RESEARCH ARTICLE

Parasite Infection Negatively Affects PHA-Triggered Inflammation in the Subterranean Rodent *Ctenomys talarum*

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ABSTRACT

Magnitude and effectiveness of immune responses vary greatly between and within species. Among factors reported to determine this variation, parasitism is a critical one, although controversial effects of parasites over immunological indices have been reported. Information regarding immune strategies in species with different life histories is crucial to better understand the role of immune defenses in an ecological and evolutionary context. Here, we examine the influence of the parasite community on immune responsiveness of a solitary subterranean rodent, *Ctenomys talarum*. To do this, we assessed the impact of the natural parasite community and the experimental infection with *Eimeria* sp. on the phytohemagglutinin (PHA)-response, as well as other immune, condition, nutrition, and stress parameters. PHA-triggered inflammation was similarly impaired by *Eimeria* sp. infection alone or co-occurring with a number of gastrointestinal nematodes. None of the other physiological parameters studied were affected by parasitism. This indicates that parasitism is a general key factor modulating immune responsiveness of the host, and in particular for *C. talarum*, it could explain the great inter-individual variation previously observed in the PHA-response. Thus, our results highlight the importance of taking the parasite community into account in ecoimmunological studies, particularly when using immunological indices. *J. Exp. Zool. 325A:132–141, 2016.* © 2015 Wiley Periodicals, Inc.

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Ecoimmunological studies have shown that magnitude and effectiveness of immune responses vary greatly between species and even within them (Martin et al., 2008). This variation has been associated with life-history traits as well as modulation by ecological, genetic, and physiological factors (Schmid-Hempel, 2003; Lee, 2006; Ardia et al., 2011). In particular, current parasite infections may enhance or suppress immune responses to a new challenge (Page et al., 2006). Interaction among different pathogens remains difficult to understand, and this is probably why very few parasitological and ecological studies focus on more than one pathogen (Cox, 2001). However, given that concomitant

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infections—a situation in which two or more infectious agents coexist in the same host—are common in nature, an effort to understand these interactions is necessary (Cox, 2001).

Previous studies have explored the effect of parasites on the immune system through the use of phytohemagglutinin (PHA), a lectin that induces a swelling response when injected subcutaneaously. Goüy De Bellocq et al. (2006) showed that Sundevall's Jirds (*Meriones crassus*) experimentally infected by their common ectoparasite *Xenopsylla ramesis* mounted lower PHA-responses than non-parasitized animals. Similarly, Christe et al. (2000) found a negative relationship between parasite load (blood sucking mites *Spinturnix myoti*) of bats *Myotis myotis* and their response to PHA during lactation. This pattern was also observed in birds: house sparrows that developed stronger PHA-responses were those with lower intensity of infection by blood parasite *Haemoproteus* sp. (Navarro et al., 2003). The negative effect of parasitism upon this immune response may be understood as a result of competition for resources by both immune challenges, since the use of host resources by a parasite — for its own maintenance and reproduction — decreases the amount of resources a host can allocate to the development of another immune response (Graham, 2008; Cornet and Sorci, 2010). Alternatively, immune components stimulated by simultaneous immune challenges could directly interact through, for example, mutual inhibition of Th1 (type 1 T helper lymphocytes) — and Th2 (type 2 T helper lymphocytes)— mediated responses (Cox, 2001), induced immunodepression by parasites (Cox, 2001) or non-restricted release of signaling molecules (e.g., cytokines; Graham, 2008). Interestingly, positive associations between parasite load and the PHA-response were reported for birds. Individuals from nests with high ectoparasitic loads showed larger swelling responses to PHA (Christe et al., 2000; Gwinner et al., 2000), and increases in infection susceptibility to *Isospora lacazei* correlated positively with the magnitude of the PHA-response in greenfinches (Saks et al., 2006), which leads to the hypothesis that parasite infection enhances the host's cell-mediated immune function, up-regulating the response to other challenges that stimulate the same pathway (Saks et al., 2006).

Even when the publication of Hamilton and Zuk's article ('82) on the role of parasites in sexual selection encouraged the study of the effects of parasites over their hosts (Norris and Evans, 2000), additional information regarding immune strategies in species with different life histories is crucial to better understand the role of immune defenses in an ecological and evolutionary context (Lee, 2006). Most ecoimmunological studies in wild populations have involved birds, probably as a consequence of their relatively complex immune system and the possibility of experimentally manipulating their life-history decisions (Norris and Evans, 2000). Here, we add information regarding the influence of host parasite community on the immune response of a mammal species, specifically of *Ctenomys talarum* Thomas ('98) (tuco-tuco), a solitary subterranean rodent.

Members of *C. talarum* are distributed in coastal grasslands and sand dunes of Buenos Aires Province, Argentina (Busch et al., '89). Tuco-tucos are successfully maintained in captivity, which allows experimentation under controlled conditions. Their endoparasite community is composed of gastrointestinal nematodes (*Trichuris pampeana*, *Paraspidodera uncinata*, *Graphidioides subterraneus*, *Pudica ctenomydis*, *Strongyloides myopotami*, *Trichostrongylus duretteae*, and *Taenia talicei*) and an intestinal protozoan (*Eimeria* sp.) (Rossin et al., 2010; Cutrera et al., 2011). Moreover, the inflammatory response of this species has been explored through the characterization and evaluation of the magnitude and energetic costs of the local swelling triggered by PHA (Merlo et al., 2014a,b). This immune response includes innate and cell-mediated components for tuco-tucos (Merlo et al., 2014a), and does not imply a significant energetic cost (Merlo et al., 2014b). Interestingly, the PHA-response varies greatly between individuals (Merlo et al., 2014a), but this variation could not be attributed to trade-offs with other activities such as reproduction or somatic growth (Cutrera et al., 2014a; Merlo et al., 2014b).

The present study was designed to explore a possible trade-off between the response to PHA and the response to parasite infections, as a potential basis for explaining the inter-individual variation previously observed in the PHA-response. To do this, we induced the response to PHA 1) in animals with their natural assemblage of parasites; 2) in animals with a known (experimentally manipulated) level of infection by one endoparasite (*Eimeria* sp.); and 3) in animals treated with antiparasitic drugs. Concomitantly, parameters of condition, nutrition, stress, and immunity were measured to assess the impact of parasite infections on tuco-tucos. We focused our analyses of parasite burden on gastrointestinal parasites given their impact on fitness attributes reported for a wide range of animal species (Albon et al., 2002; Stien et al., 2002). We chose *Eimeria* sp. to manipulate the parasitic load of tuco-tucos for several reasons: 1) *C. talarum* from the locality of Mar de Cobo show a high prevalence of *Eimeria* sp. infection and susceptibility to this protozoan was observed to be positively associated with an specific MHC allele (Cutrera et al., 2011); 2) large quantities of non-infective oocysts are shed by infected tuco-tucos (Cutrera et al., 2011), which facilitate their culture; 3) infections by *Eimeria* sp. are short in time, *ca.* 20 days (Long, '82); 4) inducing a marked damage and inflammation in intestinal epithelial cells in a restricted period, as described in chicken and rabbits (Parker and Duszynski, '86; Baker, '98; Yun et al., 2000; Allen and Jenkins, 2010); and 5) oocysts cultured in lab conditions are immunogenic (Rose, '87).

Given the generally negative impact of parasites on their hosts, we hypothesize that both natural and experimentally manipulated parasitic loads will negatively affect the magnitude of the PHA-induced response in tuco-tucos.

MATERIALS AND METHODS

Animal Capture and Captivity Conditions

Adult *Ctenomys talarum* (tuco-tucos) were live-trapped in the locality of Mar de Cobo, Buenos Aires Province, Argentina (37° 46′ S 57°27′ W) using wire tube-shaped live traps (10 cm diameter, 35 cm length). Holes were dug to access the underground burrows and traps 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 76 individuals, 39 males (163.05 ± 25.37 g) and 37 females (126.87 ± 19.92 g), were caught during the reproductive seasons (August to December) of 2013 and 2015. Immediately after capture, feces of each animal were collected and conserved in 4% formaline for future procedures (see "Culture of *Eimeria* sp. Oocysts" below). Animals were transported to the Laboratory of Ecophysiology at the National University of Mar del Plata (Mar del Plata, Argentina) where they were weighed and put in individual plastic boxes ($25 \times 32 \times 42\ cm^3$) provided with a wire-mesh lid and lined with wood shavings as bedding. Animals were fed ad libitum quantities of a mixture of chicory, lettuce, corn, sweet potatoes, and sunflower seeds. 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 (*ca*. 16 days) after which they were released at the point of capture. Field and labwork were performed according to the American Society of Mammalogists guidelines (Gannon and Sikes, 2007). The animals were cared for in accordance with the Guidelines for the Use of Animals in Behavioral Research and Teaching (ASAB/ABS, 2003).

Experimental Groups and Protocol

Individuals of each sex were randomly assigned to three groups: naturally parasitized (Natural, $n = 28$; $n_m = 15$, $n_f = 13$), deparasitized (Deparasitized, $n = 24$; $n_m = 12$, $n_f = 12$) or deparasitized and inoculated with infective *Eimeria* sp. oocysts (Inoculated, $n = 24$; $n_m = 12$, $n_f = 12$); animals among groups were equally represented along the entire season to avoid seasonality effects over the parameters measured. Immediately after arrival in the lab (day 1), animals from Deparasitized and Inoculated groups received a single oral dose of ivermectin (Iverplus®, Laboratorios Aviar S.A., Buenos Aires, Argentina; 1% suspension, 0.25 μL/g corporal weight) and toltrazuril (Toltrazol®, Mayors laboratory, Buenos Aires, Argentina; 5% suspension, 0.4 μL/g corporal weight). Ivermectin is an anthelmintic that acts primarily against gastrointestinal nematodes, while toltrazol is a broad-spectrum anticoccidial. Seven days after the administration of both drugs, their antiparasitic effect was tested in both groups by quantifying parasite eggs/oocysts present in fecal samples (see "Quantification of Parasites" below). At this time point (day 7), individuals from group Inoculated received an oral dose of 500 *Eimeria* sp. infective oocysts (200–1000 μL of oocysts–sucrose solution, depending on the oocysts concentration obtained, see "Culture of *Eimeria* sp. Oocysts" below), while animals from the other two groups received 500 μL of sugar water as a control. Also, weight was recorded for all animals. On day 14, animals from the three groups were weighed and a blood sample was collected from the retro-orbital sinus for subsequent determinations (see "Blood Determinations"). The following day (day 15), 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 that, 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, while the right hind foot was injected in the same manner with PHA (immune challenged foot; *Phaseolus vulgaris* PHA-Sigma L-8754 solution dissolved in PBS, 3 mg/mL; 0.3 μL/g corporal weight). Twenty-four hours after injections, thickness of both hind feet were measured again. Inflammatory response in each foot was calculated as the difference between pre- and post-injection thickness divided by initial thickness (response = (post – pre)/pre); Xu and Wang, 2010). Thickness measurements were always performed by the same person (JLM) in order to minimize measurement errors.

Quantification of Parasites

To determine endoparasite loads, the number of parasite eggs/oocysts present in fecal samples collected immediately after capture and at day 7 was quantified per individual. This procedure allows the detection of all taxa present in digestive tracts of tuco-tucos, with the only exception of *Taenia talicei* (Rossin et al., 2004). Eggs/oocysts present in feces were assessed using the flotation technique proposed by Sheather ('23). Briefly, 1 g of feces (fixed in formalin) per individual was ground up and suspended in 15 mL of modified Sheather's flotation solution (Sheather, '23). Fecal suspension was mixed to homogenate. Morphological identification and count of parasite eggs/oocysts was performed at 400× magnification (Olympus CX 31, Tokyo, Japan). For each egg type, fecal egg count (FEC) was estimated per gram of wet feces by counting all eggs present using a MacMaster chamber. *P. ctenomydis*, *G. subterraneus*, *S. myopotami*, and *T. duretteae* eggs could not be differentiated and were all accounted a "strongylids," given their phylogenetic proximity and their relatively low abundance and prevalence, which is consistent with those reported for the adult forms reported in this population of *C. talarum* (Rossin et al., 2010). Prevalence of each egg/oocyst type was calculated as the percentage of hosts shedding

eggs/oocysts over all hosts examined. Intensity of infection of each parasite was calculated as the median of FECs obtained (Cutrera et al., 2011).

Culture of *Eimeria* sp. Oocysts

Eimeria sp. oocysts were obtained from feces collected from three animals with high intensities of infection by *Eimeria* sp. from the Natural group, between days 1 and 7. Feces (15–65 g) were maintained in potassium dichromate with air bubbled for 48 hr. After that, the oocysts preparation was washed twice to remove the dichromate, followed by centrifugation (2500 rpm, 15 min). Quantification of the concentration of sporulated (infective) oocysts in the precipitated fraction was performed by the flotation technique described above. Sucrose solution was added to make the solution palatable and in adequate quantities to reach the desired concentrations for oral administrations.

Blood Determinations

Leukocyte Profiles. Relative counts of each leukocyte type in blood provide information about different processes that could be occurring in the individual, as stress (denoted by an increased neutrophils/lymphocytes ratio (N:L ratio)), allergies and parasitism (eosinophilia), infection, or endocrine disorders (increased basophil count), chronic infection or inflammation (increased monocyte count; Voigt, 2000). Thus, leukocyte diversity and abundance of blood smears performed on day 14 were quantified following standard protocols (Voigt, 2000). After fixation with methanol, the 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" (Voigt, 2000) was used to record the abundance of lymphocytes, neutrophils, eosinophils, monocytes, and basophils (identifications based on cell morphology described by Voigt (2000) in vertebrates; Vera et al. (2008) and Cutrera et al. (2010) for *C. talarum*) until a total of 200 leukocytes had been examined. The "wandering" technique allows a random sampling of cells, avoiding the recount of the same field. The smear is examined from one edge, across the entire monolayer, up to the other edge, moving inward a short distance and parallel to the edge for 3–5 fields, and then moving back to the first edge, repeating this procedure as often as necessary to identify the required number of cells (Voigt, 2000). 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, R. R. Zenuto, unpublished) was recorded and then standardized to 100,000 erythrocytes for comparative purposes.

Erythrocyte Sedimentation Rate (ESR). ESR is a non-specific measure of presence and intensity of disease processes in the body, which usually rises during acute infection due to tissue destruction (Blaxhall and Daisley, '73). Following Saino and Møller ('96), heparinized capillary tubes in which blood samples were collected were put in vertical position for 4 hr at 4°C. Sedimentation rate (proportion of blood 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.

Hematocrit. Hematocrit is the proportion of blood volume occupied by packed red blood cells and is considered an indicator of general health status (Hoi-Leitner et al., 2001). After ERS 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).

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) with some modifications. Briefly, we inoculated 2 mL of LB medium with *Escherichia coli* (non-pathogenic strain donated by the Microbiology Lab at University of Mar del Plata) from a glycerol stock stored at −80°C and incubate it overnight at 37°C with shaking (200 rpm). Next, we determined the optical density (OD) at 600 nm using a spectrophotometer (Ultrospec 1100 pro, Little Chalfont, Buckinghamshire, England) and diluted the culture to obtain an OD = 0.1. This new culture was incubated again for an hour at 37°C with shaking (200 rpm). To determine the optimal incubation duration prior to measuring antimicrobial capacity, the time to reach log phase growth in the absence of plasma was first quantified as described by (Liebl and Martin, 2009), which in our case was 120 min (A. P. Cutrera, unpublished). To test antimicrobial capacity of plasma for each individual, 7.5 μL of plasma was added to 62.5 μL of the *E. coli* culture in an Eppendorf tube. Sterile PBS was added to reach a total volume of 242.5 μL. This was performed in triplicates. Another tube (control) was prepared in the same way but without adding the *E. coli* (adding 235 μL of PBS instead). The last tube was the blank and contained PBS (180 μL) and *E. coli* (62.5 μL). All procedures were performed in a certified laminar flow hood. Tubes were incubated for an hour at 37°C with shaking. After that 1250 μL of LB medium were added to all tubes and we incubated them for 120 min at 37°C with shaking. When the second incubation was complete, microbial concentrations were determined by measuring the absorbance of each sample at 600 nm. The anti-microbial 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.

Albumin. In response to inflammation, serum albumin concentrations decrease dramatically (Gitlin and Colten, '87; González Naranjo and Molina Restrepo, 2010). A fraction (~60 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), read at 625 nm in a spectrophotometer (Ultrospec 1100 pro).

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.

Hemolysis-Hemagglutination Assay. The hemolysis-hemagglutination assay proposed by Matson et al. (2005) allows quantifying the natural antibody-mediated complement activation and red blood cell (RBC) agglutination (innate immune capacity measures) of the plasma sample. After blood collection, samples were centrifuged at 3000 rpm for 15 min and the resulting supernatant (plasma fraction) was stored at −20°C until used. Following Matson et al. (2005), 20 μL of each plasma sample was added to 20 μL of PBS in the first well of a 96-well microplate (Corning Star # 3798, Corning, New York, USA); this sample was then serially diluted in PBS (1:2 to 1:65,536) to fill the remaining wells in that row of the plate and six of the following. 25 μL of 1% suspension of sheep red blood cells (SRBC) was then added to each well. The plate was gently agitated for 10 sec and incubated at 37°C for 1 hr. After incubation, visual assessment of lysis and agglutination was performed, as described by Matson et al. (2005). Levels of hemolysis and RBC agglutination were expressed as the negative $\log_2$ of the minimum plasma concentration that produce visible lysis/agglutination of the sample. To increase the consistency of the data set, scoring of plates was always performed by the same person (JLM). To assess if the lack of complement lytic activity (see "Results" section) was due to improper conservation of the plasma samples, we conducted another experiment. Ten extra animals that were not part of the parasitism design were used to obtain plasma in the same way described above. Each plasma sample was immediately aliquoted in two tubes. One tube was treated exactly in the same manner as described previously, until storage. The other tube containing whole blood was immediately put on ice and then centrifuged at 4°C (3000 rpm for 15 min). After the plasma was separated, tubes were put in liquid nitrogen and transported to an ultrafreezer (−80°C), where they were stored until analysis. Finally, both sets of tubes were assayed following the protocol described above.

Cortisol. This hormone is responsive to acute stress in *C. talarum* (Vera et al., 2011). Plasma obtained as described previously (see "Hemolysis-Hemagglutination Assay" section) was used to determine levels of cortisol in each individual, using an enzyme immunoassay kit (EIA 1887 DRG® Instruments GmbH, Marburg, Germany).

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

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. Two-way ANOVAs were performed to test the effect of treatment and sex on each parameter studied. N:L ratio, total leukocyte count, cortisol and glucose values were Ln-transformed, triglyceride values were square root-transformed, and ESR values were Box–Cox-transformed ($\theta = 0.4$) to reach normality. Eosinophil, basophil, monocyte, and parasite counts could not be normalized, thus differences between treatments were analyzed by non-parametric Mann–Whitney *U* Tests or Wilcoxon matched pairs tests. Throughout the text, results are expressed as means ± standard error (±s.e.).

RESULTS

Anthelmintic and anticoccidial drugs significantly decreased egg/oocyst counts in feces after 7 days of oral administrations to individuals from Deparasitized and Inoculated groups (Wilcoxon matched pair tests; *Eimeria* sp. count: $Z = 3.97$, $P < 0.0001$; *P. uncinata* count: $Z = 5.39$, $P < 0.0001$; *T. pampeana* count: $Z = 2.71$, $P = 0.007$). Strongylid counts were not included in the analysis since most individuals did not present strongylid eggs in feces (field count: 0.3 ± 0.1; day 7 count: 0.13 ± 0.05; $n = 48$).

The swelling response was significantly greater in PHA-injected hind feet compared to control hind feet injected with PBS (paired *T*-test; $t = -11.52$, $n = 74$, d.f. = 73, $P < 0{,}0001$; $\text{swelling}_{PHA} = 0.35 \pm 0.008$, $\text{swelling}_{PBS} = 0.02 \pm 0.03$). The swelling response was significantly higher in individuals from the Deparasitized group compared to the other two groups (two-way ANOVA; $F_{(1,70)} = 6.08$; $P = 0.0037$; post-hoc Tukey test, $P < 0.05$; Fig. 1). The parasitic load of the Natural group was composed by *Eimeria* sp., *P. uncinata*, *T. pampeana*, and strongylids (Table 1). Erythrocyte sedimentation rate did not differ between treatments (two-way ANOVA, $F_{(1,70)} = 2.24$; $P = 0.11$; Fig. 2). Levels of serum albumin differed among treatments (two-way ANOVA, $F_{(1,70)} = 4.10$; $P = 0.02$), being significantly greater in the Deparasitized group than in the Inoculated group (Tukey post-hoc test, $P < 0.05$; Fig. 3).

Measures of condition, stress, nutrition, and other immunological parameters did not differ between treatments (see Table 2). Levels of lysis of erythrocytes from the hemolysis-hemagglutination assay scored 0.00 in 59 of 62 samples (0.00 ± 0.008). Thus, lysis titres from this assay were not included in the statistical analysis. This lack of lytic activity of the complement seems not

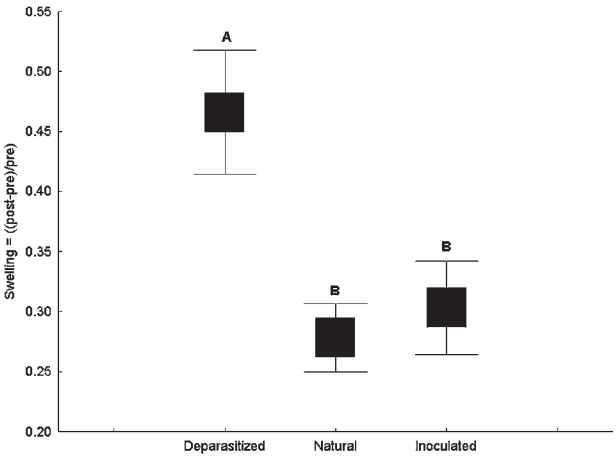

Figure 1. Mean hind feet swelling (±s.e.) of Parasitized vs. Deparasitized tuco-tucos, 24 hr after PHA injection. Letters denote statistical differences between groups.

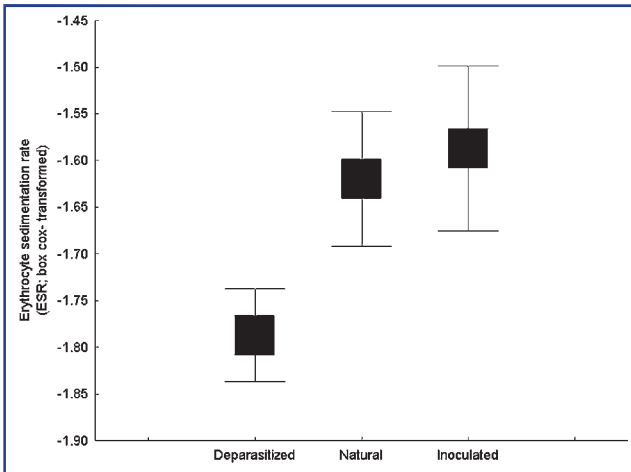

Figure 2. Box–Cox transformed mean erythrocyte sedimentation rate (ESR; ±s.e.) of different treatment groups (Deparasitized, Natural and Inoculated) of tuco-tucos.

to be explained by improper conservation of the samples. Of the 10 samples of extra animals assayed, only one showed a lysis titre different from zero. For this sample, the lysis titre obtained from the plasma sample preserved as described by Matson et al. (2005) was 1, whereas the titre for the plasma sample kept at low temperatures until stored at −80°C was 2. The rest of samples showed no lysis, irrespective of the method used to preserve the plasma. These results support the hypothesis that tuco-tucos may have low lytic activity of the complement, as described for Leach's storm petrels (Mauck et al., 2005), mourning doves, zebra finches, and waved albatrosses (Matson et al., 2005).

DISCUSSION

The results of this study support the idea that parasite infections could be a critical factor influencing the magnitude of the immune response to a new challenge. Moreover, the extent of the negative impact of parasitism on the inflammatory response verified in our study was comparable between the group artificially inoculated with *Eimeria* sp. oocysts and the naturally infected group, in which *Eimeria* sp. co-occurred with a number of gastrointestinal nematodes.

In the naturally infected animals, *Eimeria* sp. oocysts were the most prevalent and showed the highest levels of infection compared with eggs shed by other species. Although the fecal-egg counting technique has its limitations (e.g., each parasite species may have a particular life cycle with different times and volumes of egg/oocyst shedding and parasite reproductive output can be affected by the variability in host quality; Poulin, '96), it has proven particularly useful in many studies of free-living species (e.g., Harf and Sommer, 2005; Schad et al., 2005), including previous work on *C. talarum* (Cutrera et al., 2011; Cutrera and Zenuto, 2014b) because it is non-invasive. In addition to differences in parasite burden composition, naturally and artificially infected groups differed in the stages of infection by *Eimeria* sp. In the last group, before *Eimeria* sp. oocyst inoculation, animals were treated with anthelmintic and anticoccidial drugs in order to eliminate their current parasitic infections. This procedure ensures the interruption of potential protective immunity developed by chronic or low intensity-infections and makes animals become susceptible again (Gómez-Lucía, 2006). Thus, 7 days after the infective oocysts inoculation, animals from this group were in an early infection

Table 1. Summary of parasite species and their infection parameters in tuco-tucos from the "Natural" group, obtained through egg/oocyst counts in feces, at day 7.

Parameter	Prevalence (%)	Intensity of infection	Range of infection (25th–75th quartiles)
Eimeria sp.	94.9	31	5–227
P. uncinata	71.4	17	0–50
T. pampeana	32.1	0	0–2
Strongylids	21.4	0	0–0

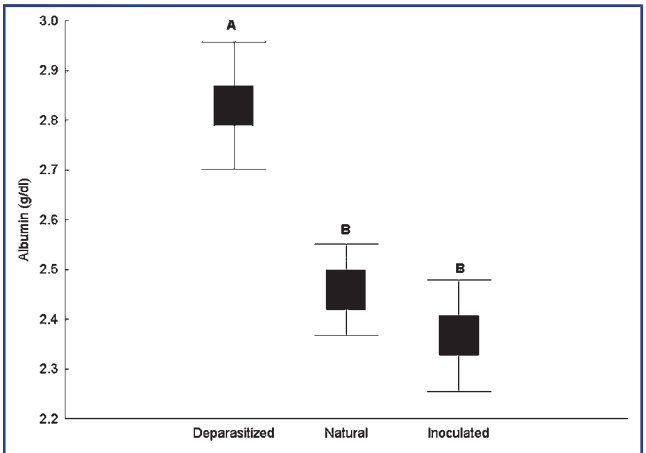

Figure 3. Mean serum albumin level (±s.e.) of different treatment groups (Deparasitized, Natural and Inoculated) of tuco-tucos. Letters denote statistical differences between treatments.

stage, while in the naturally parasitized group, animals infected with *Eimeria* sp. could be suffering different stages of infection depending on the particular time of contact with *Eimeria* sp. of each individual. This is an important fact given the differential immunogenicity of each stage of *Eimeria* sp.'s life cycle (intracellular, extracellular, asexual, and sexual stages; Yun et al., 2000). In conclusion, both groups differed in several traits of their endoparasitic infection, but their similar impairment of the inflammatory response to a new challenge indicates that parasitism is a general key factor modulating immune responsiveness of the host.

Other authors have already explored the influence of different types of parasites on the immune response to PHA in mammals. Through manipulation of the parasitic load, Goüy De Bellocq et al. (2006) found that flea-infested Sundevall's jirds had a lower PHA-response compared to non-parasitized animals, while Christe et al. (2000) observed that bats *Myotis myotis* highly parasitized by blood-sucking mites mounted lower PHA-responses. Apart from the present study, the effect of coccidians over the PHA-response was previously reported only for a bird species: greenfinches (*Carduelis chloris*) showed a positive association between the intensity of infection by *Isospora lacazei* and their capacity to mount a PHA-response (Saks et al., 2006). In addition to some protocol differences (anticoccidial treatment, quantity of oocysts inoculated, coccidian species used, time of immune challenge) between this study and ours, different outcomes observed among immune responses of greenfinches and tuco-tucos in relation to coccidian infection could arise due to differences in life histories traits and immune strategies. Our

Table 2. Mean (±s.e.) values of immunological, nutritional, condition and stress parameters from Deparasitized, Natural, and Inoculated groups of *Ctenomys talarum* and results of the two-way ANOVAs and Kruskal Wallis Tests used to test differences in these parameters between groups.

Parameter	Deparasitized group	Natural group	Inoculated group	*F*/*H*	*P*
Condition parameters					
Body weight (g)	148.29 (±4.78)	151.05 (±6.34)	143.18 (±4.97)	$F = 0.75$	0.48
Hematocrit (%)	47.63 (±0.80)	47.54 (±1.14)	47.15 (±1.27)	$F = 0.08$	0.93
Immunological parameters					
BKC (%)	4.62 (±0.86)	5.95 (±0.83)	4.59 (±0.60)	$F = 0.98$	0.39
Hemagglutination titres	2.89 (±0.18)	2.56 (±0.24)	2.81 (±0.25)	$F = 0.58$	0.56
Total leukocytes/100,000 RBC	15.92 (±1.66)	15.61 (±2.43)	13.83 (±2.10)	$F = 0.61$	0.55
Neutrophils[1]	105.60 (±7.49)	102.00 (±5.90)	94.09 (±6.79)	$F = 0.69$	0.50
Lymphocytes[1]	88.84 (±7.39)	90 (±5.71)	99.17 (±6.86)	$F = 0.64$	0.53
Eosinophils[1]	2.48 (±0.42)	4.64 (±1.34)	2.78 (±0.81)	$H = 0.83$	0.66
Basophils[1]	2.20 (±1.44)	2.43 (±0.65)	2.26 (±1.10)	$H = 2.59$	0.27
Monocytes[1]	1.52 (±0.44)	1.21 (±0.27)	1.70 (±0.37)	$H = 0.93$	0.63
Stress parameters					
Cortisol (ng/mL)	26.93 (±6.84)	28.56 (±9.25)	27.79 (±6.89)	$F = 0.96$	0.39
N:L ratio	1.75 (±0.31)	1.46 (±0.22)	1.21 (±0.19)	$F = 0.68$	0.51
Nutritional parameters					
Glucose (mg/dL)	78.44 (±6.87)	76.46 (±7.10)	80.70 (±8.18)	$F = 0.17$	0.84
Triglycerides (g/L)	4.76 (±4.01)	0.70 (±0.20)	0.30 (±0.18)	$F = 1.55$	0.22

RBC, red blood cells.
[1]Abundances based in a total of 200 leukocytes examined per individual.

results contribute to understand the ecology of immune defenses of *C. talarum*, a rodent species with subterranean habits, and mammals in general, but are also interesting to a wide range of hosts that can be infected by *Eimeria* species, including livestock, poultry and wild fauna (Yun et al., 2000; Allen and Jenkins, 2010; Pérez et al., 2010). In this sense, apart from the known coccidiosis disease that *Eimeria* can cause (Pérez et al., 2010), our results show that coccidians can cause a modulation of the host immune responsiveness.

When more than one parasitic infection is considered, the complex interaction between different parasite species and host immune system becomes more complicated to understand. For example, the immune response to PHA of blackbirds (*Turdus merula*) was explained in a significant part by the interaction of two different parasite species (*Hymenolepis* sp. and *Plasmodium* sp.; Biard et al., 2015). Results reported by Goüy de Bellocq et al. (2007) regarding the response to PHA of white-toothed shrew (*Crocidura russula*), showed that it was negatively related to cestode infection but positively related to nematode infection. These different modulating effects on immunity have been attributed to trade-offs arising from the stimulation of different arms of the immune system (mainly Th1 vs. Th2), or to the immunoregulatory capacity of helminths (Goüy De Bellocq et al., 2006; Biard et al., 2015). *C talarum* parasitized by *Eimeria* sp. or several endoparasitic species (see Table 1) showed a lower immune response compared to animals without endoparasites. In addition to the confirmed effect of *Eimeria* sp., additional studies are required to explain the particular influence of other parasite species on the immune response measured.

Albumin level in serum, an indicator of inflammatory processes, was affected by treatments. Among the acute phase proteins, albumin is a negative one, which means that its serum levels decrease during inflammatory processes (Gitlin and Colten, '87). Tuco-tucos from Deparasitized group showed higher albumin levels than artificially parasitized animals, demonstrating that parasites induce inflammatory processes in the host. Moreover, these results also confirm that the inoculation treatment was effective, in that oocysts inoculated are likely producing physical gut damage and inflammation through the penetration of epithelial cells of the intestinal mucosa (Parker and Duszynski, '86; Baker, '98; Yun et al., 2000; Allen and Jenkins, 2010). Individuals from the Natural group showed albumin levels intermediate between Deparasitized and Inoculated animals. Aside from the fact that Natural and Inoculated groups differed in their parasitic composition, differences observed in albumin levels between both groups (Fig. 2) can also be attributed to the differences in infection stages of *Eimeria* sp. An early infection, such as that induced in the Inoculated group, could be highly harmful for the host given that early endogenous stages of the parasite life cycle are considered to be more immunogenic than the later sexual stages (Yun et al., 2000). Surprisingly, ESR — a measure that increases as consequence of inflammation and/or infection processes in the body — did not differ between treatments. However, although non-significant, animals from Deparasitized group tended to present lower ERS than naturally or artificially parasitized animals (see Fig. 2). According to our results, albumin levels in serum were a better indicator of parasitic infections than ESR.

Interestingly, parameters associated to condition, nutrition, stress and immunity, except for the PHA-response, studied here were not impacted by parasitism. For the particular case of *Eimeria* sp. infections, the most harmful effects generally involve diarrhea, dehydration, weight loss and, at times, mortality (Yun et al., 2000). In the present study, diarrhea was detected only in two naturally parasitized animals (J. L. Merlo, unpublished), and no differences in body weight between parasitized (Natural and Inoculated) and Deparasitized groups were observed. Thus, it is possible that levels of infection in Natural and Inoculated animals were enough to induce an immune response (as evidenced by serum albumin levels) but not so high to cause serious disease symptoms and to negatively impact other physiological parameters studied. Another possibility is that detrimental effects of parasitism over the physiological condition were compensated by the high availability of nutrients and energy provided by the ad libitum diet and the restriction of costly activities (e.g., thermoregulation and digging) in captivity. In this sense, future studies exploring the effects of parasitism over animals under nutrient restriction or energy demanding conditions would provide valuable information regarding this hypothesis. With respect to leukocyte counts, although the PHA-response can induce significant variations in levels of circulating leukocytes in some species (e.g., Mongolian jirds, Xu and Wang, 2010; several parrot species, Tella et al., 2008), this is not the case for *C. talarum* (Merlo et al., 2014a). Therefore, although treatment affected the magnitude of the local inflammatory response to PHA, levels of leukocyte counts or total leukocyte counts in blood were not expected to be equally affected.

Finally, even when our results are in line with those reported for other mammals (Christe et al., 2000; Goüy De Bellocq et al., 2006), additional studies are needed to explain the mechanisms underlying the negative effect of parasitism over other immune challenges. The fact that natural and *Eimeria* sp. parasitism impaired the PHA-response but not the general condition of tuco-tucos could evidence a trade-off between both immune challenges through immune components instead of host resources. However, additional measures of host condition should be taken to exclude the last hypothesis. Moreover, measurements of immune components (e.g., leukocytes and cytokines) released into blood and present in affected zones during the different treatments assayed here could give more information to elucidate the mechanisms underlying the relationship between both

immune challenges via the immune system. For example, host immunodepression could be induced by parasites through production of molecules capable of direct or indirectly affect immune cells (Cox, 2001). Even when it is unknown if *Eimeria* sp. or other *C. talarum* endoparasites are capable of this type of mechanism, this possibility cannot be excluded from our results.

To sum up, the present study provided valuable information for the understanding of the inter-individual variation observed in the PHA-response of a subterranean rodent, *C. talarum*, showing that the magnitude of the inflammatory response measured in the PHA-test, a technique widely used to measure individual inflammatory and T-cell capacity in the field of ecoimmunology, is negatively influenced by parasitism.

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