RESEARCH ARTICLE

Food Restriction Affects Inflammatory Response and Nutritional State in Tuco-tucos (*Ctenomys talarum*)

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ABSTRACT

Insufficient or unbalanced food intake typically has a negative impact on immune responses. The understanding of this effect is, however, hampered by the effect that food has on general condition, which, in turn, affects immunity, and the interaction among general condition, immunocompetence, and concurrent infections. The goal of this study was to determine the effects of food restriction and methionine supplementation on immunity in tuco-tucos (*Ctenomys talarum*). Effects of diet manipulations on nutritional state, inflammatory response to phytohemagglutinin (PHA), and other immune parameters (bacterial killing capacity, natural antibodies, and leukocyte profile) were evaluated. Health and stress parameters and endoparasite loads were assessed to understand more deeply potential effects of treatments on immune status. Individuals under food restriction presented an altered nutritional state as well as increased stress levels (higher N: L ratios) compared with individuals fed ad libitum, and a marked reduction in the inflammatory response to PHA. Supplementation with methionine did not affect any of the parameters analyzed. Endoparasite loads were not affected by treatments. Our results support the idea that food insufficiency can modulate the individual's immune responsiveness through the lack of adequate essential nutrients, metabolic fuel and energetic reserves, or by a detrimental effect of the stress caused by nutrient limitation. We show that the response to PHA previously reported as nonenergetically costly for *C. talarum*, implies a nutritional cost; an opposite pattern to that previously found for the adaptive antibody response to sheep red blood cells in the same species. *J. Exp. Zool. 00:1–13, 2017.* © 2017 Wiley Periodicals, Inc.

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Environmental fluctuation in resource availability can lead to the modification of physiological responses of organisms to maintain a balanced energy budget (Antinuchi et al., 2007). Among these resource-depending physiological functions, the immune system used by vertebrates to defend themselves against pathogens, demands micro- and macronutrients for the

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Abbreviations A/G, albumin: globulin ratio; N: L, neutrophils: lymphocytes ratio; PHA, phytohemagglutinin

synthesis and proliferation of leukocytes and proteins required for triggering the immune response (Hasselquist and Nilsson, 2012). For example, the acute phase response is suggested to be the most nutritionally demanding immune response (Klasing, '98). Also, activation of immune system can reduce body weight (Lochmiller and Deerenberg, 2000) when body reserves for protein synthesis are insufficient (Schmid Hempel, 2011). Micronutrients are also critical for the normal function of immunity; carotenoids, selenium, and vitamin E availability, for example, can compromise the magnitude in which an immune response is developed (Klasing, '98; Surai, 2002). Therefore, insufficient food intake (energy and protein deficiency) as well as sufficient but unbalanced rations, typically have a negative impact on the immune system and the development of immune responses (Scrimshaw and SanGiovanni, '97; Fekete and Kellems, 2007). For example, food-restricted *Peromyscus maniculatus* produced notably less IgG against a foreign antigen (keyhole limpet hemocyanin, KLH) than their ad libitum-fed counterparts (Martin et al., 2007), and *Phasianus colchicus* (ring-necked pheasant) males under high protein content diet produced stronger secondary antibody responses to diphtheria and tetanus than males under a low protein content diet (Smith et al., 2007). However, increases in immunity or no effect in certain immune components (antibody responses, bacterial killing capacity, BKC) with food restriction have also been reported (e.g., Hangalapura et al., 2005; Poston et al., 2005; Zysling et al., 2009). However, interpretation of these results is difficult due to the effect that food restriction has on general condition, given that the effect of food manipulation on immunity may ultimately depend on a general effect on individual condition (Hasselquist and Nilsson, 2012). From studies in poultry, it is known that adding certain amino acids (e.g., arginine, methionine) to the diet improves the immune response (Swain and Johri, 2000; Soler et al., 2002; Abdukalykova and Ruiz-Feria, 2006). For example, diets supplemented with sulfur amino acids such as methionine enhance T-cell mediated immunocompetence of broilers strain chicks (Tsiagbe et al., '87; Swain and Johri, 2000), most likely because these amino acids are involved in glutathione synthesis, the intracellular concentration of which is directly linked to T-cell functioning (Grimble, '92). Positive effects of amino acid supplementation on immunity were also reported for wild birds; *Parus caeruleus* nestlings showed increased cell mediated immunity under methionine-supplemented diets, but this treatment also affected growth in a less clear manner (Brommer, 2004; Pitala et al., 2010). Additional studies in wild mammal species are necessary to better understand the influence of amino acids diet content on immune function (Hasselquist and Nilsson, 2012). Impaired immunocompetence by malnutrition leads to decreased resistance to new infections (Fekete and Kellems, 2007). For example, infected rodents accumulated worms in proportion to the intensity of exposure when fed with a low-protein diet, whereas worm accumulation declined under higher protein content diets (e.g., Slater and Keymer, '86; reviewed in Hasselquist and Nilsson, 2012). In turn, the nutritional state of the host can be altered by a decreased food intake and poor food utilization induced by parasites (Fekete and Kellems, 2007). Therefore, interaction among nutrition, immunity, and infection is such that changes in one factor directly affect the other two (Chandra and Newberne, '77; Beldomenico and Begon, 2010).

The goal of this study was to determine the effects of diet on immunity of *Ctenomys talarum* Thomas, 1898 (tuco-tucos), a South American solitary subterranean rodent. Ecoimmunological information of wild rodents, which are particularly important models for infectious biology and medical immunology (Morand et al., 2015), is scarce in relation to that available for birds and amphibians (e.g., Lindström et al. 2004; Gervasi and Foufopoulos 2008; Vinkler et al., 2010; Brown et al., 2011; Gutiérrez et al. 2011). Tuco-tucos are an interesting model to study immune function in free-living rodents since they have been extensively studied with regard to their life history traits (e.g., Zenuto '99; Busch et al. 2000), ecology (e.g., Rossin and Malizia 2002; Cutrera et al. 2006; Fanjul et al., 2006; Rossin et al. 2010), and physiology (e.g., Antinuchi et al. 2007; Vera et al., 2008; Cutrera et al., 2011); thus, an integrative study of their immune defense strategies is possible. *C. talarum* are herbivorous generalist rodents that inhabit coastal natural grasslands; they consume roots and subterranean stems, but mainly collect the aerial vegetative portion of grasses available near the burrow during brief excursions to the surface (Busch et al., 2000). In spite of their food source being abundant, changes in the vegetal composition are important for this opportunistic species, which consumes the majority of the plant species present in the grassland, changing its diet in relation to food availability (del Valle et al., 2001). Basal measures of immune components (antibody production against sheep red blood cells, SRBC; inflammatory response to phytohemagglutinin, PHA; leukocyte profile, natural antibodies, Nabs; and BKC) were studied, and some of them explored in relation to physiological condition (Cutrera et al. 2010; Merlo et al. 2014a), growth (Cutrera et al., 2014), spatial learning (Schleich et al., 2015), major histocompatibility complex (MHC) genotypes (Cutrera et al., 2011), and parasitism (Cutrera et al., 2011; Merlo et al., 2016). Also, the effect of food restriction on the level of antibodies against SRBC produced by adult tuco-tucos was assessed, finding no differences between slight and severe restriction (Schleich et al., 2015). However, the immune system is complex, involving several components that together determine the individual immunocompetence. Thus, additional information regarding how nutrition affects other immune components of tuco-tucos is needed to understand how immune defenses are regulated in this species, in relation to other physiological functions and changes in food resource availability. The present study was aimed to evaluate the effect of (1) food restriction and (2) methionine supplementation of diet

in the inflammatory response to PHA and other immune parameters (BKC, Nabs, and leukocyte profile). Nutritional parameters (plasma levels of glucose and triglycerides) were monitored to assess if the nutritional status of animals was effectively affected by treatments. Additionally, general condition of animals, monitored by health and stress parameters, was assessed to evidence possible effects of treatments on the immune status through the influence of individual condition. Levels of total proteins, albumin, and globulins in plasma, as well as albumin: globulin ratio (A/G), were studied in relation to the magnitude of the PHA response, since these parameters vary when inflammatory processes occur in the body (Gitlin and Colten, '87; Kaneko et al., 2008; González Naranjo and Molina Restrepo, 2010) and are also affected by nutrition (Kaneko et al., 2008). Finally, given that parasitism negatively influences the magnitude of the response to PHA in tuco-tucos (Merlo et al., 2016), individual endoparasite loads were also considered in our study.

We expected that food restriction would impair immune function as well as the general condition of *C. talarum*, which in turn may contribute to the decrease of immune function. Further, we hypothesized that food restriction increases endoparasite loads, which worsen the general condition of individuals. On the other hand, methionine supplementation was predicted to improve the immune response to PHA and possibly to change leukocyte profiles, since these immune parameters involve T- cells, which are affected by sulfur amino acid insufficiency (Grimble, '92). Major effects of methionine supplementation on general condition or endoparasite load were not expected.

MATERIALS AND METHODS

Animal Capture and Captivity Conditions

Adult *C. talarum* of both sexes were live-trapped in the locality of Mar de Cobo, Buenos Aires Province, Argentina (37°46' S 57°27' W). Holes were dug to access the underground burrows, and wire tube-shaped live traps (10 cm diameter, 35 cm length) were situated as an elongation of existing tunnels. Nursing females trapped were immediately released back into their burrow system so as not to deprive dependent young of maternal care. A total of 43 individuals, 29 males (163.44 ± 23.15 g) and 14 females (126.89 ± 21.07 g), were caught during the reproductive season (July to December) of 2014. Immediately after capture; feces of each animal were collected and conserved in 4% formalin for future procedures (see the section Quantification of Endoparasites). A blood smear was prepared from a single drop of blood obtained by making a small incision near the tip of the tail (Vera et al., 2008), then air-dried and fixed in 70% methanol for 10 min to preserve the sample until analysis (see the section Leukocyte Profiles). Animals were transported to the Laboratory of Ecophysiology at the National University of Mar del Plata (Mar del Plata, Argentina). There, 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 according to treatments (see below). Fresh food was provided daily to ensure water provision since *C. talarum* do not drink free water. Room conditions (temperature and photoperiod) were automatically controlled (25 ± 1°C, 12 L: 12 D). Tuco-tucos remained captive for the duration of the experimental assays (13 days) after which they were fed ad libitum until recovery of their initial body weight and released at the point of capture. Field and labwork were performed according to the Guidelines for the Treatment of Animals in Behavioural Research and Teaching (ASAB/ABS, 2003). Live captures were implemented according to the permit number 22500-21222/13 issued by the Office of Agrarian Business of the Buenos Aires Province.

Experimental Protocol and PHA Challenge

Immediately after arrival in the lab (day 1), individuals of each sex were randomly assigned to one of three diets: ad libitum ($n = 14$), composed by 70 g chicory, 50 g sweet potatoes, and 20 sunflower seeds per day; restricted ($n = 14$), consisting of 50 g lettuce and 7–15 g sweet potatoes, to lower the initial body weight in a 10%; or supplemented ($n = 15$), ad libitum diet plus five intramuscular doses (spaced in time: days 1, 3, 5, 7, and 9) of methionine (Hepatone®; 1 μL/g body weight), to increase the protein content of diet. Animals remained 12 days in treatment (diets) and were weighed once a day. On day 10, a blood sample (~ 500 μL) was collected from the retro-orbital sinus of all animals for posterior determinations (see the section Blood Determinations). The following day (day 11), fresh feces were collected from each individual cage for parasite quantification (see below), and all animals were immune-challenged with PHA as described by Merlo et al., (2014b). Briefly, thickness of both hind feet was measured with a digital micrometer (Insize®, Sao Paulo, Brazil) to the nearest 0.01 mm. Immediately after, the instep of the left hind foot was subcutaneously injected with sterile phosphate-buffered saline solution (control foot; PBS, 0.3 μL /g of corporal weight) using a 30 G needle, whereas the right hind foot was injected with PHA in the same way (immune challenged foot; *Phaseolus vulgaris* PHA-Sigma L-8754 solution dissolved in PBS, 3 mg/ml; 0.3 μL /g corporal weight). 24 hr after injections (day 12), thickness of both hind feet was measured again, and then another blood sample (~ 200 μL) was collected from the retro-orbital sinus of all animals for posterior determinations (see *Total proteins, albumin and globulins pre- and post-PHA injection* under the section Blood Determinations). Inflammatory response in each foot was calculated as the difference between pre- and postinjection thickness divided by initial thickness (response = (post – pre)/pre); Xu and Wang 2010; Merlo et al., 2014b). A timeline showing treatment period and time points of sample collections is provided in Figure 1.

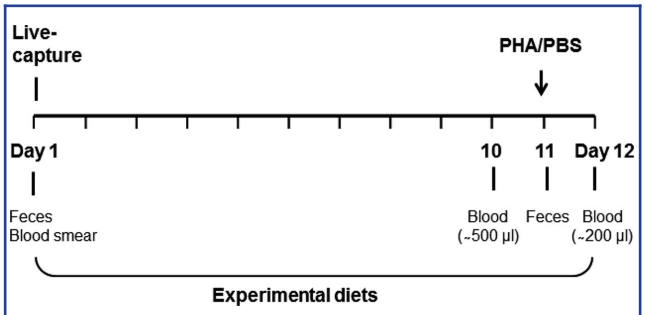

Figure 1. Timeline for different sample collections during the 12-day experiment.

Blood Determinations

From blood samples collected on Day 10, the following parameters were assessed.

Immune Parameters. *Leukocyte profiles*. Relative abundances of leukocytes in blood provide information about different processes occurring in the body, such as allergies and parasitism (eosinophilia), infection or endocrine disorders (increased basophil count), chronic infection or inflammation (increased monocyte count; Voigt 2000). Nevertheless, leukocyte profiles interpretation requires caution because other factors can cause fluctuations in blood cell counts (e.g., stress; Davis et al. 2008; Beldomenico et al., 2008a). Blood smears were immediately made and fixed with methanol after blood extraction. Slides were stained with May-Grunwald Giemsa solution and then examined under oil immersion at 1000× magnification (Olympus CX 31, Tokyo, Japan). The "wandering technique" was used to record the abundance of lymphocytes, neutrophils, eosinophils, monocytes, and basophils. Cells were identified based on morphology, as described by Voigt (2000) in vertebrates and Vera et al. (2008) and Cutrera et al. (2010) for *C. talarum*, until a total of 200 leukocytes had been examined. To calculate the total leukocyte abundance, the number of leukocytes encountered in 30 fields in which there was a single layer of erythrocytes (~20,000 erythrocytes; Roxana R. Zenuto, unpublished data) was recorded and then standardized to 100,000 erythrocytes for comparative purposes. Blood smears performed immediately after capture (day 1) were stained and examined in the same manner.

Erythrocyte sedimentation rate (ESR). ESR is a nonspecific measure of infectious processes in the body. The increment of ESR during acute infection is due to the erythrocyte aggregation caused by acute phase proteins, which delays the sedimentation process (Blaxhall and Daisley '73). In *C. talarum*, parasitized individuals showed a tendency toward higher ESRs, in comparison with uninfected individuals (Merlo et al., 2016). Following Saino and Møller ('96), blood samples were collected into heparinized capillary tubes and these were put in vertical position for 4 hr at 4°C. The sedimentation rate (proportion of erythrocytes sedimented per hour) was calculated as the volume of the capillary not occupied by red blood cells × blood volume in the capillary$^{(-1)}$ × 0.25.

Bacterial killing capacity assay. This technique quantifies the capacity of plasma components to inhibit bacterial growth in vitro, providing a functional measure of the innate immune response. We followed the methodology proposed by Liebl and Martin (2009) and the modifications done by Merlo et al. (2016) for *C. talarum*. The antimicrobial activity of plasma was calculated as 1– (absorbance of sample /absorbance of control), or the proportion of microbes killed in samples relative to positive controls.

Hemolysis–hemagglutination assay. The hemolysis–hemagglutination assay proposed by Matson et al. (2005) allows quantification in plasma of the natural antibody (NAb)-mediated activation of the complement (evidenced as lysis of SRBC added to the plasma sample) and NAb activity (evidenced as agglutination of SRBC; both measures of innate immune capacity). In tuco-tucos (Merlo et al. 2016), as in many other species (Matson et al. 2005), lytic activity of the complement is not detectable through this procedure, and thus, agglutination of SRBC is the only measure that can be determined (Merlo et al., 2016). Levels of SRBC agglutination were obtained following the methodology described in Merlo et al. (2016) and expressed as the negative $\log_2$ of the minimum plasma concentration that produces visible agglutination of the sample. To increase the consistency of the data set, scoring of plates was always performed by the same person (JLM).

Nutritional Parameters. *Glucose*. Level of glucose in whole blood collected from each individual was determined using a glucometer (Accu-Chek Active®, Basilea, Suiza) as a measure of nutritional condition.

Triglycerides. Plasma concentration of triglycerides is an indicator of nutrition and metabolic syndromes (Lee et al., 2003). Triglycerides in plasma samples were determined for each individual using a colorimetric kit (TG color ®, Wiener laboratory, Santa Fe, Argentina).

Health Status and Stress. *Hematocrit*. Hematocrit, the proportion of blood volume occupied by packed red blood cells, is considered an indicator of general health status (Hoi-Leitner et al., 2001). After ESR determination (see above), capillary tubes were centrifuged at 14,000 rpm for 15 min (Cavour VT 1224 centrifuge, Buenos Aires, Argentina). Hematocrit was assessed as the proportion of capillary tube length occupied by packed red blood cells, relative to the capillary tube length occupied by all blood components (Abaco CAV 1224, Buenos Aires, Argentina).

Cortisol. Cortisol was found to vary in response to exposition to stress factors in *C. talarum* (Vera et al., 2011). Plasma obtained as described previously was used (without dilution) to

determine levels of cortisol in each individual, using an enzyme immunoassay kit (EIA 1887 DRG® Instruments GmbH, Marburg, Germany; kit validation: F. Vera et al., unpublished results).

Neutrophils: lymphocytes ratio (N: L). N: L is a known stress indicator, which increases with glucocorticoid secretion, specially during chronic exposure (Davis et al., 2008), although more recently it is also proposed that N: L ratios and glucocorticoids would indicate different stress situations (Müller et al. 2011). In *C. talarum,* increases in N: L have been verified during acute (brief immobilization; Vera, 2011) and chronic stressful situations (e.g., captivity, Vera et al., 2008, and food restriction, Schleich et al., 2015)). N: L was calculated from neutrophils and lymphocytes counts recorded per individual (see "Leukocyte profiles" above).

Total Proteins, Albumin, and Globulins Pre- and Post-PHA Injection. Total proteins, albumin, and globulins. Albumin and globulins are the major protein components of plasma (Kaneko et al., 2008). In response to inflammation, serum albumin concentrations decrease dramatically (Gitlin and Colten, '87; González Naranjo and Molina Restrepo 2010); but these also decrease if a poor nutrition impair their synthesis (Kaneko et al., 2008). In tuco-tucos, higher levels of plasmatic albumin were verified in parasitized animals, in comparison with uninfected conespecifics (Merlo et al., 2016). Plasma globulins (α, β, and γ) are a heterogeneous family of proteins that play a role in inflammatory responses, transportation of various lipophilic compounds, homeostasis, and production of antibodies (Abdou et al., 2014). Normally, there is a little more albumin than globulins, giving an albumin: globulin ratio (A/G) slightly over 1. Because disease may affect the relative amounts of albumin and globulin, the A/G may provide additional information about the cause of the change in protein levels (Kaneko et al., 2008). A fraction ($\sim$ 30 μL) of the blood collected from each individual was stored 1 hr at 4°C to clot, centrifuged at 3000 rpm for 15 min, and the resulting supernatant (serum) was collected. Albumin levels in serum samples were determined using a colorimetric kit (Albúmina AA®, Wiener laboratory, Santa Fe, Argentina; respectively) read at 625 nm in a spectrophotometer (Ultrospec 1100 pro). Total proteins levels were determined using a colorimetric kit (Poteínas Totales AA®, Wiener laboratory, Santa Fe, Argentina; respectively) read at 540 nm. Globulin levels were calculated as the quantity of total proteins that are not albumin (globulins = total proteins – albumin; Gornall et al., '49). Albumin, total proteins, and globulins levels were calculated in the same way from blood samples taken on day 12.

Quantification of Endoparasites

The number of parasite eggs/oocysts present in fecal samples collected immediately after capture and at day 11 was quantified per individual (investigator JLM was blinded to the group origin of samples when processing them). This procedure ensures the detection of all taxa present in digestive tracts of tuco-tucos, with the only exception of *Taenia talicei* (Rossin et al., 2004; Cutrera et al., 2011). Eggs/oocysts present in feces were assessed using the flotation technique proposed by Sheather ('23), already used for *C. talarum* (Merlo et al., 2016). *Pudica ctenomyidis, Graphidioides subterraneus, Strongyloides myopotami*, and *Trichostrongylus duretteae* eggs could not be differentiated and, given their phylogenetic proximity and their relatively low abundance and prevalence (consistent with reports for the adult forms found in this population of *C. talarum*, Rossin et al., 2010), they were all grouped as "strongylids."

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 paired *t*-test was performed to compare swelling response in PHA- versus PBS-injected hind feet. Two-way analysis of variances (ANOVAs) were used to test the effect of treatment (diets) and sex on each parameter studied. Repeated-measure ANOVAs were used to test the effect of treatment and sex on parameters measured at two different time points (e.g., day 1 and 10, day 10 and 12). Tukey tests were used to assess post hoc differences among groups. Pearson correlations were used to test associations between the magnitude of swelling and body weight, albumin level$_{\text{day 12}}$ and A/G$_{\text{day 12}}$. Triglycerides, glucose, cortisol, and N: L (at days 0 and 10) values were ln-transformed, and BKC values were Box–Cox-transformed ($\theta = 0.4$) to reach normality. Eosinophil, basophil, and endoparasite eggs/oocysts counts could not be normalized; thus, the change (Δ; count at last time point measured – count at first time point measured) was calculated for each parameter, and differences among treatments were analyzed by the nonparametric Kruskal–Wallis test whereas differences between sexes, through Mann–Whitney U tests. Throughout the text, results are expressed as means $\pm$ standard error ($\pm$ S.E.).

RESULTS

Before the experiments, body weight and leukocyte profiles were homogeneous among individuals assigned to the different groups (body weight$_{\text{day 1}}$: two-way ANOVA, $F_{(2,37)} = 0.52$, $P = 0.6$; lymphocytes$_{\text{day 1}}$: $F_{(2,37)} = 0.61$, $P = 0.55$; neutrophils $_{\text{day 1}}$: $F_{(2,37)} = 0{,}92$, $P = 0{,}41$; eosinophils$_{\text{day 1}}$: Kruskal–Wallis, $X^2_{(2,42)} = 0{,}62$, $P = 0{,}73$; basophils $_{\text{day 1}}$: $X^2_{(2,42)} = 0{,}04$, $P = 0{,}98$; monocytes $_{\text{day 1}}$: $X^2_{(2,42)} = 0{,}17$, $P = 0{,}92$).

Animals in the restricted diet lost 12.8 $\pm$ 4.7% of their initial body weight by day 10 (significant weight loss: repeated-measure ANOVA; "time*diet" factor: $F_{(2,40)} = 10.89$, $P < 0.001$; Tukey test, $P < 0.001$; Fig. 2), whereas weight of individuals fed ad libitum or under the supplemented diet did not differ from

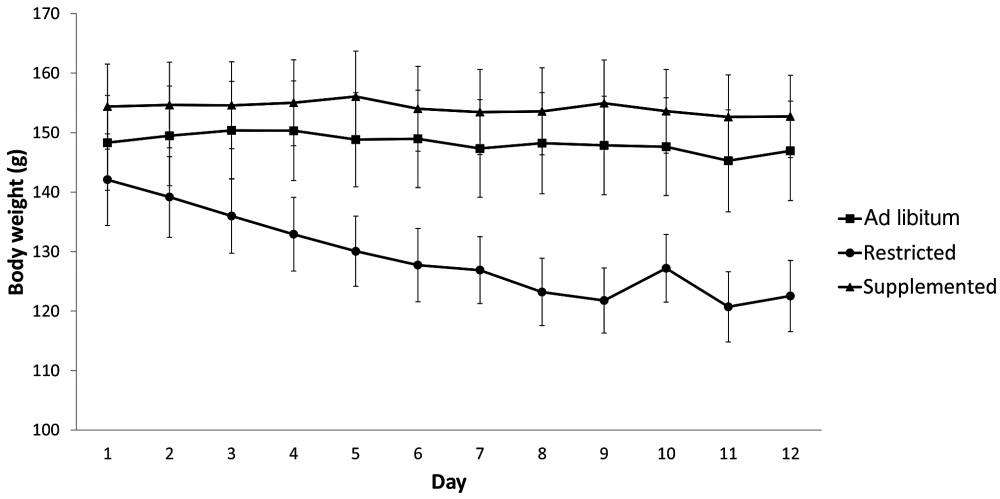

Figure 2. Mean body weight per group (ad libitum diet: $n = 14$, restricted diet: $n = 14$, supplemented diet: $n = 15$; ± S.E.) of tuco-tucos during the experimental period.

day 1 to 10 (Tukey test: ad libitum: $P = 0.50$; supplemented: $P = 0.99$; Fig. 2).

Induced Inflammatory Response to PHA

The swelling response was significantly greater in PHA-injected hind feet compared to control hind feet injected with PBS (paired t-test; $t = 12.86$, $n = 43$, $d.f. = 42$, $P < 0.001$; $\text{swelling}_{PHA} = 0.46 \pm 0.22$, $\text{swelling}_{PBS} = 0.02 \pm 0.09$). The swelling response of animals in restricted diet was significantly lower than those of animals in the other two diets (Table 1; Fig. 3). Magnitude of swelling did not correlate with body weight at day 11 in any of the three groups (ad libitum: $r = -0.22$, $P = 0.45$; restricted: $r = -0.23$, $P = 0.44$; supplemented: $r = 0.11$, $P = 0.71$).

Other Immune Parameters

ESR, BKC, and NAb titers did not differ among treatments (Table 1). Males presented lower BKC than females (Table 1). Total leukocyte counts were not significantly different among treatments or between sexes (repeated-measure ANOVA; "diet" factor: $F_{(4,72)} = 0.44$, $P = 0.78$, "sex" factor: $F_{(2,36)} = 1.28$, $P = 0.29$). Δeosinophil and Δbasophil counts did not differ among treatments or between sexes (Table 1). Monocyte counts were not included in the analysis given the low counts registered (scored 0 in 54 of 86 samples; 0.73 ± 1.16).

Nutritional Parameters

Blood glucose levels from animals in the restricted diet were lower than those from animals in the other two diets (Table 1; Fig. 4A), and females presented higher levels than males (Table 1). Triglyceride levels were significantly lower in the restricted diet than in the ad libitum diet (Table 1; Fig. 4B). Also, females showed higher levels of triglycerides than males (Table 1).

Health Status and Stress

Hematocrit did not differ among treatments (Table 1). Males presented higher hematocrit than females (Table 1).

N: L significantly increased after the experimental period in animals under restricted diet (repeated- measure ANOVA; $F_{(4,72)} = 4.81$; $P = 0.01$; Tukey test, $P < 0.001$; Fig. 5). Cortisol levels did not differ among diets (Table 1). Females showed significantly higher cortisol levels than males (Table 1).

Total Proteins, Albumin, and Globulins

Effect of Diet. Levels of albumin and total proteins were significantly lower in animals in the restricted diet compared to those fed ad libitum (albumin: two-way repeated measures ANOVA, "diet" factor, $F_{(2,37)} = 4.06$; $P = 0.03$, Tukey test, $P < 0.01$; total proteins: $F_{(2,37)} = 3.32$; $P = 0.047$; Tukey test, $P < 0.01$). Globulins and A/G were not affected by treatment (globulins: two-way repeated measures ANOVA, "diet" factor, $F_{(2,37)} = 0.37$; $P = 0.70$; A/G: $F_{(2,37)} = 0.84$; $P = 0.44$). Females showed lower $\text{A/G}_{\text{day 10}}$ than males (two-way repeated measures ANOVA, "time∗sex" factor, $F_{(2,37)} = 6.45$, $P = 0.02$; Tukey test, $P = 0.03$).

Effect of the PHA Injection. Levels of albumin and A/G decreased after the PHA challenge (albumin: two-way repeated measures ANOVA, "time" factor, $F_{(1,37)} = 14.58$; $P < 0.001$, Fig. 6A; A/G: $F_{(1,37)} = 7.93$; $P < 0.01$, Fig. 6B), but levels of total proteins and globulins were not affected (total proteins: two-way

Table 1. Mean or median (± S.E.) values of immune, nutritional, health, stress, and parasite load parameters of ad libitum, restricted and supplemented fed tuco-tucos (*C. talarum*) and results of the two-way ANOVAs, Kruskal–Wallis, and Mann–Whitney tests used to test differences in these parameters among groups and sexes. Asterisks denote significant differences.

				Diet effect		Sex effect	
Parameter	ad libitum	Restricted	Supplemented	Statistic	*P*	Statistic	*P*
Immune parameters							
ESR	0.03 (± 0.004)	0.03 (± 0.006)	0.03 (± 0.008)	$F = 0.26$	0.77	$F = 0.12$	0.73
BKC (%)[a]	1 (± 1)	2 (± 1)	3 (± 1)	$F = 0.48$	0.63	$F = 4.5$	0.04∗
Nabs	3.09 (± 0.32)	3.63 (± 0.22)	3.31 (± 0.16)	$F = 0.38$	0.70	$F = 0$	1
Total leukocytes /100,000 RBC	34 (± 5.07)	37.71 (± 4.3)	35.6 (± 4.21)	$F = 0.03$	0.98	$F = 2.3$	0.13
ΔEosinophils[b]	−5 (± 2.08)	−11 (± 2.9)	−7 (± 2.30)	$X^2 = 4.36$	0.11	U = 184.5	0.63
ΔBasophils[b]	0 (± 2.33)	−1.5 (± 1.5)	−1 (± 2.85)	$X^2 = 0.62$	0.74	U = 158	0.24
Nutritional parameters							
Glucose (mg/dL)[a]	92 (± 16.55)	63.5 (± 2.78)	101 (± 12.23)	$F = 10.6$	< 0.001∗[d]	$F = 11.18$	0.002∗
Triglycerides (g/L)[a]	1.02 (± 0.27)	0.55 (± 0.12)	0.93 (± 0.13)	$F = 4.38$	0.02∗[e]	$F = 6.11$	0.02∗
Health							
Hematocrit	45.07 (± 1.17)	49.00 (±1.39)	45.63 (±1.27)	$F = 0.89$	0.05	$F = 6.5$	0.02∗
Stress							
Cortisol (ng/mL)[a]	9.38 (± 2.57)	13.7 (± 2.29)	8.23 (± 2.01)	$F = 1.78$	0.18	$F = 14.19$	< 0.001∗
Parasites							
Δ*Eimeria* sp.[c]	−3,671.5 (± 4,347)	15,200 (± 11,388)	−3,456.5 (± 1,847,5)	$X^2 = 5.04$	0.08	$U = 135$	0.92
Δ*P. uncinata* sp.[c]	−260,5 (± 460,5)	203,5 (± 313)	−170 (± 325)	$X^2 = 0.24$	0.89	$U = 102$	0.21
Δ*T. pampeana* sp.[c]	1,560.5 (± 992)	650 (± 303)	146,5 (± 72)	$X^2 = 0.11$	0.95	$U = 110.5$	0.34

PHA: phytohemagglutinin; ESR: erythrocyte sedimentation rate; BKC: bacterial killing capacity; NAbs: natural antibodies; RBC: red blood cells.
[a]Nontransformed values.
[b]Abundances based in a total of 200 leukocytes examined per individual. Values correspond to $counts_{day10}$ – $counts_{day0}$.
[c]Values correspond to $counts_{day11}$ – $counts_{day0}$.
[d]Restricted group differed from the other two groups (Tukey test, $P < 0.001$).
[e]Restricted group differed from ad libitum group (Tukey test, $P < 0.005$) (see the text for more details).

repeated measures ANOVA, "time" factor, $F_{(1,37)} = 3.71$, $P = 0.06$, Fig. 6C; globulins: $F_{(1,37)} = 0.92$, $P = 0.34$, Fig. 6D). Albumin and A/G did not correlate with the magnitude of swelling in response to PHA (albumin: $r = 0.28$, $P = 0.065$; A/G: -0.10, $P = 0.54$). Additionally, differences in these parameters were observed between sexes: total proteins significantly decreased in males after the challenge (two-way repeated measures ANOVA, "time∗sex" factor, $F_{(1,37)} = 5.30$, $P = 0.03$; Tukey test, $P < 0.01$), globulin levels increased in females and decreased in males (two-way repeated measures ANOVA, "time∗sex" factor, $F_{(1,37)} = 7.62$, $P < 0.01$) and A/G of females decreased to values similar to males at day 12 (two-way repeated measures ANOVA, "time∗sex" factor, $F_{(1,37)} = 6.45$, $P = 0.02$; Tukey test, $P = 0.01$).

Parasite Loads. Change in egg/oocyst shedding (fecal counts after treatment – fecal counts at time of capture) did not differ among treatments or sexes (Table 1). Strongylid egg counts were not included in the analysis given the low values registered (scored 0 in 73 of 86 samples; 0.2 ± 0.94).

DISCUSSION

As expected, individuals under food restriction presented lower levels of glucose, triglycerides, total proteins and albumin in plasma, as well as increased N: L values than individuals fed ad libitum. This shows that food restriction, even for a short period of time, has a significant effect on nutritional parameters and overall homeostasis in tuco-tucos. On the other hand, supplementation with methionine did not produce significant effects on nutritional parameters and, contrary to our predictions, did not significantly affect the response to PHA nor any of the parameters analyzed here.

Tuco-tucos under food restriction mounted a lower inflammatory response to PHA (~ 54% reduction) in comparison to those fed ad libitum, supporting our hypothesis that food restriction reduces the ability to mount a PHA-induced inflammatory response. Tuco-tucos showed a 10% reduction in their body weight under the food-restricted diet, which may not only represent a decrease in total energetic intake of the animals but also in the content of minor components (such as proteins), macronu-

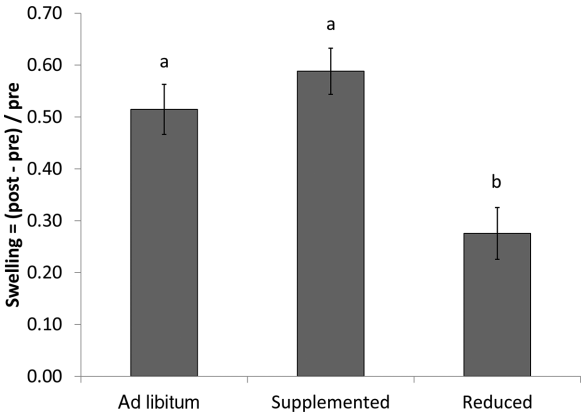

Figure 3. Mean hind feet swelling (± S.E.) of tuco-tucos under the different three diets (ad libitum diet: $n = 14$, restricted diet: $n = 14$, supplemented diet: $n = 15$), 24 hr after PHA injection. Letters denote statistical differences among groups (two-way ANOVA, $P < 0.05$).

trients (carbohydrates, lipids) and micronutrients (vitamins and minerals) in comparison to the ad libitum diet. Therefore, the reduction in the magnitude of the inflammatory response may have been induced by a caloric restriction, a lower intake of several nutritional components, or both. Previous studies have also shown a negative impact of food restriction on immunity: for example, 3-day fasted *Meriones unguiculatus* (Mongolian gerbils) mounted lower responses to PHA than those of their nonfasted counterparts (Xu and Wang, 2010). Also, a restricted dietary protein intake (but without caloric restriction) had a negative effect on the magnitude of the delayed-type hypersensitivity response of *Peromyscus leucopus* (white-footed mice; Thomason et al., 2013). Moreover, micronutrients deficiency can influence several components of innate immunity (Erickson et al., 2000). For example, synthesis or activity of several of the enzymes and reactions that are essential to neutrophil function (a key component of the swelling response to PHA in *C. talarum*; Merlo et al., 2014b) can be altered by the deficiency or supplementation of micronutrients such as certain vitamins (Kaplan and Basford, '76; Twining et al., '97). A decrease in glucose and triglyceride blood levels verified in *C. talarum* under food restriction shows that they had lower metabolic fuel and energetic reserves to face an immune challenge in comparison to animals fed ad libitum. It is known, for example, that glucose uptake and utilization is a required part of the metabolic response of T-cells to mitogenic signals, and that fatty acids are required for membrane synthesis in proliferating T-cells (Fox et al., 2005). Thus, part of the swelling reduction in tuco-tucos under a restricted diet could be consequence of the lower availability of these molecules, in addition to the reduced caloric input and micronutrient deficiencies.

Except for the inflammation in response to PHA, immune parameters studied here did not vary in response to food restriction, despite the wide array of immune components assessed, including complement system activity (BKC), NAbs, and leukocyte profiles. Although production and maintenance of these components use body resources (Fox et al., 2005), the performance of these processes did not vary in relation to nutritional deficiencies, which may evidence that these components of the immune system are less sensitive to variations in nutritional status compared to those required for an induced immune challenge, such as PHA. Similar results have been found by Zysling et al. (2009) in *Phodopus sungorus* (Siberian hamsters); food -restricted animals presented increased humoral activity against a subcutanean injection of KLH compared with animals fed ad libitum, but there was no difference in the ex vivo measure of BKC between both groups. Overall, our results support the hypothesis that

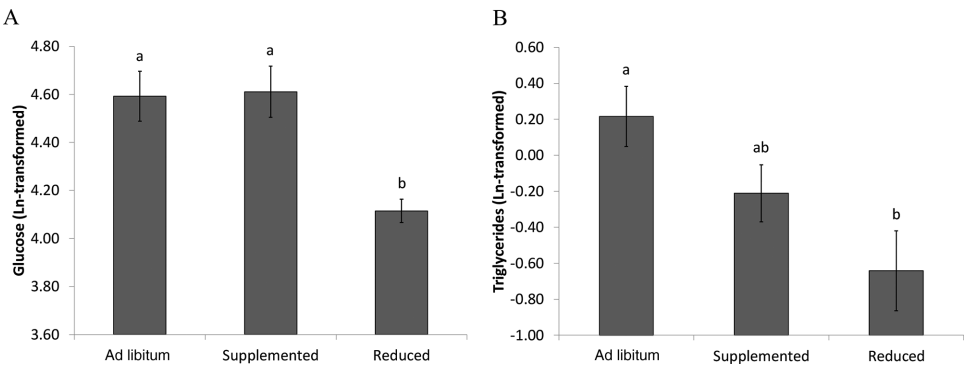

Figure 4. Mean ln-transformed glucose (A) and triglycerides (B) levels (± S.E.) in plasma of tuco-tucos under the different diets (ad libitum diet: $n = 14$, restricted diet: $n = 14$, supplemented diet: $n = 15$). Letters denote statistical differences among groups (two-way ANOVAs, $P < 0.05$).

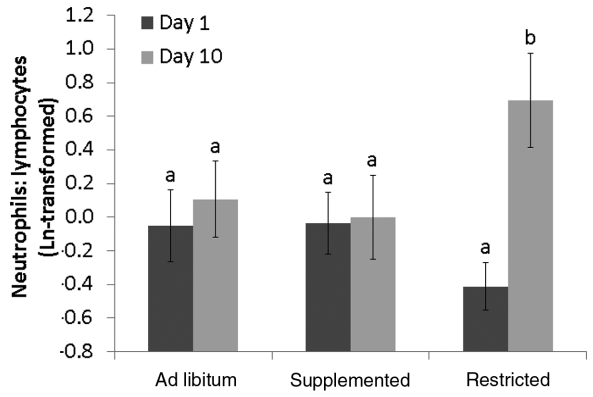

Figure 5. Mean Ln-transformed N: L (± S.E.) at day 1 and 10 in plasma of tuco-tucos under the different diets (ad libitum diet: $n = 14$, restricted diet: $n = 14$, supplemented diet: $n = 15$). Letters denote statistical differences among groups (repeated measures ANOVA, $P < 0.05$).

developmental costs of constitutive innate (e.g., phagocytic cells, complement and antimicrobial proteins) and humoral (NAbs) defenses are low, whereas an induced inflammatory local response has a high cost of use (Lee, 2006). Although the development of the PHA response does not represent a significant energetic cost for *C. talarum* (Merlo et al., 2014a), results from the present study showed that it requires sufficient nutritional resources, another currency proposed to mediate the costs of immune activation (Hasselquist and Nilsson, 2012).

Stress associated with food restriction can lead to increased levels of glucocorticoids in mammals (Murphy and Wideman, '92). Glucocorticoids, in turn, have been proved to alter immune function. As a consequence of the redistribution of leukocytes during stressful situations, N: L ratios tend to increase (Davis et al., 2008). In the present study, tuco-tucos under food restriction exhibited higher N: L than individuals under the other two diets, which is consistent with previous results for this species (Schleich et al., 2015), suggesting that this treatment induced a chronic stress over the animals. However, although cortisol levels were slightly higher in food-restricted *C. talarum*, they were

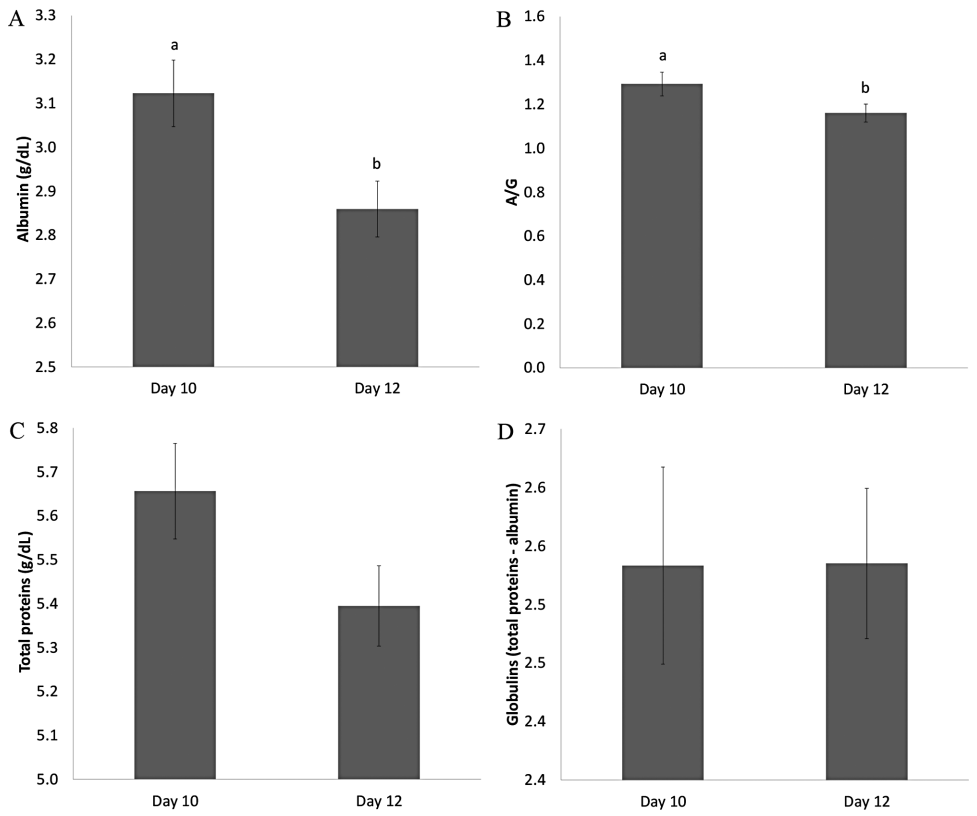

Figure 6. Mean albumin (A), A/G (B), total protein (C), and globulins (D; ± S.E.) in plasma of tuco-tucos under the different diets (ad libitum diet: $n = 14$, restricted diet: $n = 14$, supplemented diet: $n = 15$), at days 10 and 12. Letters denote statistical differences among days of treatment (repeated measures ANOVAs, $P < 0.05$).

not statistically different among experimental diets. Increases in glucocorticoid levels occur in short periods of time during restricted feeding (Zysling et al., 2009), and thus, the possibility of having missed the best time point to measure the effect of treatments on the induction of this hormone cannot be excluded from the present study.

We expected that under food restriction, the survival or reproduction of intestinal parasites would be positively affected, given the poorer nutritional status of their hosts. However, change in fecal egg/oocyst counts was not different among diets. It has been observed that malnutrition leads to decreased resistance against new parasite infections in several domestic species, but has no influence on the already present worms, including their egg production (Fekete and Kellems, 2007). Additionally, food restriction increases coccidian infection in *Hydrochoerus hydrochaeris* (capybaras), but reduces helminth intensities (Eberhardt et al., 2013), indicating that the relation between nutrition and infection may vary depending on the type of parasite considered. Therefore, the hypothesis of an additional impairment of the condition of *C. talarum* under food restriction due to an increase in their endoparasite loads was not supported by our results.

Contrary to our predictions, supplementation with methionine did not significantly improve the immune response to PHA in *C. talarum*. However, a slight increase in the magnitude of the inflammatory response (~15%) in the supplemented group in comparison with animals fed ad libitum can be appreciated from the reported results, the effect of which on the PHA-induced inflammation may be further clarified using larger sample sizes. However, it is also important to consider that coprophagy, an effective way to obtain extra protein, essential amino acids, essential fatty acids, and vitamins (Takahashi and Sakaguchi, '98; Barnes, 2009), was reported in *C. talarum* (Martino et al., 2007). Tuco-tucos are capable of increasing the frequency of this behavior in response to diet quality (Martino et al., 2007). Thus, experimental manipulation of the quality of the diet is difficult to perform in this species, since tuco-tucos can regulate the nutritional ingest themselves by engaging in coprophagy. As mentioned before, previous studies in birds (Swain and Johri, 2000; Soler et al., 2002; Brommer, 2004; Abdukalykova and Ruiz-Feria, 2006; Pitala et al., 2010) have revealed significant effects of amino acid supplementation on the response to PHA. But, these studies were performed on growing individuals (Tsiagbe et al., '87; Swain and Johri, 2000; Soler et al., 2002; Abdukalykova and Ruiz-Feria, 2006), and thus a surplus of amino acids provided in the period when immune system is developing may have strong effects on the individual immunocompetence. Additional studies in adult animals will be relevant to determine the effect of particular amino acids in the promotion of immune responses.

Interestingly, levels of albumin and A/G of tuco-tucos decreased from day 10 to 12 considering animals from the three groups together. Between day 10 and 12, two main events occurred to individuals: a blood extraction of ~500 μL and the immune challenge to PHA (Fig. 1). If the blood extraction itself contributed to decrease albumin levels and A/G, we would expect similar variation in all components of blood; but no significant changes were observed in globulins or total proteins. Therefore, the reduction of albumin levels and the concomitant decrease in A/G can be considered as a consequence of the inflammatory response induced by PHA, suggesting that protein resources are required for mounting this immune response. Therefore, lower inflammatory responses to PHA observed in animals under a restricted diet in the present study could be due to the reduction in protein availability.

Overall, our results support the idea that food quantity and quality can modulate the individual's capacity to respond to a novel immune challenge. This modulation can occur as consequence of the availability of proteins and essential nutrients needed for the activity of immune components (e.g., T cells), the lack of metabolic fuel and energetic reserves, or even by a detrimental effect of the stress produced by nutrient limitation. However, the immune system is composed of a wide variety of cells, organs, and humoral compounds, and thus, modulation of one compartment will not necessarily affect others. Findings for the present study add information regarding the immune strategies of *C. talarum*. The response triggered by PHA, which involves inflammatory and T-cell adaptive pathways (Lee, 2006), is not energetically costly in this species (Merlo et al., 2014a), but it involves a nutritional cost that compromises the development of this response under suboptimal food availability conditions. On the other hand, the energy-demanding antibody response against SRBC (Cutrera et al., 2010) did not show variation in magnitude between treatments of slight and severe food restriction (Schleich et al., 2015). This may suggest that highly energetic demanding processes are not necessarily more compromised under periods of nutrient deficiency than processes associated with lower energetic costs. The nonenergetically expensive response to PHA in *C. talarum*, in addition to be affected by nutritional condition, is also compromised by parasitism, being higher in animals not infected by endoparasites compared with animals naturally or artificially infected (Merlo et al., 2016). Therefore, the inflammatory response induced by PHA seems to be adjustable in relation to intrinsic (e.g., simultaneous infections) and extrinsic (e.g., food availability) factors in tuco-tucos, possibly as a strategy to relieve some of the demands on the organism resources during stressful times. Modulation of this response may be possible given the wide array of cells and physiological changes involved in the process of inflammation, in comparison with the more specific pathways activated during an induced antibody response. Ongoing studies of the trade-offs between simultaneous activation of the PHA (inflammatory)- and SRBC (humoral)-induced responses will add information regarding these findings.

Our findings are relevant because of the generalist and opportunistic habits exhibited by *C. talarum*, as well as other free-living mammals. For example, changes in vegetal composition and availability due to climate warming (Alward et al., '99) could compromise the acquisition of nutrients required to fulfill all physiological functions, resulting in an impaired individual immunocompetence. Ultimately, this could result in a complex ecological feedback on population dynamics, from individual displacements to population disease or decline.

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