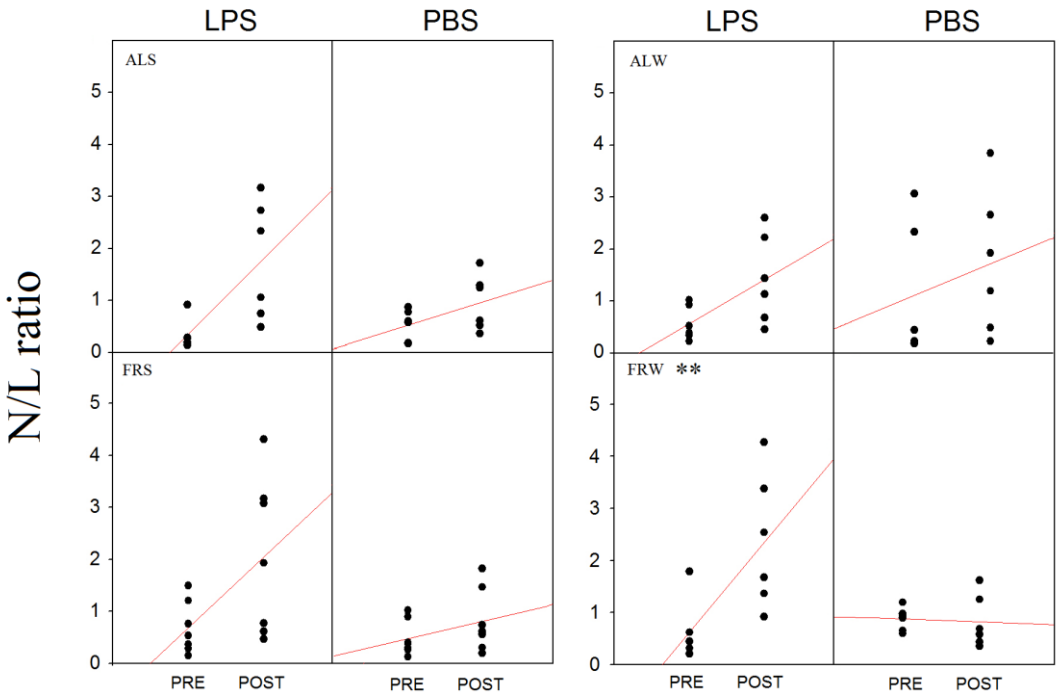

N/L ratio
LPS
PBS
LPS
PBS
ALS
ALW
FRS
FRW **
0
1
2
3
4
5
PRE
POST
PRE
POST
PRE
POST
PRE
POST

Figure 2.

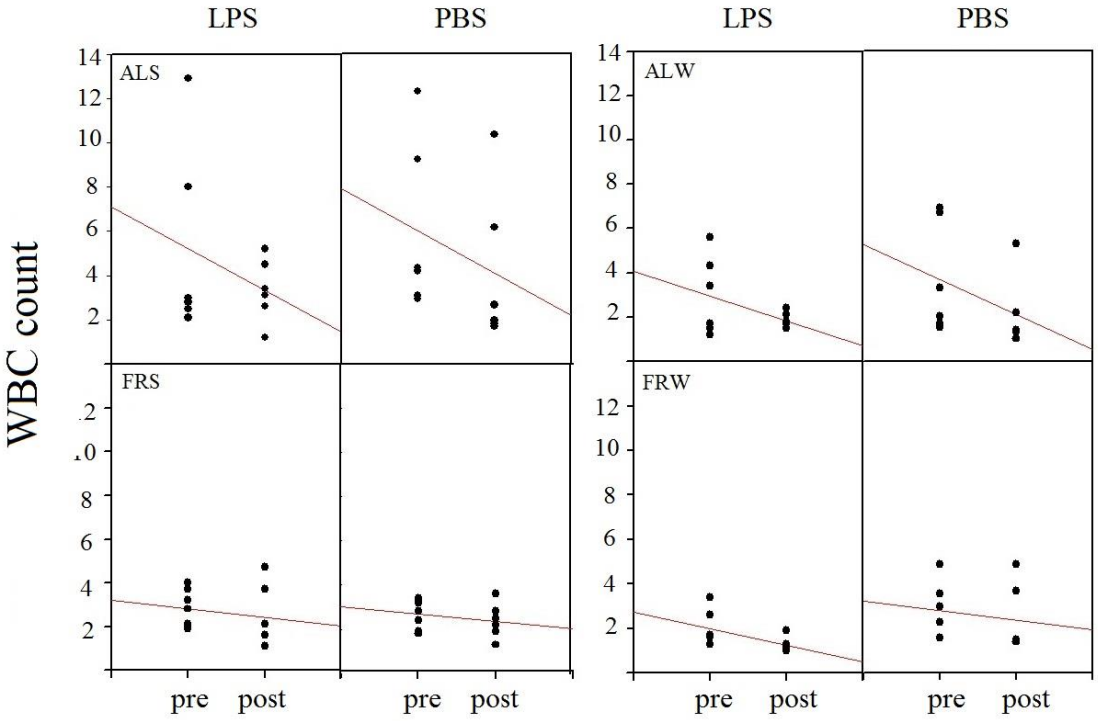

LPS
PBS
LPS
PBS
WBC count
ALS
ALW
FRS
FRW
pre
post
pre
post
pre
post
pre
post

Figure 3.

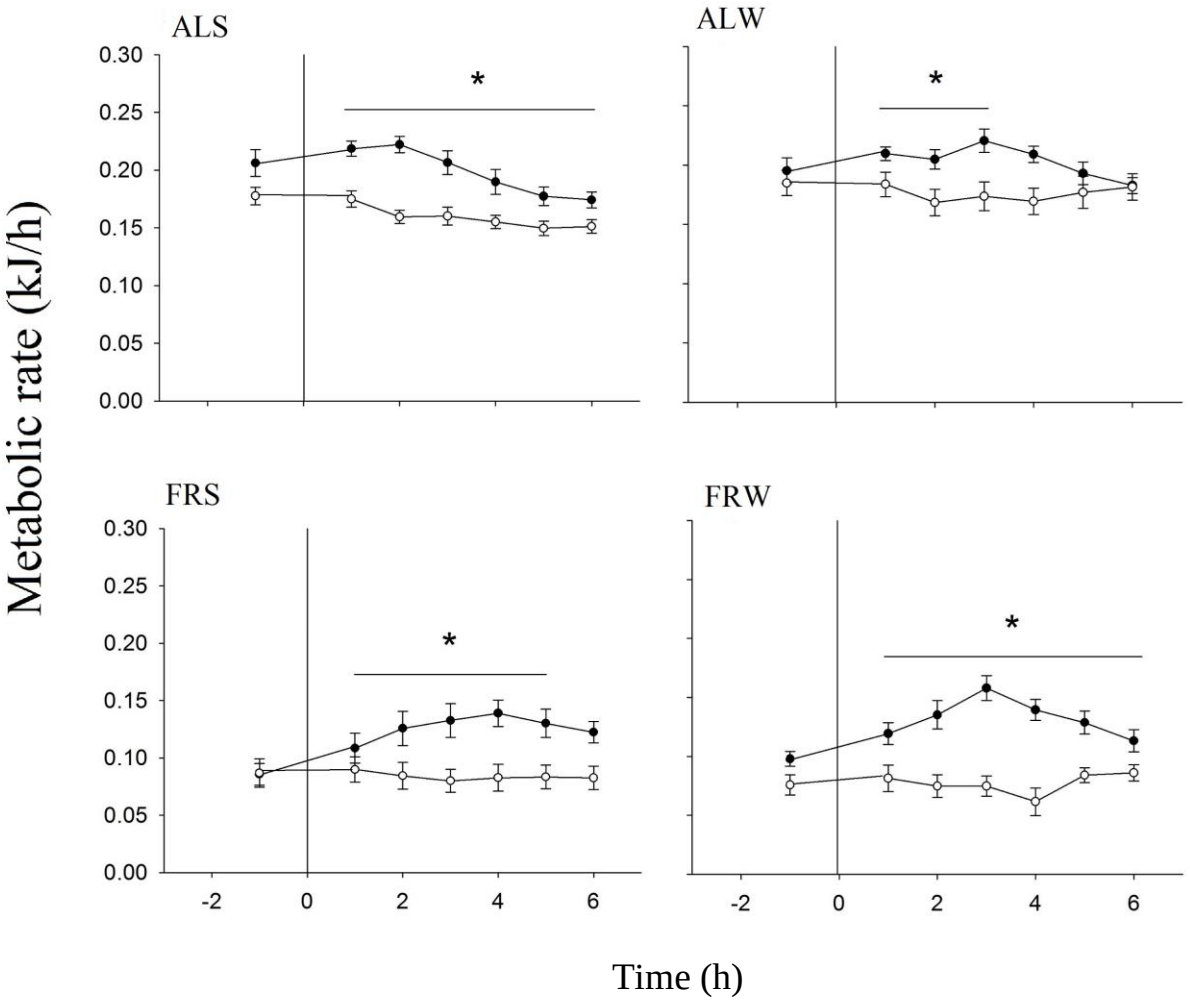
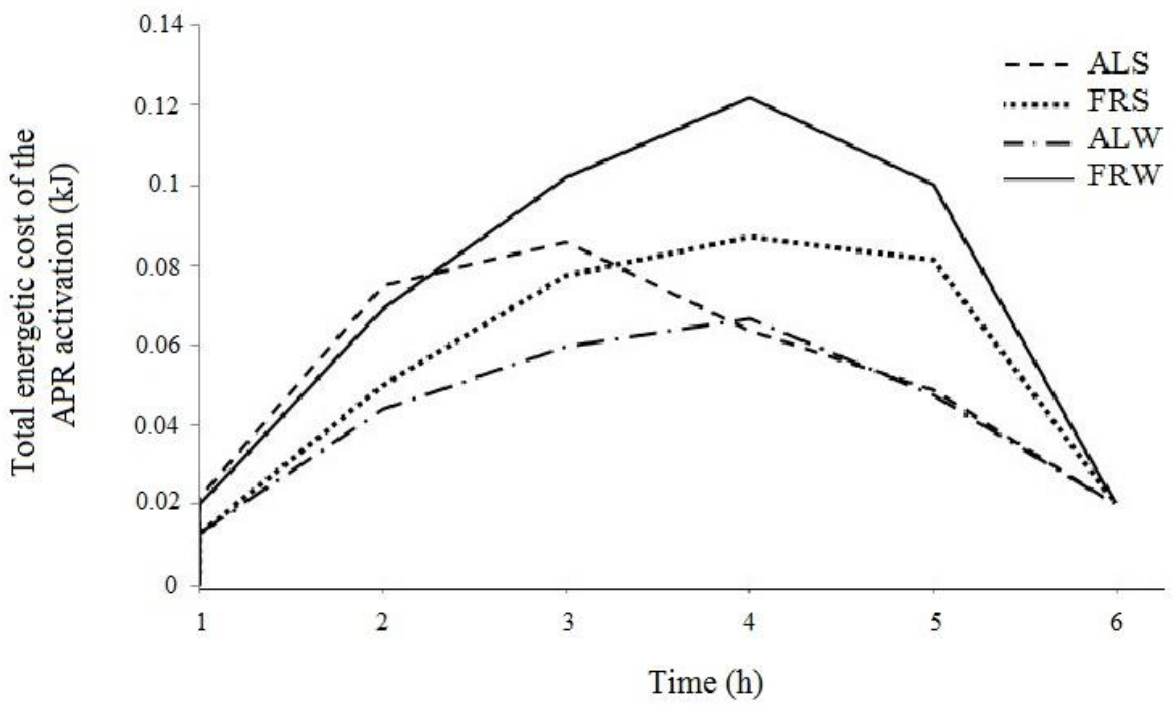

ALS
ALW
FRS
FRW
*
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-2
0
2
4
6
Metabolic rate (kJ/h)
Time (h)

Figure 4.


Total energetic cost of the APR activation (kJ)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
1
2
3
4
5
6
Time (h)
ALS
FRS
ALW
FRW