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Immune challenge but not dietary restriction affects spatial learning in the wild subterranean rodent *Ctenomys talarum*

Cristian E. Schleich *, Roxana R. Zenuto, Ana P. Cutrera

Laboratorio de Ecofisiología, Instituto de Investigaciones Marinas y Costeras (IIMyC), Universidad Nacional de Mar del Plata — Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), CC 1245, 7600 Mar del Plata, Argentina

HIGHLIGHTS

- Trade-off between the costs of the immune defense and learning abilities in *C. talarum*
- Immune-challenged tuco-tucos displayed lower spatial learning capabilities.
- Dietary restriction affected neither learning capabilities nor immune response.

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ABSTRACT

Several lines of evidence suggest that learning and triggering an immune response are both metabolically expensive and thus likely to be subject to nutritional trade-offs between them and other competing demands. Therefore, we evaluated if an immune challenge with a novel antigen affects spatial learning in the subterranean rodent *Ctenomys talarum* under two different dietary conditions. The results showed that immune-challenged animals were affected in their spatial learning capabilities, increasing the number of errors and marginally the time required to reach the goal of a complex labyrinth. No effect of the dietary restriction nor interaction between factors were observed. This work provides support for the existence of a trade-off between the costs of the immune defense and learning abilities, indicating that when investment is required to fight infection, fewer resources are available for learning. The absence of effect of nutritional condition on this trade-off suggests that other physiological processes, besides cognition, may be limited by the energetic resources necessary to the more immediately critical immune response.

1. Introduction

Spatial ability involves the ability to perceive, encode, store, retrieve, transform and integrate spatial information represented from two or three-dimensional space. Particularly, spatial learning is a hippocampus-dependent process that allows animals to navigate their environment and learn to relate particular locations with objects or experiences [1]. Enhanced learning ability requires greater allocation of energy and resources to the neural and sensory structures responsible for the acquisition, processing and storage of information [2,3]. Therefore, cognitive functions are expected to be compromised when other energetic demanding processes, such as reproduction, compete for nutritional resources, as demonstrated by the negative relationship found between fecundity and learning performance [4].

The mammalian immune system represents a complex and dynamic network that confers the individuals the capacity of protecting themselves from infections. It consists of an innate and an adaptive set of responses, the last one consisting in two arms: the humoral and the cell mediated [5–7]. Immune defense represents not only benefits in the form of resistance, but also energetic costs associated with cell migration, cytokinesis, phagocytosis, and antigen processing and presentation. Further, different effector functions (synthesis of antibodies, cytotoxicity, regulatory roles) depend directly or indirectly on cellular energy supply [8,5]. In consequence, trade-offs in resource allocation between immunity and other costly physiological processes are expected to arise [9,10]. In addition, several aspects of the immune system are condition-dependent, being affected by nutritional status [11–13] and endocrine factors (i.e., androgen and/or glucocorticoid levels are related to the magnitude of immune response) [14].

Trade-offs between immunity and life-history traits such as fecundity, growth or survival have been explored in numerous taxa. For example, in ectotherms, Uller et al. [15] showed that reproductive mallee female dragons injected with lipopolysaccharide of *Escherichia coli* suffered a reduction in their reproductive investment in terms of egg mass. Similarly, in female house sparrows (*Passer domesticus*),

* Corresponding author. Tel./fax: +54 2234753150.
E-mail address: cschleic@mdp.edu.ar (C.E. Schleich).

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Bonneaud et al. [16] found that immune challenged individuals also experienced a decrease in their reproductive success, although this reduction depended on brood size. Recently, studies have focused on the relationship between immune function and neuro-behavioral processes, such as learning. Enhanced learning abilities were detected under experimental increase of cytokines [17] while performances were lowered when facing an immunological challenge during an infection [18]. Parasitic infections affect the ability of individuals to learn spatial tasks; for example, Kavaliers et al. [19] found that *Mus musculus* infected with the protozoan *Eimeria vermiformis* displayed significantly poorer acquisition and retention of a water-maze task than control individuals. Similarly, male mice immune depressed with antithymocyte serum (ATS) performed poorly in a combined odor/spatial learning task [20]. On the other hand, Braithwaite et al. [21], using rats and mice infected with the gastrointestinal nematode *Strongyloides ratti*, did not find any effect of the inoculation on the spatial learning abilities of these species, suggesting a more complex relationship between learning and immune status.

The South American *Ctenomys talarum* (talas' tuco-tucos) is a solitary subterranean rodent that inhabits systems of closed galleries parallel to the soil surface [22]. Their foraging behavior involves an underground exploration in search for food patches and brief excursions to the surface to collect vegetation that is later consumed inside the burrows [23]. The burrow system of this species has a complex branching structure, consisting of a main axial tunnel and a variable number of lateral branches and feeding tunnels, all of them plugged [24]. In addition to their structural complexity, these burrows also present large extensions, with estimated sizes ranging from 10 to 30 m^2 from excavated burrows [24,25], although smaller than other species of subterranean rodents, particularly those of social living [23]. Highly developed spatial abilities are therefore required for an accurate orientation inside these intricate tunnel systems in order to decrease the high energetic costs associated with digging when extending the burrows for localization of food patches or to evaluate accurately the position of predators or conspecifics. This can be clearly observed in *C. talarum*, which display a highly developed ability to learn and remember structurally complex radial and longitudinal labyrinths [26–28].

Other remarkable features of burrows used by subterranean rodents in general are their physical conditions, characterized by high moisture, darkness, low ventilation, and protection from UV light, conditions that favor parasite proliferation and transmission [29,30]. Concomitantly, tuco-tucos display a high prevalence of parasitism, but low parasite richness [30,31]. Previous studies revealed significant associations between specific alleles of the major histocompatibility complex (MHC), and both parasite load and intensity of humoral immune response against a novel antigen, suggesting that parasites represent a strong selective pressure in *C. talarum* [31]. Further, even though tuco-tucos mounted a low antibody response against SRBCs (sheep red blood cells) compared with other rodent species, such antibody response was energetically costly [32], similar to the cost of lactation for female tuco-tucos (nearly 30% increase in resting metabolic rate; [33]).

Therefore, for tuco-tucos living in highly structured burrow systems, maintaining a proficient spatial performance is crucial to accomplish efficiently their daily activities. Given that spatial learning is costly, learning performance is expected to be compromised when other energy demanding process such as an immune response triggered by parasite exposure, in a context of limited nutritional resources.

In particular, the objective of the present study is to evaluate whether an experimental induction of the immune system with a non-pathogenic novel antigen that elicits a humoral response (SRBCs) affects the spatial learning capabilities of individuals of *C. talarum* under two different nutritional conditions. We hypothesize that (i) an activation of the humoral arm of the adaptive immune system will impair the capacity of tuco-tucos to learn a spatial task, increasing both the time and number of errors made until reaching the goal of a longitudinal labyrinth with respect to the control group, and (ii) this trade-off effect will be stronger in individuals under a severe nutritional restriction.

2. Materials and methods

2.1. Animals capture and maintenance

Male tuco-tucos were trapped during the breeding (July–December) and non-breeding (March–June) seasons of 2009 and 2010 in Mar de Cobo (37° 46′ S, 57° 26′ W, Buenos Aires province, Argentina) using plastic tube traps inserted into animal's burrow systems showing fresh surface mounds. Then, we transported the animals to the laboratory and housed them in individual plastic cages (25 cm × 32 cm × 42 cm) containing wood shavings for bedding and a half terra cotta flowerpot as refuge. Temperature and photoperiod in the room housing the animals were strictly controlled (25 ± 1 °C; non-breeding 12L:12D; breeding 14L:10D). Food was provided ad libitum consisting of sweet potatoes and lettuce.

2.2. Experimental treatments

After a one-week period of adaptation to captive conditions, the animals (n = 47) were randomly assigned to the different treatments involving immune challenge and food restriction. Learning trials started 66 hs after initiating the following treatments: Group a) slight food restriction (see Diet section) and injection with saline solution (n = 7), Group b) slight food restriction and injection with SRBCs (immune-challenged; n = 13); Group c) severe food restriction and injection with saline solution (n = 10); and Group d) both severe food restriction and immune challenged (n = 17). Blood samples were obtained twice, 66 h before starting the learning trials (immediately before the injection with the antigen or saline solution) and 1 h after the end of the last trial, as explained below. Antibody titers, leukocyte profile, hematocrit and cortisol concentration were obtained from blood samples, as detailed in Section 2.2.

2.2.1. Diet

Animals under slight food restriction were maintained at ≈85% (mean body weight loss: 14.54 ± 1.5%) of the initial body weight during the experiments, condition required to ensure their motivation to explore and learn the maze. This slight restriction treatment consisted in daily feeding the animals with 6–7 g of sweet potatoes and two lettuce leaves, while in the severe food restriction treatment individuals were provided with 3–4 g of sweet potato and two lettuce leaves in order to maintain their body weight around ≈75% (mean body weight loss: 22.1 ± 1.9%) of their initial value. Animals that did not reach the experimental weight at the beginning of the spatial learning period or suffer from large variations in their body weight (≥4%) during trials were excluded from experiments. The period of time the test animals were subjected to food restriction in this study did not affect their general condition and mobility but longer exposition to food restriction usually affects individuals' health and even survival. Once concluded the experiments, animals were fed ad libitum until recovery of their initial body weight. After that, they were returned to the field and released at the site of capture.

2.2.2. Immune challenge tests

We used sheep red blood cells (SRBCs), a non-pathogenic antigen known to trigger a Th2 and B-lymphocyte response [34], resulting in antibody production in several vertebrates [35–37]. The magnitude of this antibody response is thought to reflect an individual's ability to mount an acquired immune response to a novel antigen as well as its ability to resist extracellular infections (e.g. bacteria, macroparasites) [38,39]. Previous research has shown that *C. talarum* produces significant antibody titers in response to injection with SRBC (10% suspension) while control animals injected with saline solution do not mount a response [32]. Also, immune challenge tests indicated that antibody response to SRBC is detectable at 7 days post injection and mounting the antigenic response was associated with a significant increase in resting metabolic

rate (RMR) 4 days post injection [32]. Hence, first learning trials -when tuco-tucos display higher improvements in their spatial learning performance [28] coincide with the start of humoral response which is critical in an energetic sense. The increment in the RMR extends until 10 days after injection, comprising therefore the complete learning period. Briefly, on day 7 of captivity, we injected animals intra-peritoneally with SRBC (Sigma R3378, 15% suspension, 1.5 μl/g of animal weight). Immediately after injection, we collected ~200 μl of blood from the retro-orbital sinus of each animal. Seven days after the injection with the antigen or saline solution, a final blood sample was collected at late afternoon, after the last trial, to evaluate immune response. Antibody production was assessed by a hemagglutination assay in 96-well microplates (Corning Star Catalog No. 3798). Immediately after collection, blood was kept at 4 °C until centrifuged at 3000 rpm for 15 min. Plasma was separated, heat-inactivated at 56 °C for 30 min and stored at −20 °C until used in the hemoagglutination assay. Following the protocol of Cutrera et al. [32], 20 μl of heat-inactivated plasma was added to 20 μl of phosphate-buffered saline (PBS) in the first well of the plate; serial dilutions of PBS (1:2–1:256) were then carried out, followed by the addition of 20 μl of a 1% suspension of SRBC to each well. The plates were gently agitated for 1 min and then incubated at 37 °C for an hour. After that, plates were put still at 4 °C for 2 h before macroscopic examination for agglutination was performed. Antibody titers were expressed as the negative $\log_2$ of the minimum plasma concentration that contained enough antibodies to agglutinate the antigenic SRBC.

2.3. Learning performance

A longitudinal maze was built with white PVC tubes and transparent acrylic sheets on the top to allow the observation of the animals. The maze was composed of a series of dead-end paths and one correct path leading to the goal point at the opposite end of the start point (Fig. 1). A food reward (2 g of sweet potato) was placed at the goal point. A spatial error occurred when the animal entered a dead-end path during the test trial. Total length of the artificial labyrinth (9.70 m) was within the range of burrow lengths of individuals of *C. talarum* from Mar de Cobo (mean 14 ± 8 m, [24]), being therefore representative of the natural burrows of this subterranean rodent.

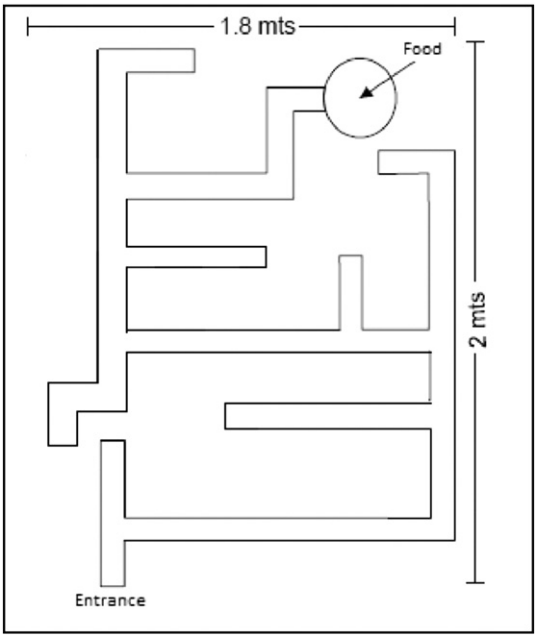

Fig. 1. Longitudinal maze used to evaluate spatial learning performance in tuco-tucos suffering an induction of their immune system under two different nutritional conditions.

Animals were trained in two daily trials (one in the early morning and one in the late afternoon) until ten trials were completed, which is the number of trials required to learn longitudinal mazes in *C. talarum* [26–28]. Before starting each trial, the animal was transported in a transfer tube from its home cage to the start point of the labyrinths, where it remained in the dark for a habituation period of 2 min. Then, the study animal was allowed to enter the maze and the trial ended when the animal reached the food reward or when 10 min had elapsed, if the reward was not obtained. At the end of each trial, the animal was weighed and returned to its home cage in the transfer tube. Later, the labyrinth was dismantled and washed with tap water and odorless detergent, wiped with ethanol and then allowed to air dry to ensure that no odors from previous trials remained. Spatial learning performance was assessed recording the time spent to complete the task (trial duration) and the number of errors made by individuals (spatial performance parameters) was recorded during each trial.

2.4. Physiological parameters

2.4.1. Leukocyte profile

Because leukocytes are directly involved in the mammalian immune response to pathogens [40], estimates of levels of circulating leukocytes provide a global estimation of immune activity. Additionally, the ratio of neutrophils/lymphocytes (N:L) is a known chronic stress indicator [41] that could serve to monitor the animals' stress status in captivity (see [42]). Leukocyte abundance and diversity were quantified following standard protocols [43]. Following initial and final blood samples collection, smears were fixed in 70% methanol for 10 min, then stained with May-Grunwald Giemsa solution and examined under oil immersion at 100 x magnification (Olympus CX 31). The cells were counted only across the entire monolayer area of the slide ("wandering technique"; [43]). Leukocytes were identified according to the characteristics of their morphology: lymphocyte, neutrophil, basophil, eosinophil and monocyte. Leukocyte counts were obtained by recording the number and type of each cell type until a cumulative number of 200 cells was reached; then the N:L ratio was calculated. Also, in a single pass along the slide, we recorded both the number of erythrocytes and leukocyte encountered in 30 fields and the number of total leukocytes was standardized to 100,000 erythrocytes [44].

2.4.2. Hematocrit

The proportion of blood volume occupied by packed red blood cells is considered to reflect the animal's condition since it is thought to be affected by ecological conditions, exercise and blood parasites [45,46]. Following initial and final blood samples collection, a small blood volume (ca. 40 μl) was collected in a heparinized capillary tube, which was then centrifuged at 14,000 rpm for 15 min (Giumelli Z12D centrifuge). The hematocrit was assessed as the proportion of capillary length occupied by packed red blood cells in relation to capillary length occupied by all blood components (Abaco Giumelli). Hematocrit was determined in duplicates and the resulting values were averaged.

2.4.3. Stress hormone essays

In *C. talarum* cortisol, and not corticosterone, was found to vary in response to exposition to stress factors [47]. Therefore, cortisol determinations were performed using plasma samples from initial and final blood extractions stored at −20 °C (no heating required) since no substances in plasma samples that interfere with cortisol were detected in *C. talarum* [47]. Cortisol was measured using the Coat-A-Count procedure (Siemens Medical Solutions Diagnostics), which is a solid phase radioimmunoassay (RIA, catalog number: TKCO1) in which ^{125}I-labeled cortisol compete with cortisol in the samples for antibody sites. The assay is capable of measuring cortisol concentrations up to 200 ng/mL. Detection limit is 2 ng/mL, as informed by the manufacturer. Intra and inter-assay coefficients of variation were 4.8% and 5.2% respectively, as determined by running duplicates of plasma samples and controls of

known cortisol concentrations. Cross-reactivities of the antibody used in this assay with other structurally-similar molecules are very low, as reported by the manufacturers (e.g., corticosterone: 0.94, 11-deoxycorticosterone: 0.26, 11-deoxycortisol: 11.4, dexamethasone: 0.04).

2.5. Statistical analysis

Student's t-test was used to evaluate if antibody titers differed between tuco-tucos subjected to the two different dietary conditions. A first examination of the learning pattern of tuco-tucos in this study allowed us to recognize that it was not lineal, with a critical trial number after which the rate of learning changes. Therefore, we used the SegReg software (segmented regression analysis; www.waterlog.info) to statistically identify the existence of a critical breakpoint in the learning pattern curve of *C. talarum*, so that it could be described by two straight lines with different slopes. The results of the evaluation of the learning performance of the control individuals indicated that there was an optimal breakpoint between trials 5 and 6 ($x = 5.4 \pm 0.6$ trials, 90% confidence intervals) dividing it into an initial sloping segment followed by a nearly horizontal line; thus we proceeded to analyze the learning performance of this rodent in two separate regressions: a) from trials 1 to 5 and b) from trials 6 to 10. The number of errors and the time needed to reach the goal in the spatial learning trials were compared between the different groups using Generalized Linear Models (Statistica 9.0) for the two different trial blocks. When data did not meet the assumptions of normality and homoscedasticity, transformations obtained from the utilization of the Box–Cox method were applied. The Box–Cox identifies an appropriate exponent (λ) to transform data into a normal shape. The λ value indicates the power to which all data should be raised. According to the Box–Cox results, trial duration was transformed with $\log_{10}$ while the number of errors was raised to 0.2. Considered factors were treatment (injected with SRBCs or saline), diet (slight or strict restriction) and number of trials (as a repeated measure).

To evaluate if there was an effect of the immune challenge or the dietary restriction on the leukocyte, N/L ratio, hematocrit and cortisol levels before and after the experiments, we calculated the individual's difference in these physiological parameters levels (final–initial) and used these values to perform a two-way Anova, with injection of SRBC/saline and diet as factors.

2.6. Ethical note

Once concluded the experiments, animals were fed ad libitum until recovery of their initial body weight. After that, they were returned to the field and released at the site of capture. All field and laboratory procedures conformed to institutional and national guidelines (Argentine National Council for Scientific and Technological Research: PIP 2787, Argentine Agency for Scientific Promotion: PICT 1295-2008). The animals were cared for in accordance with the Guidelines for the Use of Animals in Behavioral Research and Teaching (ASAB/ABS 2003).

3. Results

3.1. Immune response

With the exception of two individuals in the high dietary restriction group, all animals injected with SRBC under both diet conditions mounted an immune response. Antibody titers did not differ between dietary groups (Student's t-test, $n = 30 - 13$ individuals at 15% diet and 17 individuals at 25% diet, $p = 0.52$, Fig. 2). As expected, individuals injected with saline solution did not display any immune response to the antigen.

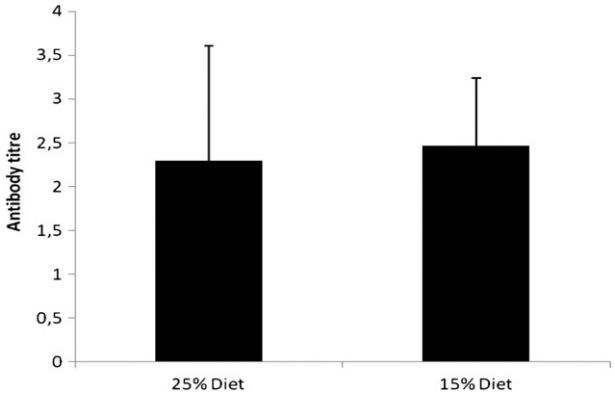

Fig. 2. Titers of antibodies (mean + s.d.) against sheep red blood cells (SRBCs) in tuco-tucos under slight (15% diet) or severe (25% diet) food restriction at the end of the spatial learning period. Antibody titers are expressed as $-\log_2$ of the minimum plasma concentration that contained enough antibody to agglutinate the antigen.

3.2. Learning experiments

3.2.1. Trials 1–5

Exposure of individuals to the antigen did not affect the time spent and number of errors made until reaching the end of the labyrinth (GLM, $n = 47$, trial duration: $F_{1,43} = 0.98$; $p = 0.32$; errors: $F_{1,43} = 0.73$; $p = 0.39$, Fig. 3). No effect of the dietary restriction was observed on these parameters (GLM, trial duration: $F_{1,43} = 0.129$; $p = 0.72$; errors: $F_{1,43} = 0.001$; $p = 0.97$). No interaction between both factors was observed (GLM, errors: $F_{1,43} = 3.7$, $p = 0.06$, trial duration: $F_{1,43} = 1.27$, $p = 0.26$).

3.2.2. Trials 6–10

Tuco-tucos injected with SRBC committed more errors and displayed a clear trend to require more time – albeit differences were not significant – to reach the goal with respect to the saline groups (GLM, $n = 47$, errors: $F_{1,43} = 4.85$, $p = 0.032$, trial duration: $F_{1,43} = 3.34$, $p = 0.07$; Fig. 3). No effect of the dietary restriction (GLM, errors: $F_{1,43} = 2.53$, $p = 0.11$, trial duration: $F_{1,43} = 0.62$, $p = 0.43$) nor interaction between both factors were observed (GLM, errors: $F_{1,43} = 0.003$, $p = 0.95$, trial duration: $F_{1,43} = 0.03$, $p = 0.86$).

3.3. Physiological parameters

3.3.1. Leukocyte profile

No effect of diet or immune challenge on total leukocyte count was verified (two-way Anova, $n = 46$, immune challenge: $F_{1,43} = 0.65$, $p = 0.42$; diet: $F_{1,43} = 0.628$, $p = 0.43$; Fig. 4A).

Regarding the N/L ratio, a significant effect of the diet was observed, with higher values found in individuals under strict dietary restriction (two-way Anova, $n = 46$, $F_{1,43} = 6.6$, $p = 0.01$). Mounting an immune response did not have an effect on this parameter (two-way Anova, $F_{1,43} = 0.22$, $p = 0.63$; Fig. 4B).

3.3.2. Hematocrit

No significant effects of mounting an immune response or being subjected to a strict dietary restriction on hematocrit levels were observed after finishing the learning period (two-way Anova, $n = 39$, immune challenge: $F_{1,36} = 0.87$, $p = 0.35$; diet: $F_{1,36} = 1.87$, $p = 0.18$; Fig. 4C).

3.3.3. Cortisol

Values of cortisol were not affected by immune challenge or dietary regime (two-way Anova, $n = 43$, immune challenge: $F_{1,40} = 1.63$, $p = 0.2$; diet: $F_{1,40} = 0.132$, $p = 0.25$; Fig. 4D).

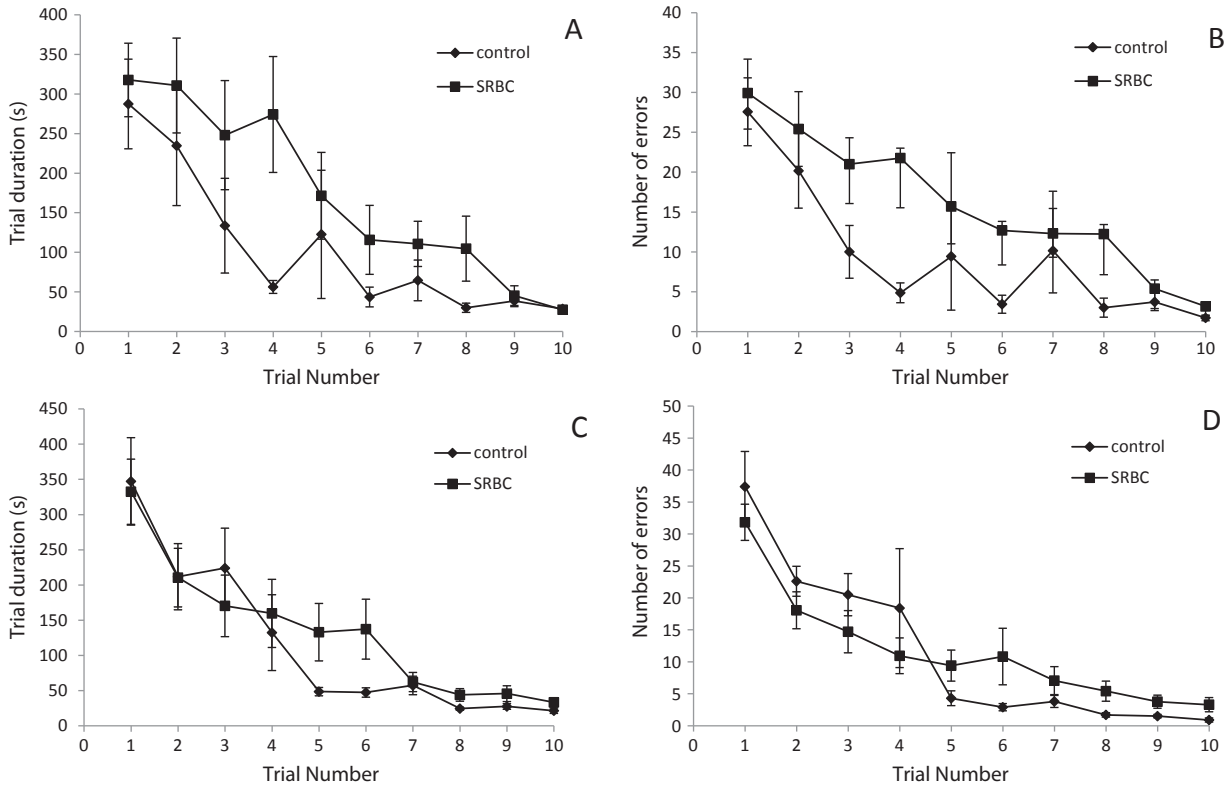

Fig. 3. Time spent (trial duration) and number of errors (means ± s.e.m.) made by individuals of *C. talarum* injected with saline (control) or the antigen (SRBC) and under slight (15%, A and B) or severe (25%, C and D) food restriction, during each trial of spatial learning in the longitudinal labyrinth.

4. Discussion

Learning allows animals to adjust their behavior in an adaptive way to a changing environment and is deeply related to the memory, which constitutes the ability to remember experiences previously learned [48]. The formation and consolidation of the memory depend on the synaptic plasticity of neurons, which in turn requires de novo protein synthesis needed to synaptic growth and remodeling [49]. As a consequence, learning is considered an energetically costly process. Spatial learning, and subsequently spatial memory, represents the ability of an individual to recognize, store and retrieve knowledge about spatial features of its environment [50]. This is highly relevant for most of the vital behavioral activities performed by animals, such as localization of food, avoiding neighbors or predators, or finding mates during the reproductive period. An enhanced spatial learning capability is crucial for species living in structurally complex habitats or in environments deprived or restricted of sensorial cues. In fact, both conditions are present in the ecotope of subterranean rodents, where individuals must orientate inside their

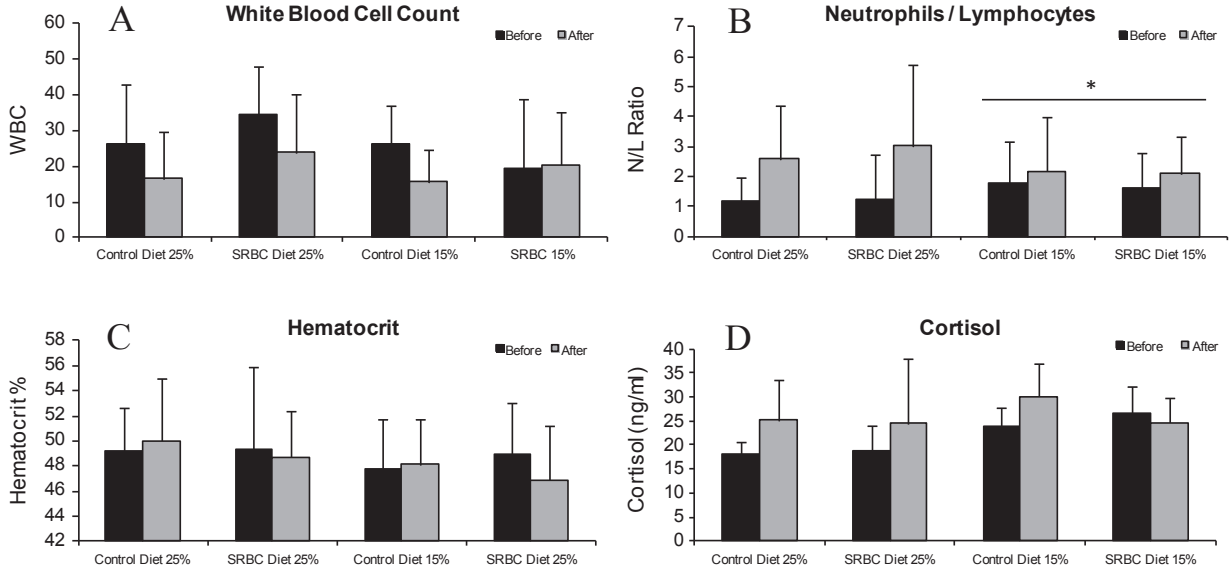

Fig. 4. Means (+s.d.) of (A) total white blood cell (WBC) counts, (B) neutrophils/lymphocyte (N/L) ratio, (C) hematocrit and (D) plasma cortisol levels before and after the spatial learning period in tuco-tucos injected with saline (control) or the antigen (SRBC), and under slight (15% diet) or severe (25% diet) food restriction. Asterisk indicates significant differences in N/L ratio in individuals under the two different dietary conditions ($p < 0.05$).

large and intricate tunnels without the use of visual cues and with limited access to odor and acoustic cues [51,26,27]. Therefore, any deterioration of the spatial capabilities in subterranean rodents may have a profound impact on their survival and reproduction.

There is evidence that learning ability is influenced by immune status, indicating a competition for nutritional resources between both traits. However, in some of these studies, the effects on learning were confounded with the ones caused by the parasite on the motor abilities or the overall health status of the hosts [19]. For example, manipulation of host behavior, such as an increase in activity [52] and a decrease in fear of novelty [53] through formation of parasitic cysts in the brain of rodents has been proved for *Toxoplasma gondii* [54]. Also, immune stimulation by bacterial endotoxin (lipopolysaccharide, LPS) caused deficits in learning and memory [55,56], although this impairment was consistent with sickness and lowered locomotor performance [57]. In the present work, we demonstrated that the spatial performance of the subterranean rodent *C. talarum* was lowered, both considering time required and the number of errors made to reach the goal of the maze, in individuals suffering an induction of their immune system with a non-pathogenic antigen. Although immune challenged tuco-tucos only displayed a tendency to require more time to complete the labyrinth, the fact that number of errors, deeply related to trial duration, was clearly significant, provides support for the biological significance of the increment in this spatial performance parameter. This decrease in spatial learning occurred in the absence of any symptoms of illness (absence of movement) or motor impairments (difficulties in walking), suggesting for the existence of a trade-off between learning capacity and immunity status in this species. This result is in line with previous findings that presented initial evidence of reduced learning ability under conditions of immunodepression or infection [19,62,20,63], indicating that when investment is required to fight infection, fewer resources are available for learning. Besides this energetic trade-off, in the last years many research has been devoted to study and untangle the complex interactions between the immune, endocrine, and nervous systems. These interactions occur at multiple levels, involving brain cells showing immune function (microglia and astrocytes), immune cells (T-cells and macrophages) and neural cells. Between others, the cytokines, major signaling molecules of the immune system that regulate, for example, the innate and adaptive immunity and inflammatory responses [58,59], were shown to impair cognitive functions by disrupting the acquisition of a learned response [60,61]. Although this effect of cytokines, particularly of the pro-inflammatory ones (e.g. IL-1, IL-6, TNFα), were observed to be associated with the appearance of sickness behaviors, it would be interesting to realize experimental studies in tuco-tucos measuring variations in the levels of cytokines to see whether some of these signaling molecules could be responsible for altering the learning performance of this species without the manifestation and influence of sickness behaviors.

Food quantity and quality can modulate the organism's capacity to respond to infectious challenges, and even minor differences in the level or kind of nutrients can cause variations in the immune response [12]. Usually, deficiencies of micronutrients, and in lesser degree macronutrients, produce a weaker response of the immune system [12]. Moreover, energy restriction in the diet, if prolonged, can lead to the suppression of the immune system [64,11,65]. For example, deer mice fed 70% of their ad libitum diet two weeks after the first antigen presentation produced 95% less IgG against a novel protein after a second antigen challenge than mice fed ad libitum [66]. Contrary to this, we did not find differences in the immune response between individuals of *C. talarum* under slight or severe food restriction. Moreover, tuco-tucos under severe dietary restriction displayed a similar spatial learning performance than individuals subjected to slight restriction, indicating that there was not a further redistribution of energetic resources between the immune and cognitive systems to maintain similar levels of antibody titers after immunization. One possible explanation is that under severe nutritional restriction, other physiological processes besides cognition – like growth or reproduction – may suffer a reduction in their energetic budget in favor of the more immediately critical immune response. Besides any priority in energy distribution, immune system is directly involved in memory, learning and neural plasticity. Although conceptual and mechanistic topics are partially verified in model animals like rats — using an experimental approach and humans — using correlation studies, much more studies are needed to better understand this relationship but avoiding other confounding factors [58].

Neutrophils and lymphocytes constitute the majority of white blood cells in mammals [48]. While neutrophils are the primary phagocytic leukocyte and proliferate in circulation in response to infections, lymphocytes are mainly involved in immunoglobulin production and immune defense modulation [48]. Contrary to what was expected, injection of SRBCs did not affect total WBC or N/L ratio in *C. talarum*. This species usually displays lower numbers of WBC and an increase in the N/L ratio in response to chronic stress associated with captivity [32,47]. These variations were also observed in this work, probably masking the effects of the immune challenge on these parameters. Only severe food restriction induced an increment in the N/L ratio, a trend also previously observed in this species [42], reflecting the effect of this chronic stressor on *C. talarum* physiological condition. Hematocrit level, an estimator of the animal's condition, was neither influenced by immune challenge nor food restriction, indicating that the experimental treatments did not have a strong effect on tuco-tucos' oxygenation carrying capacity. Finally, glucocorticoids are considered indicators of stress response with low threshold levels, so that even minor perturbations trigger increments in cortisol. Besides being used as a stress marker, glucocorticoids have strong effects on the immune system, inducing redistribution of lymphocytes from the blood to different organs [67]. In the present work, we did not observe any effect of the immune challenge or dietary condition on plasmatic cortisol levels. However, and similar to what previously observed in tuco-tucos kept in captivity [68], values of this hormone were sometimes very low (less than 20 ng/mL), possibly hiding any effect of the experimental treatments on this physiological parameter. Nevertheless, the low cortisol levels along the experiments suggests for the absence of immunosuppressive effects of this hormone that could have interfered with immune response to the injected antigen, although further studies are needed to confirm for this possibility in this species.

5. Conclusion

In conclusion, this work provides support for the existence of a trade-off between the costs of the immune defense and learning abilities in the subterranean rodent *C. talarum*. Learning impairment was detected without clear symptoms of illness or stress due to immune challenge. Under parasitic or infectious diseases, this species may redistribute energetic resources towards the immediately vital immune response in detriment of other important, but not urgent, physiological processes like learning. Although the immune challenge used in this study is non pathogenic, it elicits a humoral immunity, which involves storage of information about pathogen recognition [69]. It has been proposed that such immunological memory may be especially important for “slow-living-pace” species, such as tuco-tucos (with long life span, time to reach maturity, and strategy of pup development; see [23]) because repeated infections are likely in these species, and hence immune memory may lower the time and resources needed for fighting common pathogens [70].

In contrast to our prediction, resource limitations, as implemented in this study, did not affect learning performance, revealing the complexity of the relationships among these factors. Besides this, other factors such as time under food restriction as well as food quality (i.e. micronutrients availability) must be further considered.

While previous works on the subject searched for trade-offs among diverse traits using rodent model species like *Mus*, this is the first study

that provides evidence for a relationship between the immune system and learning in a wild rodent, measuring a cognitive trait (spatial learning) highly relevant for the survival and reproduction of this species. Further studies that measure the immune response, both cellular and humoral, to pathogenic antigens and including diverse life-history traits in an integrative approach, are needed to continue advancing in our understanding of the costs, benefits, and net fitness consequences of the activation of the immune system.

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