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PHA-induced inflammation is not energetically costly in the subterranean rodent *Ctenomys talarum* (tuco-tucos)

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ABSTRACT

Immune activity has been proposed to be associated with substantial costs, due to trade-offs with other functions or activities that share common resources and contribute to an animal's fitness. However, direct estimates of the cost of mounting an immune response are few and have been performed mainly in birds. Thus, further work is needed to clarify the relative costs of different components of the immune system and the role of environmental and life-history traits in modulating the costs of resistance. Within the components of immunity, inflammation is considered to be associated with a larger energetic expenditure. Here, we evaluated the energetic cost of the inflammatory response to phytohemagglutinin (PHA) in a wild population of a subterranean rodent, *Ctenomys talarum*, and the trade-offs between immune activity and reproduction. *C. talarum* develops an inflammatory response to PHA, but contrary to our predictions, this response was not associated with an increase in oxygen consumption regardless of reproductive status or sex. Our study shows that an immune challenge may not always result in a detectable energetic cost. We discuss the possibility that other currencies could be underlying the cost, such as micro-or macronutrients requirements, autoimmunity or oxidative stress.

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1. Introduction

The vertebrate immune system provides defense against pathogens, cell vigilance to eliminate potential tumor cells and helps maintain individual homeostasis (Gómez-Lucia et al., 2007). These fitness benefits may be associated with substantial costs (Lochmiller and Deerenberg, 2000). Traditionally, immune costs have been indirectly assessed as trade-offs with other functions or activities that share common resources and contribute to an animal's fitness (Sheldon and Verhulst, 1996; Lee, 2006), such as reproduction, growth and development (see Klasing et al., 1987; Norris and Evans, 2000; Gervasi and Foufopoulos, 2008; Schwanz et al., 2011; Hasselquist and Nilsson, 2012). Most of these studies support the assumption that immunological defenses are costly. However, the mechanisms that mediate these trade-offs are more enigmatic (Hasselquist and Nilsson, 2012) and direct estimates of the cost of the immune response are required to elucidate this. Four currencies are generally considered as potential mediators of these trade-offs: energetic costs, nutrient costs, autoimmunity and oxidative stress (Hasselquist and Nilsson, 2012). Energy is the most common currency predicted to underlie trade-offs between immune responses and other life history traits (Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000). However, direct estimates of the energetic cost of mounting an immune response are few and have been performed mainly in birds (reviewed by Klasing, 2004; Hasselquist and Nilsson, 2012; but see Cooper et al., 1989; Demas et al., 1997; Råberg et al., 2002; Derting and Virk, 2005). Although frequently assumed, the existence of a positive relationship between the magnitude of the energetic cost and the strength of immune response needs to be verified (Saino et al., 2002). From the few available studies, mainly involving phytohemagglutinin (PHA) and sheep red blood cells (SRBC) as antigens, the relationship between immune response and the change in minimal energy metabolism is not yet clear (e.g. Svensson et al., 1998; Nilsson et al., 2007). Further work is needed to clarify our knowledge about the relative costs of different components of the immune system (Klasing, 2004) and the role of environmental and life-history traits in modulating the costs of resistance (Sandland and Minchella, 2003).

Within the components of immunity, inflammation, which is primarily triggered by the entry of microorganisms or tissue damage, is considered to be associated with a large energetic expenditure, since it involves the production of cytokines by activated macrophages and the subsequent recruitment and infiltration of leukocytes (Klasing and Leshchinsky, 1999). In contrast, the energetic cost of the production of lymphocytes required for an adaptive immune response is assumed to be very low (Klasing and Leshchinsky, 1999). The present study was

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designed to evaluate the cost of the inflammatory response in a wild population of a subterranean rodent, *Ctenomys talarum* (tuco-tuco), and the trade-offs between immune activity and reproduction. The inflammatory response was induced by the subcutaneous injection of PHA, a plant lectin widely used in ecological studies (Martin et al., 2006). This lectin triggers both the innate immune system (inflammation) and the cell-mediated arm of the adaptive immune system (Goto et al., 1978), nevertheless the relative importance of these two systems in the reaction differs between species (Martin et al., 2006; Vinkler et al., 2014). The use of PHA allowed us to specifically assess the cost of the inflammatory response, without triggering additional costs associated with pathogen replication and tissue damage for the host. Several reasons make *C. talarum* an interesting study subject to analyze the costs of the inflammatory response. Much is known about the natural history, ecology, and physiology of this species (Busch et al., 2000; Zenuto et al., 2002; Antinuchi et al., 2007). Particularly relevant to this study is that a) the energetic costs of a large number of their activities are budgeted (Antinuchi et al., 2007), b) a significant energetic cost has been reported associated with the adaptive humoral response, which varies in relation with reproductive condition (Cutrera et al., 2010), and, finally, c) an inflammatory response to PHA is detectable in tuco-tucos as an increase in thickness of the affected area and the cellular processes underpinning this response have been described (Merlo, 2011). Tuco-tucos develop an inflammatory response to PHA 6 h after immunization and it lasts 48 h. However, the peak of infiltrating leukocytes in the zone is verified at 12 h post-injection, and it is composed mainly by neutrophils (innate-arm cells; Merlo, 2011). However, whether the inflammatory response is associated with a substantial energetic cost remains unknown. Based on the available information regarding the dynamics of the inflammation response at the cellular level in *C. talarum*, we hypothesize that the PHA-induced inflammatory process in this species entails a substantial energetic cost revealed by an increase in oxygen consumption in animals exposed to the antigen with respect to control individuals, a technique used in several studies – including *C. talarum* (Antinuchi et al., 2007) – to assess the cost of many physiological activities (Goldstein, 1988). Therefore, we expect 1) a significant increase in oxygen consumption associated with the inflammatory response to PHA, differing at 6 and 12 h post-injection given that the cellular processes acting at these time points are different, and 2) the investment in the inflammatory response to vary between seasons (reproductive vs. non-reproductive) and sexes, because energetic demands for individuals in the reproductive season are higher, particularly for females.

2. Material and methods

2.1. Animal capture and captivity conditions

A total of 65 adult *C. talarum* (tuco-tucos) were live-trapped in the locality of Mar de Cobo, Buenos Aires Province, Argentina (37°46′ S 57°27′ W) using wire tube-shaped live traps (10 cm diameter, 35 cm length) set at fresh surface mounds. Specifically, 11 males (151 ± 22 g) and 10 females (107 ± 9 g) were caught during the reproductive season (September to early December 2010) and 22 males (156 ± 28 g) and 22 females (118 ± 15 g) were caught during the non-reproductive season (April to early May 2012 and 2014). Body weight of animals caught did not differ between years of capture (two-way ANOVA, $F_{(1,62)} = 0.55$, $p = 0.58$). When nursing females were trapped, they were immediately released back into their burrow system so as not to deprive dependent young of maternal care. Animals were transported to the Laboratory of Ecophysiology at the National University of Mar del Plata (Mar del Plata, Argentina) where they were weighed and put in individual plastic boxes (25 cm × 32 cm × 42 cm) provided with a wire-mesh lid and lined with wood shavings as bedding. Animals were fed ad libitum quantities of a mixture of chicory, lettuce, corn, sweet potatoes and sunflower seeds. Room conditions (temperature and photoperiod) were automatically controlled (25 ± 1 °C, 14L: 10D). Tuco-tucos remained captive for the duration of the experimental assays (ca. 10 days) after which they were released at the point of capture. Field and labwork were performed according to the American Society of Mammalogists guidelines (Gannon and Sikes, 2006).

2.2. Immunization and experimental protocol

Individuals of each sex were randomly assigned to two groups: control (C; reproductive season: $n_m = 6$, $n_f = 5$; non-reproductive season: $n_m = 10$, $n_f = 10$) and immune challenged (IC; reproductive season: $n_m = 5$, $n_f = 5$; non-reproductive season: $n_m = 12$, $n_f = 12$; Table 1). Animals remained in captivity for 7 days before beginning the experiments because this is the necessary time lapse to acclimatize to captivity conditions and, hence, lower their stress levels (Vera et al., 2008). Therefore, 7 days after capture, oxygen consumption ($\dot{V}O_2$) of animals from both groups was measured as described below. The following day, the thickness of the right hind foot was measured with a digital micrometer (Insize®) to the nearest 0.01 mm following Merlo (2011). Immediately after that, control animals were injected subcutaneously in the instep of the right hind foot with sterile phosphate-buffered saline solution (C group: PBS; 0.3 μL/g of mass) using a 30G needle. Immune challenged animals were injected in the same manner with phytohemagglutinin (IC group: *Phaseolus vulgaris* PHA-Sigma L-8754 solution dissolved in PBS, 3 mg/mL; 0.3 μL/g of mass). Prior to injection, the area was treated with an antiseptic solution (PERVINOX®, Phoenix laboratories). The $\dot{V}O_2$ of 10 males (5 C and 5 IC) and 10 females (5 C and 5 IC) from the non-reproductive season was measured 6 h after these treatments. For the rest of the animals, the second $\dot{V}O_2$ measurement was performed 12 h after injection (n = 23 males and n = 22 females). Oxygen consumption measurements were scheduled 6 or 12 h post-injection because tuco-tucos mount a detectable inflammatory response that starts 6 h after the PHA-injection, but the leukocyte peak in the affected zone occurs at 12 h post-injection, according to a previous study (Merlo, 2011). In this way, measuring $\dot{V}O_2$ at 6 h provided us with information about the cost of the onset of inflammatory response, while at 12 h we measured the energy demand associated with the maximum recruitment of leukocytes to the affected area.

Table 1
Mean body mass (g ± SD) of male and female *Ctenomys talarum* at 0, 6 and 12 h post phytohemagglutinin (PHA)- or phosphate buffer (PBS)-injection during the reproductive and the non-reproductive seasons. *N* for each group is shown between parentheses.

Season	Treatment	Sex	Time (h)	Body mass (g)
Reproductive	PHA	Male	0	146.8 ± 30.5
		(n = 5)	12	148.3 ± 28.1
		Female	0	105.3 ± 3.6
		(n = 5)	12	103.2 ± 6.0
	PBS	Male	0	154.7 ± 13.1
		(n = 6)	12	161.6 ± 8.0
		Female	0	109.7 ± 13.1
		(n = 5)	12	110.3 ± 11.1
Non-reproductive	PHA	Male	0	167.3 ± 22.0
		(n = 7)	12	169.4 ± 22.2
		Female	0	118.3 ± 10.0
		(n = 7)	12	117.8 ± 12.3
	PBS	Male	0	137.1 ± 25.7
		(n = 5)	12	136.5 ± 25.7
		Female	0	114.0 ± 16.5
		(n = 5)	12	115.3 ± 17.1
	PHA	Male	0	160.1 ± 32.1
		(n = 5)	6	159.3 ± 31.2
		Female	0	118.0 ± 16.3
		(n = 5)	6	121.2 ± 19.2
	PBS	Male	0	154.8 ± 31.0
		(n = 5)	6	153.1 ± 25.3
		Female	0	121.8 ± 22.6
		(n = 5)	6	123.4 ± 27.8

Thickness of the right hind foot of all animals was measured again 24 h post-injection. Inflammatory response was calculated as the difference between pre- and post-injection thickness divided by initial thickness ((response = (post − pre) / pre); Xu and Wang, 2010).

2.3. Measurement of $\dot{V}O_2$

Oxygen consumption was measured using a computerized positive-pressure open-flow respirometry system (Sable System, Las Vegas, NE, USA) as described by Luna et al. (2009, 2012) and Cutrera et al. (2010). $\dot{V}O_2$ was measured in the morning or in the afternoon, because *C. talarum* exhibits an arrhythmic pattern of daily activity (Luna et al., 2000). The metabolic system consisted of a transparent acrylic cylinder of 1.8 L volume. Temperature was adjusted automatically by a computerized system (nearest 1 °C). The chamber received dry and CO_2-free air at 1400 mL min^{-1} from a mass flow controller (Sierra Instruments, Monterey, CA, USA) to ensure chamber equilibrium (Lasiewski et al., 1966). Air passed through a CO_2 absorbent (IQB, Quimica Kubo, Mar del Plata, BA, Argentina) and a water scrubber (Silica Gel, Quimica Kubo) before and after passing through the chamber. Excurrent air from the metabolic chamber was sub-sampled at 180 ± 10 mL min^{-1}, and $\dot{V}O_2$ was obtained from an Oxygen Analyzer FC-1B every second set by an ExpeData software (Sable System). Animals were allowed to habituate in the respirometry chamber, for at least 30 min. The baseline of the respirometry system was set in 20.95% of oxygen before the beginning of each experiment. Rate of oxygen was calculated using Eq. (4a) from (Withers, 1977). Oxygen consumption was calculated as the 5-min lowest steady-state values of a 60-min trial (Luna and Antinuchi, 2007), and expressed as mL O_2 consumed per hour (mL O_2 h^{-1}). All results are presented as means ± S.D.

2.4. Statistics

All tests were performed in Statistica (Statsoft, Tulsa, OK, USA) using $\alpha > 0.05$ to reject the null hypothesis. The normal distribution of variables was tested using Kolmogorov–Smirnov tests, and variance equality was verified using Levene's tests. A three-way ANOVA was used to evaluate the effect of season (reproductive vs. non-reproductive), sex and treatment (C vs. IC) on the inflammatory response. A one-way repeated measures ANCOVA was used to test the effect of treatment (PHA vs. PBS) on body weight at 0 and 12 h. A two-way repeated measures ANCOVA (with body weight as the covariate) was used to assess the effect of treatment (C vs. IC) and the time of the respirometry measurement (6 vs. 12 h) on the $\dot{V}O_2$ of animals captured during the non-reproductive season. A three-way repeated measures ANCOVA (with body weight as the covariate) was used to test the hypothesis that oxygen consumption did not differ between seasons, sexes or treatments. In order to evaluate if the strength of the inflammatory response was positively correlated with the animal's oxygen consumption, the effects of body mass on thickness measurements and $\dot{V}O_2$ 12 h post PHA-injection were first removed using the residuals of a linear regression of thickness measurement and $\dot{V}O_2$ 12 h post PHA-injection on body mass. Residual thickness measurements were correlated with residual $\dot{V}O_2$ measurements.

3. Results

The swelling response was significantly greater in PHA-injected animals ($n_{rep} = 10$, $n_{non\text{-}rep} = 24$) compared to PBS-injected ones ($n_{rep} = 11$, $n_{non\text{-}rep} = 20$) (three-way ANOVA, $F_{(1,57)} = 69.27$, $p < 0.001$; Fig. 1). No differences were detected in the swelling response between seasons or sexes (three-way ANOVA, season factor: $F_{(1,57)} = 0.27$, $p = 0.60$; sex factor: $F_{(1,57)} = 0.002$, $p = 0.97$; Fig. 1).

Body weight at 0 and 12 h did not differ between PHA-injected and control animals (one-way ANCOVA, $F_{(1,43)} = 0.77$, $p = 0.39$; Table 1). $\dot{V}O_2$ did not differ at 6 and 12 h post-injection (two-way ANCOVA, $F_{(1,39)} = 0.31$, $p = 0.58$; Fig. 2). Oxygen consumption of PHA-injected animals did not differ from that of PBS-injected animals (three-way repeated measures ANCOVA, $F_{(1,35)} = 2.83$, $p = 0.10$; Table 2; Fig. 2). Moreover, sex or season did not have significant effects on $\dot{V}O_2$ (three-way repeated measures ANCOVA, sex: $F_{(1,35)} = 0.06$, $p = 0.81$; season: $F_{(1,35)} = 3.15$, $p = 0.08$; Table 2; Fig. 2). Residuals of the regression between body weight and inflammation did not correlate with residuals of the regression between body weight and increase in $\dot{V}O_2$ 12 h after injection ($r = 0.20$, $p = 0.36$, $n = 24$).

4. Discussion

Mounting an immune response is expected to require resources that could otherwise be allocated to other biological functions (e.g., growth, reproduction). Several studies have provided evidence for the energetic costs of immunity using a variety of antigenic stimuli (as reviewed by Muehlenbein et al., 2010). Here, we assessed the energetic cost of mounting an inflammatory response against PHA in the subterranean rodent *C. talarum*, finding that it was not associated with an increase in oxygen consumption. Our findings are in line with results from

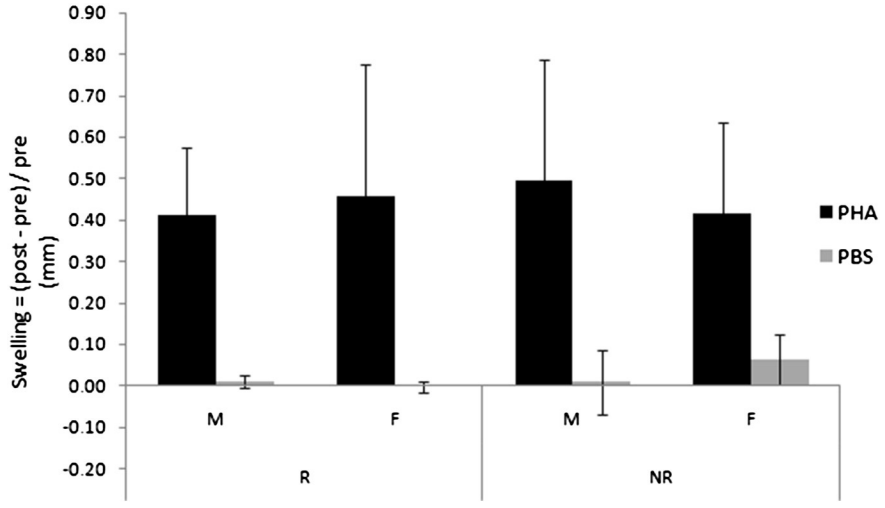

Fig. 1. Mean hind feet swelling (±SD) of males (M) and females (F) *Ctenomys talarum* caught during the reproductive (R) or the non-reproductive (NR) season, at 24 h post phytohemagglutinin (PHA) or phosphate buffer saline (PBS) injection. Letters denote statistical differences between treatments (PHA vs. PBS).

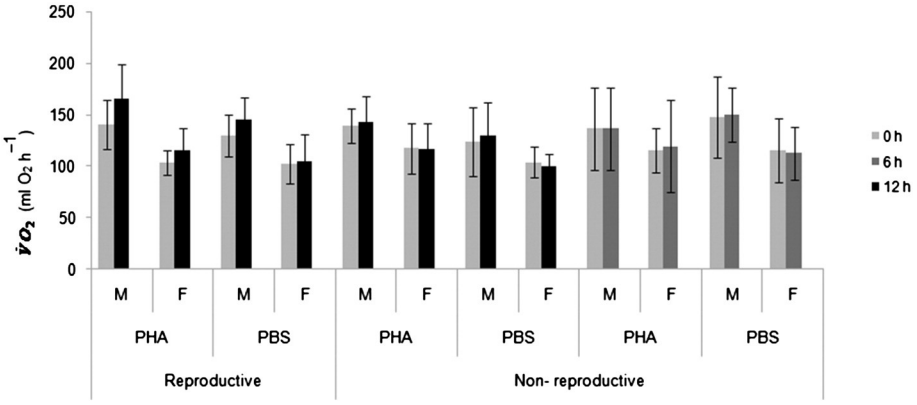

Fig. 2. Mean oxygen consumption ($\dot{V}O_2$) (mL O_2 h^{-1} ± SD) of male (M) and female (F) *Ctenomys talarum* caught during the reproductive (R) and the non-reproductive (NR) season, at 0, 6 and 12 h post phytohemagglutinin (PHA) or phosphate buffer saline (PBS) injection.

studies performed in other species of rodents. Mongolian gerbils and white-footed mice injected with PHA had similar RMRs to PBS injected ones (Derting and Compton, 2003; Zhiqiang et al., 2011). In birds, the energetic cost of the PHA-response seems to be variable among species. House sparrows (*Passer domesticus*) increased their RMR in a 29–32% (Martin et al., 2003) when injected with PHA, while great tits (*Parus major*) only showed an increase of 5% (Nilsson et al., 2007). In contrast, metabolic rate of Japanese quail (*Coturnix coturnix*) did not change in response to this immune challenge (Boughton et al., 2007), and even a 20–25% decrease in RMR was reported in PHA-injected tree sparrows (*Passer montanus*, Lee et al., 2005). Tuco-tucos, in addition to maintaining their metabolic rate when injected with PHA, did not show a detectable energetic trade-off between immunity and reproduction: oxygen consumption and inflammation of PHA-injected animals was similar in reproductive and non-reproductive animals, and differences were not observed between sexes. In contrast, trade-offs between immunity and reproduction were detected in other rodent species. Pregnancy and lactation both suppress humoral immunity in long-day exposed Siberian hamsters (Drazen et al., 2003). Also, serum bactericidal capacity and size of the thymus were suppressed in lactating compared with non-reproductive Mongolian gerbils, but the PHA response was not reduced (Yang et al., 2013). Contrary to this evidence, the PHA response was not affected by the increased investment in reproduction of lactating voles, and even the humoral and the innate immunity were enhanced under this physiological condition (Yang et al., 2013). Thus, not all studies performed in rodent species showed a negative relationship between reproductive and immunological functions, as usually expected. Given the complexity of the immune system and the diversity of antigens, whose effects over the host vary in intensity and duration, generalizations regarding the costs of resistance are difficult to state (Klasing, 2004).

Reduced activity during an immune challenge is a common strategy to reallocate energy and thus, maintain the daily energy expenditure (Hõrak et al., 2003). Even when digging was not possible for our caged animals, levels of activity could vary between individuals. Nonetheless, a significant increase in RMR associated with the activation of an artificially-induced humoral response in *C. talarum* challenged with sheep red blood cells (SRBCs) was detected under the same laboratory conditions used in the present study (Cutrera et al., 2010), suggesting that reduced activity, could not solely explain the absence of an increase in RMR in PHA-injected animals. Based on the present results, we can conclude that the inflammatory response in *C. talarum* does not entail a significant increase in energetic requirements. However, other currencies not tested yet could be underlying the cost of the PHA- response, such as micro-or macronutrients requirements, autoimmunity or oxidative stress.

The synthesis and proliferation of leukocytes and cytokines responsible of the inflammatory response can potentially induce an increase demand for micro and macronutrients of the diet (Klasing, 2004). On one hand, diet affects the general condition of the individual (e.g. by modulating stress and activity levels), and through this effect, diet may affect the immune system (Hasselquist and Nilsson, 2012; Schneeberger et al., 2013). On the other hand, some specific compounds of the diet (e.g. Vitamins A, D, E, polyunsaturated fatty acids, iron) directly regulate the immune response, frequently enhancing it (Klasing, 1998). The present study was conducted under conditions of ad libitum food availability. Animals did not experience a weight loss after the immune challenge, but it is possible that an increase in the requirements of specific nutrients was masked under conditions where resources were not limiting. To further explore the influence of diet on immunocompetence, future work will involve experimental manipulation of diet characteristics (amount of food supplied, protein supplementation, or glucose availability by means of 2-deoxi-D glucose administration) during the immune challenge.

Other costs of immune responses that should be considered are tissue damage or risks of autoimmune reactions (Theofilopoulos, 1995) and oxidative stress (Costantini and Møller, 2008). Tissue damage and release of oxidative particles are observed particularly during a chronic inflammation (Sorci and Faivre, 2009). For example, Schneeberger et al. (2013) found an increase in oxidative stress in wild bats that developed

Table 2
Results of three-way repeated measures analysis of covariance (ANCOVA) performed to evaluate the hypothesis of no differences in the oxygen consumption (mL O_2 h^{-1}) at 0 and 12 post-injection of individuals *Ctenomys talarum* between experimental groups, sexes and seasons.

Effect	F	p
Weight 0 h	3.60	0.07
Weight 12 h	0.05	0.83
Sex (males vs. females)	0.06	0.81
Season (reproductive vs. non-reproductive)	3.15	0.08
Treatment (IC vs. C)	2.83	0.10
Sex × season	0.62	0.44
Sex × treatment	0.27	0.61
Season × treatment	1.41	0.24
Sex × season × treatment	2.25	0.14
Time (within effect)	1.05	0.31
Time × weight 0 h	1.01	0.32
Time × weight 12 h	2.06	0.16
Time × sex	0.04	0.85
Time × season	5.35	0.00*
Time × treatment	0.63	0.43
Time × sex × season	0.02	0.90
Time × sex × treatment	0.25	0.62
Time × season × treatment	2.88	0.10
Time × sex × season × treatment	0.37	0.55

Degrees of freedom for each factor: 1; degrees of freedom for error: 35. IC: immune challenged; C: control. Asterisks denote significant effects.

an inflammatory response to the endotoxin LPS. Even though the PHA-response consists of a short time-induced inflammation (with swelling dissipating at 48 h after PHA-injection in *C. talarum*), the possibility that this response entails a cost in the manner of oxidative stress cannot be excluded.

During an energy-demanding period, the immune system may be adaptively down-regulated if there is a trade-off between immunity and, for example, reproduction. On the other side, in a case when the immune system needs to be activated, it could be adaptive to reduce the allocation to other costly activities (Råberg et al., 1998). Evidence suggests that the most frequent outcome of this trade-off is the first mentioned (Nordling et al., 1998; Ardia et al., 2003). Resource competition between reproduction and immunity may occur as an obligate response – resulting in all reproductive individuals suffering a fairly similar degree of immunosuppression – or as a facultative response — where reproductive demands combined with limited resources result in a variable immune response according to individual energy balance condition (French et al., 2007; Zysling et al., 2009). As in most vertebrates, reproduction in *C. talarum* includes many costly activities; lactation for females represents the highest daily energetic cost (lactating females increase in a 151% their RMR relative to non-reproductive females; Antinuchi et al., 2007). Pregnancy, although less costly in a daily basis – representing 128% RMR, a value that did not differ statistically from non reproductive females – also represents an energetically expensive process, even more considering that it extends by nearly 100 days (Antinuchi et al., 2007). In the present study, no differences were found in the magnitude of the PHA-swelling response between seasons or between sexes. Thus, the investment on innate immunity seems to be the same during the breeding and non-breeding season for *C. talarum*, suggesting that there may not be a trade-off between innate immunity and reproduction. These findings differ from those reported for the adaptive (humoral) response for this species, where an antibody induced response to SRBC represents an energetic cost for *C. talarum* (Cutrera et al., 2010). Therefore, in tuco-tucos, as well as in other vertebrates (see Lee, 2006), the innate immune response seems to be less costly than the adaptive response, thus not being subject to trade-offs with other energy-demanding processes, such as reproduction. It is worth noting, however, that only pregnant females were included in the experiment. Thus, whether there is a trade-off between lactation, the most demanding process of the reproduction in female tuco-tucos, and the inflammatory remains unknown.

The fact that the antibody production represents a major investment of energetic resources compared with the inflammatory response in *C. talarum* could be understood as a consequence of the natural history of this species. Particularly in the adulthood, tuco-tucos are frequently exposed to wounds received during fights for territory or mates (in the case of males), as well as to injuries related to subterranean activities (e.g. digging). Under this scenario, an energetically cheaper inflammatory response could have evolved in this species. Studies about the costs of resistance in relation to life-history traits are valuable since they contribute to our understanding of the ecological and evolutionary forces driving immunological variation (Lee, 2006). It is fundamental to continue our efforts to assess the various types of costs that may be associated with different branches of the immune system, beyond those strictly energetic, to get insight into the evolution of immunity in natural populations of wild species.

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