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ARTICLE

Inflammation in response to phytohemagglutinin injection in the Talas tuco-tuco (*Ctenomys talarum*): implications for the estimation of immunocompetence in natural populations of wild rodents

J.L. Merlo, A.P. Cutrera, and R.R. Zenuto

Abstract: The immunological variation in wild populations and its relation to life-history traits has recently become a central topic in the field of evolutionary biology, considering the critical contribution of immunity to an individual's fitness. A common technique used by ecologists to estimate immunocompetence in wild populations is the phytohemagglutinin (PHA) – skin test. In this test, the degree of local swelling triggered by PHA is usually considered an estimate of T-lymphocyte activity, although there is an ongoing debate regarding this interpretation. Here, we coupled the PHA–skin test with a histological analysis to examine the temporal development of the cell-mediated response in the subterranean rodent Talas tuco-tuco (*Ctenomys talarum* Thomas, 1898). The inflammation response involved lymphocytes, neutrophils, eosinophils, and macrophages at the site of injection, achieving an increase of total leukocytes from 12 to 48 h after injection. However, the abundance of any of the leukocytes observed did not correlate with the degree of swelling at any time studied, suggesting that caution should be taken when interpreting the results of the PHA-induced swelling response. Particularly, the magnitude of macroscopic swelling should not be considered a priori as indicative of T-lymphocyte activity in wild-caught rodents. Our results highlight the importance of avoiding oversimplified approaches to measuring immunocompetence.

Key words: cellular immune response, *Ctenomys talarum*, histological analysis, subterranean rodents, Talas tuco-tuco.

Résumé : La variabilité immunologique dans les populations sauvages et son lien avec les caractères du cycle vital sont récemment devenus un sujet d'intérêt central en biologie de l'évolution, au vu de la contribution essentielle de l'immunité à l'aptitude d'un individu. Le test cutané à la phytohémagglutinine (PHA) est une technique couramment utilisée par les écologues pour estimer l'immunocompétence dans des populations sauvages. Dans ce test, le degré d'enflure locale provoquée par la PHA est habituellement considéré comme fournissant une estimation de l'activité des lymphocytes T, bien que cette interprétation demeure controversée. Nous avons jumelé le test cutané à la PHA à une analyse histologique afin d'examiner le développement temporel de la réaction à médiation cellulaire chez le rongeur souterrain Talas tuco-tuco (*Ctenomys talarum* Thomas, 1898). La réaction d'enflure mettait en cause des lymphocytes, des neutrophiles, des éosinophiles et des macrophages au site d'injection, produisant une augmentation des leucocytes totaux dans l'intervalle de 12 à 48 h suivant l'injection. L'abondance des différents leucocytes observés n'était toutefois pas corrélée avec le degré d'enflure à quelque moment que ce soit, donnant à penser que la prudence est de mise dans l'interprétation des résultats de la réaction d'enflure provoquée par la PHA. En particulier, la magnitude de l'enflure macroscopique ne devrait pas être considérée « a priori » comme étant une indication de l'activité des lymphocytes T chez des rongeurs capturés à l'état sauvage. Nos résultats soulignent l'importance d'éviter les approches trop simplistes pour mesurer de l'immunocompétence. [Traduit par la Rédaction]

Mots-clés : réaction immunitaire cellulaire, *Ctenomys talarum*, analyse histologique, rongeurs souterrains, Talas tuco-tuco.

Introduction

The vertebrate immune system integrates innate and acquired responses (Janeway et al. 2004) that efficiently prevent and control pathogen infections (Roitt et al. 1996; Wakelin 1996). The innate response comprises the nonspecific first line of defense, which mobilize cells or release cytokines that generate the inflammatory response and instruct the induced (adaptive) response. In turn, the adaptive response consists of two arms: the humoral and the cell-mediated, which act against extracellular (e.g., bacteria and macroparasites) and intracellular (e.g., viruses) pathogens, respectively (Janeway et al. 2004).

The study of individual and specific variation of the immune response in natural populations of wild species represents a unique opportunity to evaluate immunity in an ecological and adaptive context, and is a powerful tool in the field of evolutionary biology, considering the critical contribution of immunity to an individual's fitness (Lochmiller and Deerenberg 2000). Because the immune system plays a crucial role in defending an animal

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against attack by pathogens and parasites, such a reduction in immunocompetence (i.e., an animal's ability to mount an effective immune defense; sensu Norris and Evans 2000) is likely to reduce fitness, so trade-offs involving immune defense could be crucial in determining optimal life-history decisions. In the past decade, ecologists have made progress exploring immunological variation in wild populations and its relation to life-history traits (reviewed by Ardia et al. 2011). Thus, acquired responses, which require substantial time and resources and are mostly beneficial against repeated infections, have been proposed to be favored in "slow-living-pace" species—with high per-offspring investment, more extensive developmental times, and longer lifespans—rather than in "fast-living-pace" species (Lee 2006). However, most studies have used techniques to measure a single indicator of immunocompetence or "immune responsiveness" (sensu Vinkler and Albrecht 2011), but how informative about the immune system these measurements are remains relatively unexplored. In this sense, researchers using these techniques need to be aware of (*i*) their usefulness as well as their limitations and (*ii*) the fact that their results are not representative of overall immunological competence with implications about disease resistance, but rather are informative of a particular aspect of immune function (Smits 2007). Specifically, one of the most common techniques used by ecologists to investigate the cell-mediated immune response in wild populations is the phytohemagglutinin (PHA) – skin test. Its popular use stems from its simplicity, little requirement of training and laboratory facilities, and its feasibility under field conditions (Martin et al. 2006). PHA is a vegetal lectin extracted from the red kidney bean (*Phaseolus vulgaris* L.); when injected subcutaneously, it is expected to trigger a local T-lymphocyte stimulation that results in significant swelling (Martin et al. 2006). The degree of swelling is considered an estimate of the adaptive cellular immune response (Goto et al. 1978; Blount et al. 2003). However, little is known about the immunological mechanisms underpinning the swelling response that follows the application of PHA in vivo (Kennedy and Nager 2006; Vinkler et al. 2010). Previous studies in birds have shown that the response induced by PHA is highly complex, involving both cells of the adaptive and innate immunity (Goto et al. 1978; Martin et al. 2006; Turmelle et al. 2010; Vinkler et al. 2010; Brown et al. 2011). These findings suggest that the PHA–skin test measures the general inducibility of proinflammatory signaling leading to cellular infiltration at the site of inflammation (Campbell and Ellis 2007). As shown by Tella et al. (2008), there is no doubt that lymphocytes (including T-lymphocytes) are involved in this process, but other blood-cell types (particularly neutrophils, macrophages, and eosinophils; Bonforte et al. 1972; Elgert 1996) also might be involved in the vasodilation, infiltration, and edema response (Goto et al. 1978). Consequently, to further our understanding of what PHA swelling represents in a functional sense, Turmelle et al. (2010) suggest identifying the leukocytes responsible for localized infiltration following the PHA injection.

Most experimental studies employing the PHA–skin test on wild populations have involved birds (e.g., Lindström et al. 2004; Vinkler et al. 2010; Gutiérrez et al. 2011), with fewer studies involving other vertebrates groups (amphibians: Wood Frog, *Lithobates sylvatica* (Le Conte, 1825) (Gervasi and Foufopoulos 2008), and Cane Toad, *Rhinella marina* (L., 1758) (Brown et al. 2011); fishes: Siamese fighting fish, *Betta splendens* Regan, 1910 (Clotfelter et al. 2007); reptiles: Galápagos marine iguana, *Amblyrhynchus cristatus* Bell, 1825 (Berger et al. 2005)). Comparatively, mammals have not been as widely studied. The few studies in this group of vertebrates were focused on the evaluation of trade-offs between immunity and other ecological factors, and results mostly support the hypothesis that immunocompetence is involved in trade-offs with life-history traits as diet (Mongolian jird, *Meriones unguiculatus* (Milne-Edwards, 1867); Xu and Wang 2010), parasitism (mouse-eared bat, *Myotis myotis* (Borkhausen, 1797) (Christe et al. 2000); several desert rodents species (Gouy de Bellocq et al. 2006*a*, 2006*b*)), reproduction (mouse-eared bat; Christe et al. 2000), and roosting habits (Brazilian free-tailed bat, *Tadarida brasiliensis* (I. Geoffroy, 1824); Allen et al. 2009). However, only the study by Turmelle et al. (2010) has validated the linkage between the swelling response and T-lymphocyte recruitment, describing the temporal development of the cellular response underlying the injection of PHA in Brazilian free-tailed bats. Thus, given the wide use of the PHA–skin test and the numerous trade-offs reported to be associated with its application, more studies are required to understand which components of the immune system are stimulated when PHA is injected subcutaneously in wild mammals.

In the present study, we evaluated the cell response to PHA injection in a species of subterranean rodent, the Talas tuco-tuco (*Ctenomys talarum* Thomas, 1898). Members of this genus are distributed in southern South America (Woods 1993). Although Talas tuco-tuco is a wild-living species, a great amount of information regarding its ecology, physiology, and behavior is available (Busch et al. 2000; Zenuto et al. 2002; Antinuchi et al. 2007). Also, individuals of Talas tuco-tuco are successfully maintained in captive conditions, making this species a good model to evaluate the implications of the PHA–skin test to study immunity in a wild-caught population. They are mostly sedentary (Busch et al. 1989) and territorial, living solitarily in permanently sealed burrows (Busch et al. 2000) with a polygynous mating system (Zenuto et al. 1999). Males are reproductively active all year round, while females reach the maximum intensity of breeding between August and November (Malizia and Busch 1997). Talas tuco-tucos live "slow-pace" reproductive lives, with delayed sexual maturity (6 months for females and 9 months for males) and females having long gestation periods (95 days) and giving birth to altricial pups (Zenuto et al. 2002) only twice a year (Busch et al. 1989). This species was characterized as a generalist and opportunistic herbivore because it consumes most of the plant species present in the grassland community, changing its diet in relation to food availability (Del Valle et al. 2001). Parasite fauna found in Talas tuco-tuco is little diverse (Rossin and Malizia 2002; Cutrera et al. 2011), but the high prevalence of it has led Rossin and Malizia (2002) to suggest that burrow systems provide physical conditions (e.g., moisture, low ventilation, protection from UV light) that favour parasite transmission. Endoparasites found in Talas tuco-tuco include gastrointestinal nematodes (*Trichuris pampeana* Suriano and Navone, 1994, *Graphidioides subterraneus* Rossin, Timi, and Malizia, 2005 (Rossin et al. 2005), *Pudica ctenomydis* Rossin, Timi, and Malizia, 2006 (Rossin et al. 2006), and *Paraspidodera uncinata* (Rudolphi, 1819)) and an intestinal protozoan (genus *Eimeria* Schneider, 1875), while ectoparasites identified are fleas (genus *Polygenis* Jordan, 1939), mites (families Laelapidae and Listrophoridae), and anoplurid lice (genus *Eulinognathus* Cummings, 1916) (Cutrera et al. 2011). Subterranean habitats provide their occupants a thermally stable environment (Nevo 1999) and are hypothesized to have favored low basal metabolic rate observed in Talas tuco-tuco (Busch et al. 1989; Luna et al. 2002). Adaptive immune function in Talas tuco-tuco was first assessed using the nonpathogenic antigen SRBC (sheep red blood cells), which triggers a humoral immune response (Bacon 1992) and has been considered indicative of resistance to extracellular infections (Deerenberg et al. 1997). Talas tuco-tuco mounted a detectable antibody response that was associated with a significant energetic cost, comparable with the cost of lactation for females (Cutrera et al. 2010). The study of other immune responses (e.g., inflammation, cell-mediated immunity) is required to better comprehend the immune strategies of this species whose "pace of life" differs markedly from most rodent species studied (typically "fast-living-pace" species). The inflammatory response is particularly interesting to study in this species, because Talas tuco-tucos, in addition to being exposed to a high parasite prevalence (Rossin and Malizia 2002), are frequently involved in fights for territory or mates (in the case of males), or may suffer predatory attacks as well as injuries related to subterranean

activities (e.g., digging); all of these events may stimulate the innate inflammatory response to ensure the animal's health. Therefore, our aim was to assess the immune response to PHA at both macroscopic and histological levels to determine the temporal development and cellular composition of this response. Specifically, we aimed to evaluate the ability of Talas tuco-tucos to mount a swelling response to a subcutaneous injection of PHA and examine the cellular processes underlying this response, both at local and peripheral levels. To achieve this, we (*i*) recorded the degree of swelling following subcutaneous injection with PHA or saline (control), at time points 6, 12, 24, 48, and 72 h after injection to determine the maximum time of swelling response, (*ii*) examined which leukocytes infiltrate to the injection area at these different time points, (*iii*) evaluated the relationships between leukocyte infiltration and measurements of macroscopic swelling, and finally, (*iv*) examined the effect of the PHA injection in the circulating (peripheral) blood on leukocyte diversity and abundance. To perform these experiments, we worked with wild-caught animals transported to the laboratory for a short period of time. Even though immunocompetence of captive animals could differ from their free-living counterparts (Calisi and Bentley 2009), our study performed in a laboratory allowed us to obtain data from individuals in a similar ambient condition (with controlled temperature, photoperiod, and diet; reduced strenuous activities; and avoided natural injuries or infections), facilitating the interpretation of the mechanism underlying the immune response to PHA. This study provides evidence that will contribute to the correct interpretation of the PHA– skin test in ecoimmunological studies. Also, knowledge about the PHA response in a "slow-living" species is valuable in an ecological and evolutionary context, as differences can arrive between Talas tuco-tucos and typically "fast-living" surface-dwelling rodents studied up to date, which may contribute to understand the modulation of a single immune response in relation to life-history traits.

Materials and methods

Animal capture and housing

Adult Talas tuco-tuco of both sexes were live-trapped in Mar de Cobo, Buenos Aires Province, Argentina (37°46′S, 57°27′W), from August to October 2010. Although all animals used were reproductively mature adults, their exact age was unknown and therefore was not considered in the following analyses. Animals were caught using wire tube-shaped live traps (10 cm diameter, 35 cm length) set at fresh surface mounds. Holes were dug to access the underground burrows and traps were situated as an elongation of existing tunnels. When nursing females were trapped, they were immediately released back into their burrow system so as not to deprive dependent young of maternal care. A total of 55 animals (n_{males} = 41; $n_{females}$ = 14) were transported to the Laboratory of Ecophysiology at the National University of Mar del Plata (Mar del Plata, Argentina) where they were individually housed in plastic cages (25 cm × 32 cm × 42 cm) with a wire-mesh lid and lined with wood shavings as bedding. The animals were fed ad libitum quantities of a mixture of grasses, corn (*Zea mays* L.), sweet potatoes (*Ipomoea batatas* (L.) Lam.), chicory (*Cichorium intybus* L.), and sunflower (*Helianthus annuus* L.) seeds. Fresh food was provided daily to ensure water provision because Talas tuco-tuco do not drink free water. Significant loss in mass was verified in captivity for males ($mass_{field}$ = 164.49 ± 12.14 g (mean ± SD); $mass_{captivity}$ = 153.20 ± 13.81 g; Student's *t* test, $t_{[18]}$ = 3.39, *P* = 0.003) and not for females ($mass_{field}$ = 131.85 ± 11.33 g; $mass_{captivity}$ = 127.74 ± 10.24 g; Student's *t* test, $t_{[7]}$ = 0.73, *P* = 0.49). However, the loss in mass for males represents less than 10% of the mean body mass. Animal-room conditions (temperature and photoperiod) were automatically controlled (25 ± 1 °C; 14 h light : 10 h dark). Animals remained captive for the duration of the experimental assays (approximately 2 weeks) after which they were released at the point of capture. The scar present at the tip of their tail (see Characterization of peripheral cellular response within the Materials and methods) allowed us to recognize animals that had been released in the field, preventing their recapture. We adhered to the Canadian Council on Animal Care guidelines for the capture, handling, and use of mammals (Canadian Council on Animal Care 1984).

Immune challenge tests

Fifty animals were randomly assigned to two groups: control (*n* = 25) and immune-challenged (*n* = 25). The remaining five animals were used to assess the peripheral leukocyte frequencies in a before-treatment state (not injected or naïve animals), as explained below (see Characterization of peripheral cellular response). The experiments started at day 10 of captivity because this is the necessary time lapse for animals to acclimatize to captive conditions and, hence, lower their stress levels (Vera et al. 2008). Individuals were uniformly distributed among the different treatments to minimize the possible effects of seasonality. Talas tuco-tucos were injected subcutaneously in the instep of the left hind foot with sterile phosphate-buffered saline solution (control group: PBS) or phytohemagglutinin (immune-challenged group: *Phaseolus vulgaris* PHA Sigma L8754 solution dissolved in PBS, 3 mg/mL) using a 30 gauge needle. PBS-injected animals served as control of the cellular response that could trigger the solution used to dissolve PHA or the injury provoked by the needle. Foot thickness increases with body mass in Talas tuco-tuco (*r* = 0.24, *P* = 0.027, *n* = 85; J. Merlo, unpublished report); therefore, we used a mass-dependent PHA dose (0.3 μL/g). Moreover, the dose chosen is large enough to cause visible expansion of the instep of the foot when successfully injected, but small enough to dissipate within a few seconds (Brown et al. 2011). Prior to injection, the area was treated with an antiseptic solution (Pervinox; Phoenix Laboratories, Buenos Aires, Argentina). The sex of the individuals was not considered because preliminary work showed that the PHA-induced swelling did not differ between females (*n* = 10; $swelling_{females}$ = 0.4 ± 0.24 mm, mean ± SD) and males (*n* = 10; $swelling_{males}$ = 0.5 ± 0.3 mm) in the study species (Student's *t* test, $t_{[18]}$ = −0.68, *P* = 0.503).

Characterization of local cellular response

Prior to injection, the thickness of the left hind foot was measured with a digital micrometer (Insize, Sao Paulo, Brazil) to the nearest 0.01 mm. Measurements were repeated twice and averaged and they were always performed by the same person (A.P.C.). At time intervals of 6, 12, 24, 48, and 72 h (±30 min) after injection, swelling measurements of five PBS-injected animals and five PHA-injected animals were performed (*n* = 5 animals/time after injection per treatment; Table 1). Thus, each animal was measured at two time points: before injection (0 h) and after injection (6, 12, 24, 48, or 72 h). Measurements were taken in the morning or in the afternoon, because Talas tuco-tuco exhibits an arrhythmic pattern of daily activity (Luna et al. 2000). The PHA or PBS swelling response was calculated as the difference between before-injection and after-injection thickness divided by initial foot thickness, i.e., response = (after injection − before injection)/before injection (Gouy de Bellocq et al. 2006*b*; Xu and Wang 2010). Immediately after the after-injection swelling measurement, a small tissue sample from the instep of the foot of each animal was taken by aseptic biopsy using a 3 mm dermal punch (Stiefel; Stiefel Laboratories Argentina, Buenos Aires, Argentina). Each animal was biopsied once, after which it was removed from the experiment. Following biopsy, the cored area was treated with an anti-inflammatory and antibiotic powder (Pharm-x; Felipe Bajer Laboratories, Buenos Aires, Argentina). All tissue biopsies were immediately fixed in 10% formalin. Tissue samples were sent to Duchené Laboratory (Buenos Aires, Argentina) where they were embedded in paraffin, sectioned (2 μm wide), stained with hematoxilin and eosin, and mounted on slides following standard procedures (Geneser 2000).

Table 1. Sample sizes of each group of Talas tuco-tuco (*Ctenomys talarum*) individuals used in the study.

		Time after injection (h)					
Level of cellular response assessment	Treatment	0	6	12	24	48	72
Local and peripheral	Phytohemagglutinin (PHA) injection	—	5	5	5	5	5
	Phosphate-buffered saline (PBS) injection	—	5	5	5	5	5
Only peripheral	Naïve (not injected)	5	—	—	—	—	—

To assess the infiltration response, leukocytes in fixed tissue samples were identified in five categories—lymphocytes, neutrophils, macrophages, eosinophils, and basophils—based on their morphology as described by Voigt (2000) and previous studies of Talas tuco-tucos (Vera et al. 2008; Cutrera et al. 2010). Total white cells were identified and counted in five fields under oil immersion at 1000× magnification (Olympus CX 31; Olympus Corp., Tokyo, Japan) from the best-stained tissue section from the slide. The five fields were randomly located along the tissue section, but areas containing hair follicles, blood vessels, and epidermis were avoided. For each individual, the five fields of tissue section were counted and mean (±SE) cell counts were calculated for each cell type.

Characterization of peripheral cellular response

To assess the possible effect of PHA injection in the peripheral circulating blood, leukocyte diversity and abundance were quantified for PHA-injected and PBS-injected animals (n = 5 animals/time after injection per treatment; Table 1) following standard protocols (Voigt 2000). Immediately after biopsy, a blood smear was made from a small sample of peripheral blood obtained from the tip of the tail. To assess the peripheral leukocyte frequencies in a before-treatment state, leukocyte counts in the peripheral blood of a group of naïve animals (not injected; n = 5; Table 1) were recorded. After fixation with methanol, the slides were stained with a May–Grunwald–Giemsa solution and then examined under oil immersion at 1000× magnification. To determine the abundance of lymphocytes, neutrophils, eosinophils, monocytes, and basophils, the number of each type encountered following the "wandering technique" was recorded (identifications based on cell morphology described by Voigt 2000; Vera et al. 2008; Cutrera et al. 2010) until a total of 200 leukocytes had been examined. Then, abundance of each cell type present in 200 leukocytes was determined. Neutrophil/lymphocyte ratio (N/L ratio) was calculated for each individual because it is a known stress indicator (Davis et al. 2008). To calculate the leukocyte abundance, the number of erythrocytes and leukocytes encountered in 30 fields in a single pass along the slide was recorded and, following the methods of Bachman (2003), the number of total leukocytes was standardized to 20 000 erythrocytes.

Statistical analyses

All analyses were performed in R version 2.15.3 package (R Development Core Team 2008) using $P < 0.05$ to reject the null hypothesis. The normal distribution of variables was tested using Shapiro–Wilk's tests and variance equality was verified using Levene's tests. Prior to the analysis, swelling-response data was $\log(x + 0.5)$-transformed to reach normality. A general linear model (GLM) was formulated to analyze the swelling response as a function of the abundance of each cellular type (lymphocytes, neutrophils, eosinophils, and macrophages) at the site of injection as covariates and the interaction with treatment (PHA vs. PBS) and time after injection (6, 12, 24, 48, and 72 h). Differences in N/L ratio in peripheral blood between naïve and PBS-injected (at 6 h time point) animals were assessed using a Student's t test. Another GLM was used to analyze the swelling response as a function of the N/L ratio and the abundance of total leukocytes and the interaction with treatment (PHA vs. PBS) and time after injection (6, 12, 24, 48 and 72 h) in the peripheral circulating blood as covariates. Throughout the text, results are shown as mean ± SE unless otherwise indicated.

Results

Macroscopic swelling and histological analysis of cellular infiltrate to PHA injection

Swelling response was significantly affected by the treatment (GLM, $\chi^2_{[1,40]} = 44.28$, $P < 0.001$), being greater in PHA-injected than in PBS-injected animals at 6, 12, 24, and 48 h after injection (GLM, $\chi^2_{[1,40]} = 5.22$, $P < 0.05$; Fig. 1*a*). A marked infiltration of leukocytes was recorded in the tissue samples of PHA-injected animals compared with samples from control individuals at all times (Figs. 2*a*, 2*b*). We did not find significant relationships between abundance of lymphocytes, neutrophils, eosinophils, or macrophages and the inflammatory response (lymphocytes: GLM, $\chi^2_{[1,46]} = 51.82$, $P < 0.001$; neutrophils: GLM, $\chi^2_{[1,45]} = 46.41$, $P < 0.001$; eosinophils: GLM, $\chi^2_{[1,44]} = 5.52$, $P < 0.05$; macrophages: GLM, $\chi^2_{[1,43]} = 5.55$, $P < 0.05$). At 12 h after injection, abundances of total leukocytes (Fig. 1*b*), lymphocytes (Fig. 1*c*), neutrophils (Fig. 1*d*), eosinophils (Fig. 1*e*), and macrophages (Fig. 1*f*) at the injection site of PHA-injected animals were significantly greater than in control animals (Figs. 1*b*–1*f*); abundances of total leukocytes, lymphocytes, and neutrophils peak at 12 h after PHA injection (Figs. 1*b*, 1*c*, 1*d*), while eosinophils and macrophages did not show this pattern (Figs. 1*e*, 1*f*). Infiltration of neutrophils in the PHA-injected animals was significantly greater than that observed in the PBS-injected animals also at 24, 48, and 72 h after injection (Fig. 1*c*).

Peripheral leukocyte response to PHA injection

N/L ratio did not differ between naïve and PBS-injected (6 h time point) animals (Student's t test, $t_{[8]} = -0.15$, $P = 0.88$). N/L ratios in the peripheral blood of PHA-injected and PBS-injected animals had no relationship with the swelling response (GLM, $\chi^2_{[1,47]} = 2.67$, $P = 0.75$). A significant relationship was found between total leukocyte count and swelling response (GLM, $\chi^2_{[1,48]} = 18.39$, $P < 0.01$), but there was no relationship between total leukocytes and swelling response in relation to the treatment (GLM, $\chi^2_{[1,42]} = 1.01$, $P = 0.96$). Abundances of each leukocyte type, total leukocytes, and N/L ratios in the peripheral blood of PHA-injected, PBS-injected, and naïve individuals are shown in Table 2. Basophils were not found in peripheral blood.

Discussion

The PHA-induced skin-swelling test has been widely used as a technique to measure immunocompetence, specifically T-lymphocyte activity; studies have focused mainly on birds (Moreno et al. 1999; Blount et al. 2003; Navarro et al. 2003) and recently also in other vertebrates (Berger et al. 2005; Allen et al. 2009; Xu and Wang 2010). Its popularity in ecoimmunological studies arise from its easy use and little laboratory requirements (Kennedy and Nager 2006; Martin et al. 2006). However, only a few studies have tested whether macroscopic measurements of swelling actually correlate with the abundance of T-lymphocytes (or at least of lymphocytes in general, given the methodological difficulty of distinguishing between different types of lymphocytes; Martin et al. 2006; Tella et al. 2008; Turmelle et al. 2010; Brown et al. 2011; Vinkler et al. 2012). Using the

Fig. 1. Box plots of swelling (*a*) and of cell counts of total leukocytes (*b*), lymphocytes (*c*), neutrophils (*d*), eosinophils (*e*), and macrophages (*f*) in tissues of Talas tuco-tuco (*Ctenomys talarum*) individuals injected with phytohemagglutinin (PHA) or phosphate-buffered saline (PBS) and biopsied at 6, 12, 24, 48, or 72 h after injection. Each box encloses the 25th and 75th percentiles, with the central white square representing the median (50th percentile) and whiskers showing the 5th and 95th percentiles.

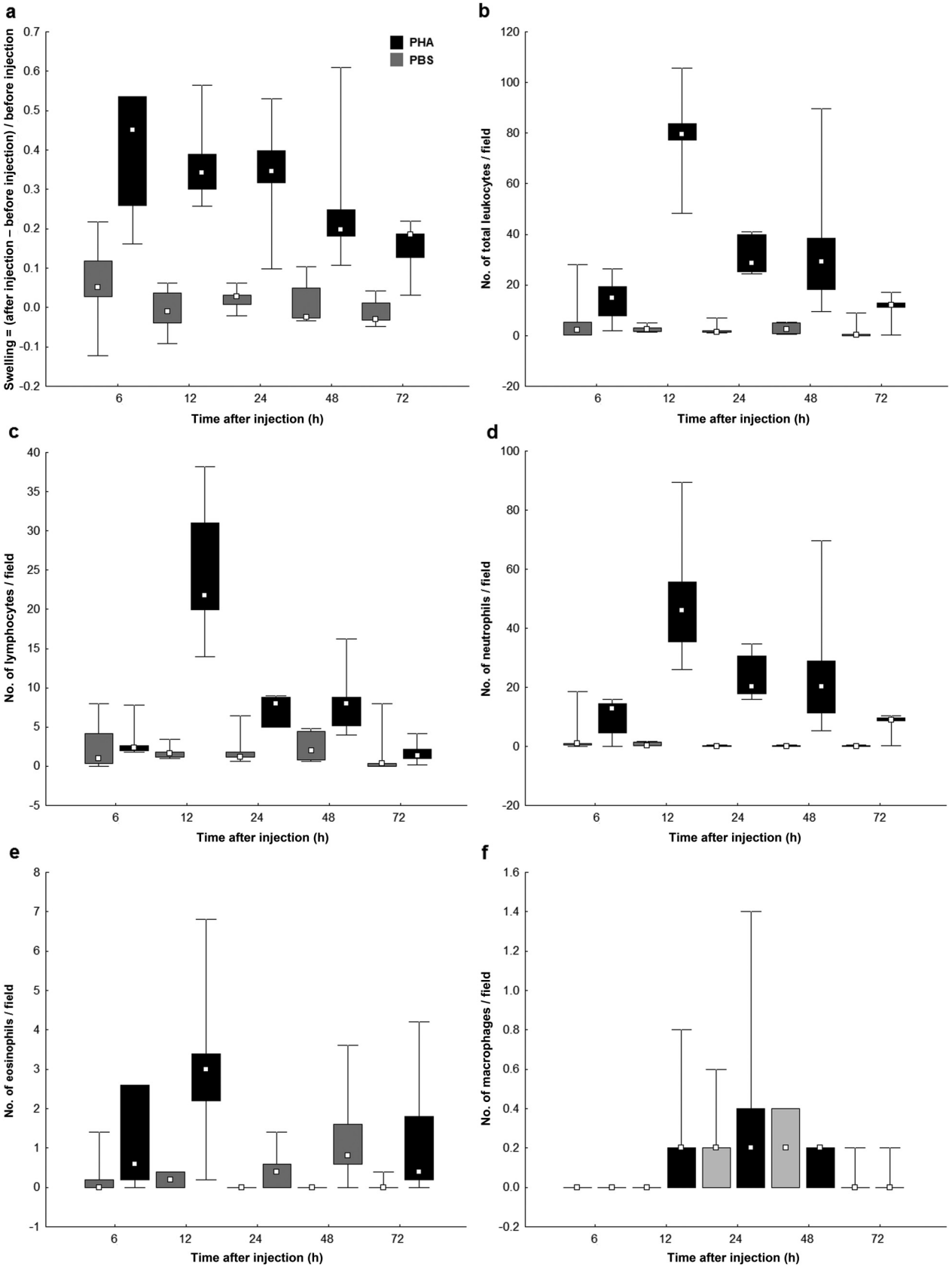

Fig. 2. Tissue sections taken 12 h after injection, viewed at 1000× magnification, from (*a*) an individual Talas tuco-tuco (*Ctenomys talarum*) injected with phytohemagglutinin (PHA) showing cellular infiltration of neutrophils (A), lymphocytes (B), eosinophils (C), and macrophages (D), and from (*b*) an individual Talas tuco-tuco injected with phosphate-buffered saline (PBS) showing no cellular infiltration. Scale bars = 10 μm. May–Grunwald–Giemsa stain.

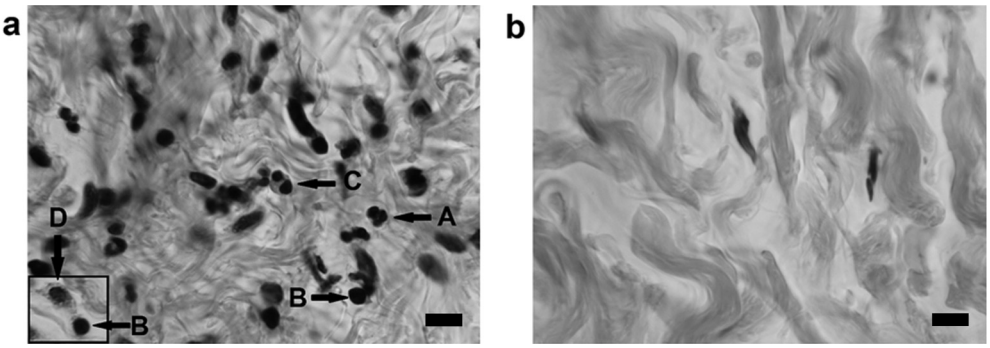

Table 2. Abundances of each type of leukocytes (in 200 leukocytes), total leukocyte count (relative to 20 000 erythrocytes), and neutrophil/lymphocyte ratio (N/L ratio) in the peripheral blood of phytohemagglutinin (PHA) injected ($n = 25$), phosphate-buffered saline (PBS) injected ($n = 25$), and naïve (not injected) ($n = 5$) Talas tuco-tuco (*Ctenomys talarum*) individuals.

	Mean (±SE) count		
Cell type	PHA-injected *C. talarum*	PBS-injected *C. talarum*	Naïve *C. talarum*
Neutrophils	119.1±34.2	128.4±28.1	91.4±55.2
Lymphocytes	75.7±34.5	67.3±27.9	104.8±56.8
Eosinophils	2.4±3.9	2.9±5.2	1.6±1.9
Monocytes	2±2.8	0.9±1.8	2±1.22
Total leukocytes	9.9±6.8	8.1±7.3	14.2±12.2
N/L ratio	2.2±1.8	2.5±1.9	1.5±1.6

Note: For PHA-injected and PBS-injected animals, abundances of each leukocyte type, total leukocytes, and N/L ratios were pooled across all time intervals (6, 12, 24, 48, and 72 h).

PHA–skin test coupled with a histological analysis of the inflammation area allowed us to describe the temporal development of the cell-mediated immune response in Talas tuco-tucos. Furthermore, our results add data to the recent debate about the scope of this technique in ecological studies. Particularly, the inflammatory response observed in this species involved an array of different cell types. However, the abundance of lymphocytes recorded in the site of injection did not explain the degree of swelling, suggesting that in Talas tuco-tucos, the PHA-induced swelling response should not be used as a direct indicator of activity mediated by T-lymphocytes.

Our findings demonstrate the ability of individual Talas tuco-tucos to mount a detectable swelling response to PHA injection from 6 to 48 h after injection. Similar times of PHA response have been described in other species of rodents (Gouy de Bellocq et al. 2006*b*; Xu and Wang 2010), as well as in bats (Turmelle et al. 2010), birds (Navarro et al. 2003; Martin et al. 2006), amphibians (Gervasi and Foufopoulos 2008; Brown et al. 2011), fishes (Clotfelter et al. 2007), and reptiles (Berger et al. 2005), where the inflammation was also detected between 6 and 48 h after injection in all cases. However, time of maximum swelling response to PHA injection differs between species (see Table 3). As reported by Goüy de Bellocq et al. (2006*b*), desert rodent species showed two types of PHA response: one type was rapid (approximately 6 h after PHA injection), while the second type was delayed (approximately 24 h after PHA injection). Furthermore, rodents that responded promptly had a lower maximum response than rodents with a delayed response. Goüy de Bellocq et al. (2006*b*) also found a relationship between type of PHA response and parasite load. Besides Talas tuco-tucos, mouse-eared bats, Brazilian free-tailed bats, Mongolian jirds, and Scarlet Rosefinches (*Carpodacus erythrinus* (Pallas, 1770)) show a rapid PHA response, while other species from many taxa had a delayed response (Table 3). However, given the different doses and methods used to calculate the mean swelling response in each study (see Table 3), comparisons about the magnitude of the PHA response between species are difficult. Therefore, we suggest using a PHA dose that is mass-dependent and selecting a single method to report measures of inflammatory response, to be able to compare different studies.

The histological analyses performed in this study showed the temporal development and cellular composition of the PHA-induced inflammation response in Talas tuco-tucos. After the subcutaneous injection with PHA, different leukocyte types arrived at the injected area, achieving a peak of cellular infiltration at 12 h after injection. At this time, the infiltrate was composed mainly of neutrophils and lymphocytes, but also contained eosinophils and macrophages in lower quantities. A similar pattern was found by Turmelle et al. (2010) in bats, although the levels of neutrophils and lymphocytes were more similar in that case. In Talas tuco-tucos, lymphocytes decreased immediately after the 12 h time point, while neutrophils, which were the most abundant cell type at all time points, remained at high levels until 72 h after injection. Surprisingly, basophils were not found in the tissue samples at any time point. In contrast, basophils were found in the cellular composition of the PHA-induced infiltrates of bats (Turmelle et al. 2010), House Sparrows (*Passer domesticus* (L., 1758) (Martin et al. 2006), and Scarlet Rosefinches (Vinkler et al. 2012). Nevertheless, basophils are poorly represented in the circulating blood of wild Talas tuco-tucos in their natural habitat (Vera et al. 2008), and it has been found that captivity-induced stress decreases the frequency of rare cell types (e.g., eosinophils, monocytes, and basophils; Cutrera et al. 2010), which may explain the absence of basophils in the tissue of PHA-injected Talas tuco-tucos. Given this evidence, it is important to mention that the infiltration of the rare cell types in response to PHA could have been underestimated in our study. The temporal development of the infiltration response in Talas tuco-tucos differed from that described in House Sparrows (Martin et al. 2006) and Cane Toads

Table 3. Comparative values of mean swelling response to phytohemagglutinin (PHA) subcutaneous injection and the time of response peak for different species.

Species	Mean swelling (mm)	Swelling peak (h)	Histology	Reference
Birds				
House Sparrow, *Passer domesticus*	0.67*,†	48	Yes	Martin et al. 2006
Scarlet Rosefinch, *Carpodacus erythrinus*	—	6‡	Yes	Vinkler et al. 2012
Yellow-legged Gull, *Larus cachinnans* (Pallas, 1811)	0.40*,†	24‡	No	Alonso-Alvarez and Tella 2001
Zebra Finch, *Taeniopygia guttata* (Vieillot, 1817)	0.62*,§	24‡	No	Love et al. 2008
Common Blackbird, *Turdus merula* L., 1758	2.39*,†	24	No	Biard et al. 2009
Pied Flycatcher, *Ficedula hypoleuca* (Pallas, 1764)	0.17*,†	24‡	No	Moreno et al. 1999
Amphibians				
Cane Toad, *Rhinella marina*	0.53*,†	24	Yes	Brown et al. 2011
Wood Frog, *Lithobates sylvatica*	0.11*,†	24	No	Gervasi and Foufopoulos 2008
Fish				
Siamese fighting fish, *Betta splendens*	0.09*,†	24‡	No	Clotfelter et al. 2007
Reptile				
Wall Lizard, *Podarcis muralis* (Laurenti, 1768)	0.05±0.005†	24‡	No	Oppliger et al. 2004
Mammals: bats				
Brazilian free-tailed bat, *Tadarida brasiliensiss*	0.63*,†	6	Yes	Turmelle et al. 2010
Mouse-eared bat, *Myotis myotis*	0.78±0.04§	10‡	No	Christe et al. 2000
Mammals: rodents				
Mongolian jird, *Meriones unguiculatus*	0.55*,‖	6	No	Xu and Wang 2010
Cairo spiny mouse, *Acomys cahirinus* (É. Geoffroy, 1803)	0.42±0.02‖	6	No	Gouy de Bellocq et al. 2006*b*
Golden spiny mouse, *Acomys russatus* (Wagner, 1840)	0.29±0.03‖	6	No	Gouy de Bellocq et al. 2006*b*
Anderson's gerbil, *Gerbillus andersoni* (de Winton, 1902)	0.69±0.05‖	6¶	No	Gouy de Bellocq et al. 2006*b*
Wagner's gerbil, *Gerbillus dasyurus* (Wagner, 1842)	0.84±0.02‖	24	No	Gouy de Bellocq et al. 2006*b*
Lesser Egyptian gerbil, *Gerbillus gerbillus* (Olivier, 1801)	1.01±0.05‖	24	No	Gouy de Bellocq et al. 2006*b*
Greater Egyptian gerbil, *Gerbillus pyramidum* (Geoffroy, 1825)	1.17±0.12‖	24	No	Gouy de Bellocq et al. 2006*b*
Sundevall's jird, *Meriones crassus* (Sundevall, 1842)	0.71±0.03‖	24	No	Gouy de Bellocq et al. 2006*b*
House mouse, *Mus musculus* L., 1758	0.25±0.02‖	6¶	No	Gouy de Bellocq et al. 2006*b*
Fat sand rat, *Psammomys obesus* (Cretzschmar, 1828)	0.56±0.01‖	24	No	Gouy de Bellocq et al. 2006*b*
Talas tuco-tuco, *Ctenomys talarum*	0.39±0.17‖	6¶	Yes	Present study

*Data extracted from published graphs using Plot Digitizer software (Huwaldt 2005).
†Calculated as thickness after injection – before injection.
‡Only measured at one time point after injection.
§Calculated as thickness (after PHA injection – before PHA injection) – (after PBS injection – before PBS injection).
‖Calculated as thickness (after injection – before injection)/before injection.
¶First time point of the maximum period of inflammation detected.

(Brown et al. 2011). In birds, heterophils (a neutrophil parallel in birds) appeared early and then disappeared, but lymphocytes were abundant throughout much of the response (Martin et al. 2006) and basophils and macrophages increased over time in the PHA-injected tissue. In Cane Toads, eosinophils, macrophages, and neutrophils appeared and peaked earlier than did lymphocytes (at 24 h after injection). Also, as described above, the abundance of each leukocyte type in bats (Turmelle et al. 2010) differed from that found in Talas tuco-tucos. Therefore, it follows that the response to a subcutaneous injection of PHA varies in terms of cellular infiltration among species. Such differences can arise because the immune system has been shaped over time by different aspects of the life history of each species, such as varying levels of parasite exposure, different per-offspring investment and lifespan, or distinct developmental strategies (Lee 2006; Martin et al. 2006). Nonetheless, only four studies in vertebrates are available at present that describe the temporal dynamics of the cellular response in relation to the macroscopic swelling (birds: House Sparrows (Martin et al. 2006); amphibians: Cane Toads (Brown et al. 2011); mammals: Brazilian free-tailed bats (Turmelle et al. 2010) and Talas tuco-tucos (this study)), which prevents us from evaluating possible evolutionary factors modeling inflammation. These efforts should be continued to elucidate a theory relating immunity and life-history traits, especially in "slow-living-pace" species.

A considerable degree of interindividual variation was found along the temporal pattern of cell infiltration in Talas tuco-tucos, as shown by the variability in the magnitude of the thickness measurements and the quantification of the cellular abundances. Individual variation in the swelling response was also reported for bats (Turmelle et al. 2010), which supports the hypothesis that the ability of an individual to respond to a novel antigen can be affected by its condition, which in turn may vary seasonally and over the course of an animal's life. Several studies have aimed at increasing the knowledge about individual variation in PHA response. For example, Allen et al. (2009) reported that roosting ecology (roost type and colony size) of bats significantly impacts the magnitude of the response to PHA, while Gutiérrez et al. (2011) found that food-restricted birds showed lower values of wing-web swelling after PHA injection and a different time course of this immune response compared with birds fed ad libitum. Huyghe et al. (2010) showed an up-regulation of the PHA response at the end of the breeding season in polymorphic lizards and different intensity of response between morphs. Furthermore, from a large number of studies performed in birds (see Martin et al. 2006), it seems clear that there is a trade-off between PHA-induced swelling and other physiological functions, indicated by the fact that PHA swellings are weaker when this challenge overlapped with other costly activities, such as moulting (Martin 2005), parental care (Fargallo et al. 2002), or breeding (Ardia 2005). Given this evidence, future studies of possible trade-offs between immunity and reproduction in Talas tuco-tucos will include the evaluation of the energetic costs associated with the PHA-response in relation to reproductive seasonality. Besides, the influence of genetics on the intensity of the immune response is probably another important factor shaping individual variation in the magnitude of the

response to PHA (Charbonnel et al. 2010). For example, for Talas tuco-tucos, significant associations have been found between specific major histocompatibility complex (MHC) alleles and both parasite load and intensity of humoral immune response against sheep red blood cells (Cutrera et al. 2011). These results add to a growing body of data reporting an interaction between immune responsiveness and genetic background.

Martin et al. (2006) proposed that part of the effects of PHA found in some studies may be due to the induction of both local and peripheral immune activities, the last one probably as a consequence of an acute stress response to PHA treatment, and hence they encouraged the study of such possibilities. In our study, we did not find a relationship between N/L ratios and total leukocytes in circulating blood with the swelling response in relation to the treatment (PHA subcutaneous injection). However, total leukocytes showed a relationship with the swelling response if treatment (PBS vs. PHA) was not considered. The absence of linkage between abundances of total leukocytes in peripheral blood and treatments could be a consequence of the great interindividual variation observed in the magnitude of swelling response in the PHA-injected group, where there were individuals that raised high swelling responses (e.g., 0.61 mm), while others developed lower responses (e.g., 0.03 mm) that were similar to PBS thickness measurements. Our results are in line with those of Xu and Wang (2010) in Mongolian jirds, who reported a positive correlation between PHA-induced swelling response and total leukocyte counts in peripheral blood. Also, results of Tella et al. (2008) showed an increase in T-lymphocyte concentration in peripheral blood after the first PHA injection and a more evident increase following a second injection. Thus, our results agree with previous findings in that there is a relationship between the leukocytes of the circulating blood and the swelling response. However, performing a previous histological analysis of the response to PHA in the species of interest, both at the local and peripheral levels, is important to arrive at a correct interpretation of the results of this test.

In conclusion, this study provides additional evidence refuting the interpretation of the PHA-induced skin-swelling test as a direct indicator of T-lymphocyte activity, as previously suggested (Kennedy and Nager 2006; Martin et al. 2006; Turmelle et al. 2010; Vinkler et al. 2010). The macroscopic swelling response of Talas tuco-tucos, despite finding a clear peak of lymphocyte abundance at 12 h after injection, was not associated with the abundance of this cell type. Also, the temporal pattern of the PHA response shows that the PHA-induced swelling persists beyond the 12 h peak of lymphocytes. The histological analysis revealed that other leukocyte cells were also involved in the infiltrating area (neutrophils, eosinophils, and macrophages). In this way, the results of our study agree with the two previous histological assessments of the PHA-induced response (Martin et al. 2006; Turmelle et al. 2010) in showing that the cell-mediated immune response triggered by this lectin is a dynamic cellular process involving multiple leukocyte populations. Also, Vinkler et al. (2014), based on molecular analyses at the site of PHA injection, revealed that PHA induces expression of inflammatory cytokines, but not those related to T-cell proliferation. Together, these results highlight the importance of taking into account the complex nature of the vertebrate immune system and avoiding simple approaches to estimate immunocompetence. Therefore, future studies involving the PHA–skin test in Talas tuco-tucos should be interpreted in light of these findings, considering the magnitude of the local swelling response (measured between 6 and 48 h after subcutaneous injection) as an estimate of the inflammatory immune capacity of the individual, which is part of its innate immune defense. The study of the PHA response allowed us to assess the nature and temporal development of one branch of the immune defenses of our species of interest. However, this is only one aspect of the immunocompetence of Talas tuco-tucos, and thus an integral study of different immune responses is necessary to reach a deeper understanding of its immunological competence (Smits 2007). Also, more studies in slow-living mammals are required to elucidate relationships between nature and temporal development of the immune response and life-history traits. In this sense, the relative investment in each type of immune response will depend on the costs and different benefits it provides; for example, how appropriate is it to invest in a costly response that provides immunological memory versus a rather less costly and more immediate response, according to the probability of needing this immune defense again during the individual's life.

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