



Effects of Acute and Chronic Exposure to Predatory Cues on Spatial Learning Capabilities in the Subterranean Rodent *Ctenomys talarum* (Rodentia: Ctenomyidae)

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Abstract

Spatial learning, the cognitive ability that allows animals to efficiently locate food, partners or avoid predators, can be affected by various factors such as stress. Stressors represent a threat to the animal's homeostasis and trigger a set of physiological and behavioral changes known as stress response. The herbivorous rodent *Ctenomys talarum* (tuco-tuco) inhabits underground complex gallery systems. Dispersal as well as food collection occurs on the surface, where they are exposed to terrestrial and aerial predators. We evaluated the effect of a natural stress factor, predation, on spatial learning of *C. talarum*. Additionally, we collected data on neutrophil/lymphocyte ratio, hematocrit, blood glucose, and plasma cortisol to assess physiological levels of stress. The acute exposure to direct cues indicating the presence of a predator (immobilization + cat urine) increased the time needed to reach the goal in a longitudinal labyrinth. The number of errors also increased albeit the difference was only marginally significant. The chronic exposure to predator cues (immobilization, cat urine, and cat fur) presented in an unpredictable order significantly increased both time and errors committed by tuco-tucos during learning trials in the labyrinth. Results show the strong impact of predatory stress on the spatial abilities of *C. talarum*, a key cognitive process involved in most of their vital activities. They also highlight the importance of investigating wild species as regards the development of antipredatory defensive behaviors that contribute both to the avoidance of deleterious consequences of a direct attack and the influence on food search and mate localization performance.

Introduction

Learning is a key cognitive process that allows organisms to modify their subsequent behavior and is deeply related to memory, which constitutes the ability to remember experiences previously learned (Kandel et al. 2000). In particular, spatial learning represents the ability of an individual to design a direct trajectory between two places, while spatial memory allows him to recognize, store, and recall the spatial structures in its habitat (Machatschke et al. 2011). Both spatial learning and memory allow the animals to construct representations of their surroundings, achieving an

adequate spatial orientation and navigation, particularly in structurally complex habitats (Kimchi & Terkel 2001). Spatial navigation 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.

Spatial learning can be affected by different factors, including stress. Stressors can be of different nature (e.g., physiological: presence of parasites and/or infections, hypothermia; psychological: presence of a predator; social: interaction with a dominant conspecific; Reeder & Kramer 2005) and may vary in their

form of presentation (acute single exposure or chronic-repeated exposure) as well as in their degree of predictability (according to the ability of the organism to anticipate the appearance of such aversive stimuli; Armario 2006). In nature, stressors can also be subdivided into whether they are reactive (direct physiological challenges that do not require cognitive processing: e.g., temperature) or anticipatory (any perceived threat, requiring cognitive processing: e.g., high predation risk; Boonstra 2013). Regardless of their nature, all of them represent a threat to the animal homeostasis and trigger a set of physiological and behavioral changes known as stress response (Sapolsky et al. 2000; Reeder & Kramer 2005). The adaptive response to stress in vertebrates is mediated by the activation of the hypothalamic–pituitary–adrenal (HPA) axis and comprises an initial secretion of catecholamines and glucocorticoids that result in immediate modifications such as mobilization of energy, changes in behavior, analgesia, and improvements in sensory and cognitive functions (Sapolsky et al. 2000; Nelson 2005; Reeder & Kramer 2005). However, if the stress factor is particularly intense or prolonged in time, adverse consequences like cognitive or organic failures, that may compromise reproduction and even survival, are triggered (Nelson 2005; but see Boonstra 2013 for a chronic adaptive response in nature).

Predators can induce a stress response in their prey not only by physically attacking them but also by making them fearful of a possible attack through the presence of predatory signals (Fletcher & Boonstra 2006). The stress response includes the development of behaviors essential for the survival of the prey, like changes in activity patterns, reductions in non-defensive behaviors, or home-range displacements (Apfelbach et al. 2005). However, and besides the development of these defensive behaviors, it has been observed that stress response also exerts a modulatory effect on certain cognitive processes like learning and memory. For example, previous studies on arctic squirrels detected either positive or negative modulations in learning and memory in an active avoidance task depending on the degree of aversion shown to the stress factor, with learning and memory enhanced with high and low aversive stimulus, respectively (Zhao et al. 2004). Moreover, exposition to chronic stressors or exogenous glucocorticoids influenced negatively spatial learning in rats (Endo et al. 1996; Nishimura et al. 1999), while a single exposure to predatory stress prior to training produced deficient spatial learning in *Mus musculus* (El-Hage et al. 2006).

The majority of subterranean mammals perform most of their vital activities, such as localization of food, mate finding, and defense or escape from predators, in their dark and structurally complex underground tunnels. Spatial orientation in these tunnels must be based on a restricted sensory input due to the limited use of auditory and olfactory signals, and the impossibility of using visual cues for orientation in the dark underground burrows (Reichman & Smith 1990). This restriction of sensory stimuli requires the development of an efficient orientation mechanism required for proper orientation in the complex tunnel structures and for the reduction in high energetic costs associated with digging while extending the burrows for foraging (Vleck 1979; Luna et al. 2002).

Ctenomys talarum (Talas tuco-tuco; Thomas 1898) is a herbivorous subterranean rodent found in coastal sandy soils of Buenos Aires Province (Argentina). This species inhabits a system of closed galleries parallel to the soil surface that generally consists of a main axial tunnel and a variable number of lateral branches and feeding tunnels. In nature, individual burrows show no subterranean connections (Antinuchi & Busch 1992). Both sexes at all ages (except the offspring until dispersal) are sedentary and maintain exclusive territories (Busch et al. 1989). The foraging behavior of *C. talarum* involves an underground exploration in search for food patches and frequent excursions to the surface to collect vegetation that is later consumed inside the burrows (Busch et al. 2000). During these brief periods at the surface, and also when individuals disperse (Malizia et al. 1995), both sexes become vulnerable to aerial and terrestrial predators. Previous studies on *C. talarum* showed that this species is frequently preyed by owls, foxes, and wild cats (Vassallo et al. 1994; Busch et al. 2000; Canepuccia 2005). Moreover, in suburban areas, predatory events by dogs and cats have also been detected (C. E. Schleich, pers. obs.).

The ability to learn and memorize a tunnel system is crucial for *C. talarum* to successfully obtain food, reproduce and defend a territory within their complex subterranean burrows (Antinuchi & Schleich 2003). Concomitantly, it has been observed that this species displays a highly developed capability to learn structurally complex radial and longitudinal labyrinths, with individuals clearly improving their spatial performance after 5–6 trials (Antinuchi & Schleich 2003; Mastrangelo et al. 2009, 2010). This spatial ability could be modified by multiple factors like the risk of predation. Previous work on *C. talarum* showed that acute exposure to direct predatory cues produces a short-term stress response that negatively affects its spatial reference memory (Mastrangelo et al. 2009). As most stud-

ies evaluating the effects of predation on learning come from model organisms (mainly rats) under artificial conditions (Park et al. 2001; El-Hage & Belzung 2002), it is necessary and relevant to include new studies in wild species under controlled laboratory conditions, where natural selective pressures occurring in their own environment are resembled. Moreover, experiments in seminatural and natural conditions, where multiple challenges can be considered, allow assessing not only cognitive performance but also changes in space use and activity patterns.

Therefore, the aim of this paper was to examine the effects of exposure to predatory cues on the spatial learning performance of *C. talarum*. In particular, we assessed (1) the effect of acute exposure to predatory cues (direct: immobilization + indirect: cat urine) at the beginning or in the middle of the learning period, and (2) the effect of a chronic variable exposure (direct: cat fur odor and immobilization + indirect: cat urine) on the ability of tuco-tucos to learn a longitudinal labyrinth. Once the last learning trial concluded, physiological parameters, namely neutrophil/lymphocyte (N/L) ratio, hematocrit, blood glucose, and plasmatic cortisol, were obtained for each individual.

We predicted that the acute exposure to signals indicating the presence of a predator would induce a stress condition in individuals of *C. talarum* that would affect their spatial learning performance, increasing both the time and number of errors made until reaching the goal of the labyrinth. As regards chronic exposure, we predicted that it would provoke a more severe stress condition in individuals of *C. talarum*, strongly affecting the spatial capabilities of this subterranean rodent in comparison with the acute stressed group.

Material and Methods

Animal Capture and Housing Conditions

Between May 2011 and May 2012, we captured adults of both sexes (46 males and 40 females) in Mar de Cobo (37°46'S, 57°26'W, Buenos Aires province, Argentina) using live traps placed at the entrances of tuco-tuco burrows. Then, the individuals were transported to the laboratory and placed in individual plastic cages (25 × 32 × 42 cm) provided with wood shavings. Food was supplied daily and consisted of carrots, sweet potatoes, lettuce, corn, alfalfa, mixed grass, and sunflower seeds. Temperature in the laboratory remained constant (23 ± 1°C) with a light–dark cycle of 12:12 h. Following the methodology used in previous studies (Mastrangelo et al. 2009,

2010), animals were maintained at 85% of their initial body weight during the experiments to increase their motivation to explore and learn mazes. Animals arrived to the laboratory 1 wk before the beginning of the learning trials. Once experiments finished, they were maintained for an extra 1-wk period to secure that animals reached their capture weight. Hence, animals stayed for 3 wk in captivity and then they were released at the site of capture in good physical condition.

Longitudinal Labyrinth

We used a labyrinth constructed with white PVC tubes (10 cm diameter) provided with transparent acrylic sheets on the top to enable the observation of the animals. The longitudinal maze, previously used to study spatial learning in *C. talarum* (Mastrangelo et al. 2010), comprised eight dead-end paths and 15 turns leading to the goal point at the opposite end of the start point. The length of the artificial labyrinth (9–10 m) was within the range of burrow lengths of individuals of *C. talarum* from Mar de Cobo (mean 14 ± 8 m, Antinuchi & Busch 1992), being therefore representative of the natural burrows of this subterranean rodent.

After each experiment, the labyrinth was completely dismantled and washed with odorless detergent and water, cleaned with ethanol and then allowed to air dry to ensure that no odor of previous treatments remained. Latex gloves were used when handling the animals and the labyrinth minimizing the transfer of human odors.

Procedure

Animals were randomly assigned to each experimental condition. In the case of females, only those obtained during the non-reproductive period (December–May) were used in the experiments. The reproductive season of *C. talarum* entirely depends on the reproductive cycle of females given that males do not undergo regression of testicles after reaching sexual maturity, and epididymides contain sperms throughout the year (Malizia & Busch 1991).

Acute Exposure to Predatory Cues

Acute exposure of each individual to predatory cues was simulated through immobilization and exposure to cat urine, a methodology successfully used in a previous study (Mastrangelo et al. 2009). We combined immobilization and urine, imitating a sequence of

predator attack and prey escape. While urine, which does not necessarily indicate the proximity of a predator, generates a state of anxiety and the need to adopt defensive behaviors in the prey (Dielenberg & McGregor 2001), immobilization constitutes a strong stressor that induces both behavioral and endocrine responses (Marquez et al. 2002; Buynitsky & Mostofsky 2009; Vera et al. 2011).

Immobilization consisted in placing each individual in a plastic cage (25 × 32 × 42 cm), holding it firmly to restrict its movement and intermittently releasing it for a period of 2 min (simulating a sequence of a predator attack and prey escape). Immobilization was carried out by pressing the animals with the hand wearing a clean leather glove. Exposure to cat urine consisted in leaving the animal in the same plastic cage with a pot containing wood shavings impregnated with domestic cat urine (obtained 24 h before starting the experiments and conserved in sealed bags at 4–6°C). Exposition to cat urine lasted 5 min, 3 min prior to, and 2 min during immobilization.

Chronic Exposure to Predator Cues

Chronic exposure of tuco-tucos to predatory cues was simulated by the synchronized exposure to cat urine and immobilization pressing the animal with a cloth impregnated with cat fur odor to intensify predatory cues similar to situations of predator approach (Apfelbach et al. 2005). Although generating greater anxiety response (Muñoz-Abellán et al. 2009; Staples 2010), fur stimulates a minor activation of the stress axis (Muñoz-Abellán et al. 2008) if compared to single immobilization (Muñoz-Abellán et al. 2008).

For that reason, each individual was transferred to a plastic cage and immobilized as explained above, but now individuals were pressed by hand with a cloth impregnated with cat fur odor. This odor-impregnated

cloth was obtained after allowing the cat to rest on it for a period of 7 d before the experiment. Similarly, exposure to cat urine consisted in leaving inside the plastic cage a pot containing wood shavings impregnated with domestic cat urine for 5 min, 3 min prior to, and 2 min during immobilization.

Spatial Learning Experiments

One morning and one evening trial were conducted during five consecutive days until completing 10 tests. That is, the period proved to be required by *C. talarum* to learn longitudinal mazes (Antinuchi & Schleich 2003; Mastrangelo et al. 2009, 2010). To evaluate spatial learning after an acute exposure to predatory cues, 43 animals (23 males and 20 females) were exposed to immobilization and cat urine just before the first trial (T1: Treatment stress trial 1, 12 males and 10 females, Fig. 1a) or prior to the sixth trial on the third day of the experiment (T2: Treatment stress trial 6, 11 males and 10 females, Fig. 1b), when a clear improvement in tuco-tucos learning performance was observed (Mastrangelo et al. 2010).

To assess the effect of chronic exposure to predatory cues on spatial learning performance, 21 tuco-tucos (11 males and 10 females) were exposed to cat urine before the first trial, and immobilization (with cloths impregnated with cat fur odor) prior to the second trial. On the third day of the experiment (trials 5 and 6), the order of presentation of stressors was reversed to avoid predictability (Fig. 1c). The combination of different types of stressors on different days corresponds to the category of ‘chronic variable stress’ (Armario 2006).

Control individuals (10 males and 12 females) were never exposed to direct or indirect predatory cues. Assignment of individuals to control or treatment groups was made at random.

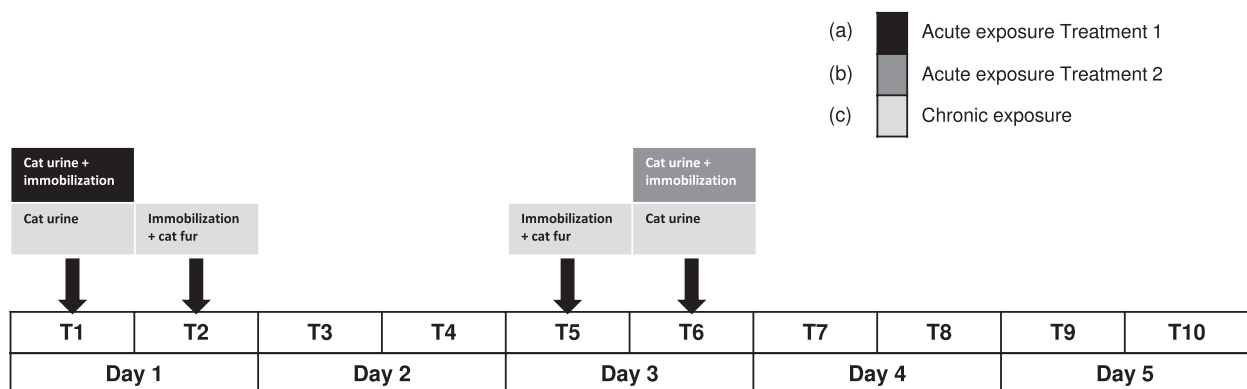


Fig. 1: Sequence of application of stress factors: (a) Acute exposure Treatment 1, (b) Acute exposure Treatment 2, and (c) Chronic exposure.

Spatial learning performance was assessed using two variables: time (latency) and errors committed (number of errors) by tuco-tucos to reach the goal of the labyrinth. To do this, animals were carried from their cages to the start point of the labyrinth, where they were kept in darkness for a habituation period of 2 min. Then, they were allowed to enter the labyrinth; latency (using stopwatches) and the number of errors made by tuco-tucos to reach the food reward (one small piece of sweet potatoes) at the end of the labyrinth were registered by means of direct observation during each of the ten trials.

Physiological Stress Measures

Diverse physiological parameters typically change during stress response and are commonly used as stress indicators (Armario 2006; Johnstone et al. 2012). To estimate the levels of chronic stress caused by trapping, captivity conditions and food restriction, and to assess the physiological stress induced by acute or chronic exposure to predatory cues, we employed parameters used in previous studies on *C. talarum*: neutrophil/lymphocyte (N/L) ratio, hematocrit, blood glucose, and plasma cortisol levels (Vera et al. 2008, 2011; Cutrera et al. 2010). Differences in the N/L ratio (low in unstressed individuals, high in stressed ones) are interpreted as a response to environments that are becoming riskier for the individuals (for example, by an increase in the activity of predators) or when the capacity to avoid injuries is low (for example, when the organism is in poor physiological condition; Johnstone et al. 2012). The N/L ratio is also recognized as responsive to chronic stressors (Davis et al. 2008). Regarding the proportion of red cells in blood, it can be considered as an estimation of the physiological condition of an organism (Johnstone et al. 2012). While changes in levels of blood glucose were previously reported as sensitive to stressors of intermediate level, with response thresholds higher than those found for catecholamines and glucocorticoids (Armario et al. 1986), glucocorticoids are now considered indicators of stress response with low threshold levels. In this case, even minor perturbations trigger increments in cortisol, corticosterone, or both, depending on the taxonomic group (Armario 2006).

To determine these physiological stress parameters, a sample of blood (300 µl) was taken from the retro-orbital sinus in all individuals 30 min after the end of trial 10 (Vera et al. 2011). Blood sampling did not take more than 3 min to guarantee that glucocorticoid levels were not affected by the extraction proce-

dure (Vera et al. 2011). Immediately after extraction, blood glucose was determined using an Accu-Check Active® Glucometer. Hematocrit level was obtained by capillary centrifugation during 15 min at 14 000 rpm. After this, blood smears were performed, fixed in 70% methanol for 10 min, and stained with May-Grunwald-Giemsa. The number of lymphocytes, neutrophils, eosinophils, basophils, and monocytes was identified after microscope examination at 1000×. All cell types were counted until the cumulative total was 200 cells and then the N/L ratio was calculated (Vera et al. 2008). Finally, the blood sample was centrifuged at 3000 rpm, and the obtained plasma was sent to a specialized laboratory for the quantification of cortisol by radioimmunoassay (RIA; validation of this technique for *C. talarum* is provided in Vera et al. 2011).

Statistical Analysis

The number of errors and latency to reach the goal in trials of spatial learning were compared between control, acute, and chronic stressed groups using generalized linear models. Considered factors were treatment (control vs. acute stress: T1 and T2; control vs. chronic stress) and sex, with trials as the repeated measure. When data did not meet the assumptions of normality and homoscedasticity, transformations obtained from the utilization of the Box-Cox method were applied.

The N/L ratio, hematocrit, blood glucose, and plasma cortisol levels between the control group and the stressed groups were compared by a two-way ANOVA (with sex and treatment as factors). Tukey tests were utilized *a posteriori* to identify differences between groups when statistical significant results were obtained.

Results

Effects of Single Exposure to Predatory Cues on Spatial Learning Performance

Tuco-tucos showed a clear improvement in their spatial learning performance after the first trials. The number of errors and latency to reach the goal in the longitudinal labyrinth decreased as the number of trials increased (*errors*: Tukey test, Trial 1 vs. Trial 10, females control: $p = 0.007$, females treatment1: $p = 0.004$, females treatment2: $p = 0.023$, males control: $p < 0.001$, males treatment1: $p = 0.003$, males treatment2: $p = 0.022$; *latency*: Tukey test, trial 1 vs. trial 10, females control: $p = 0.0006$, females treatment1: $p = 0.03$, females treatment2: $p = 0.08$, males

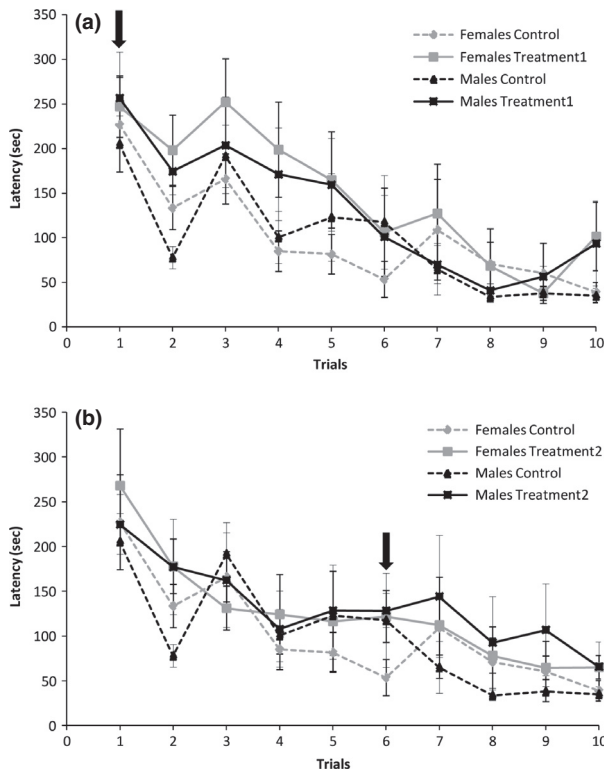


Fig. 2: Latency ($\bar{x} \pm SD$) of individuals of *Ctenomys talarum* to reach the goal during the spatial learning trials in treatments 1 (a) and 2 (b). The arrow indicates the trial that followed the exposition to predatory cues.

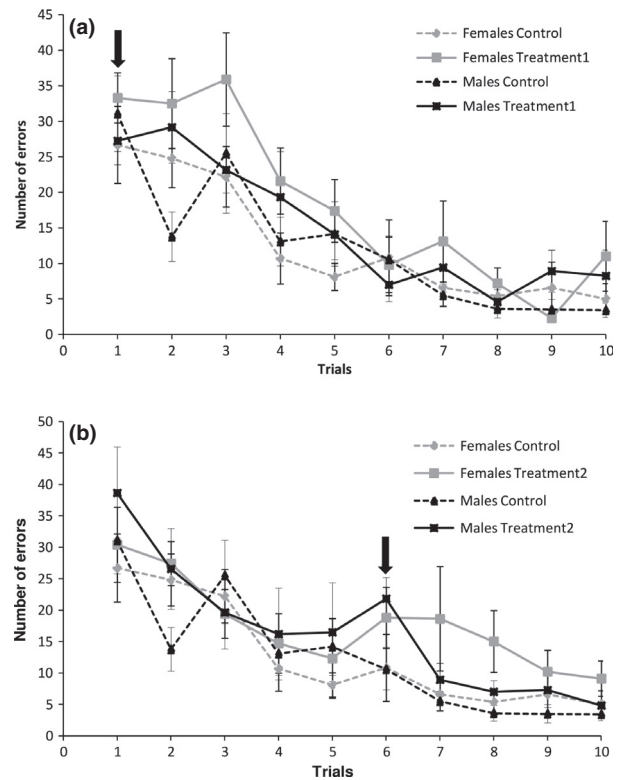


Fig. 3: Number of errors ($\bar{x} \pm SD$) made by individuals of *Ctenomys talarum* to reach the goal during spatial learning in treatments 1 (a) and 2 (b). The arrow indicates the trial that followed the exposition to predatory cues.

control: $p < 0.001$, males treatment1: $p < 0.001$, males treatment2: $p < 0.001$) (Figs 2a, b and 3a, b).

Exposure of individuals to predatory cues at the beginning or in the middle of the learning period increased the time required to reach the goal with respect to the control group (GLM, $F_{2,65} = 3.21$, $p = 0.047$). Neither effect of sex nor interaction of both factors on this parameter were observed (GLM, sex: $F_{1,65} = 0.61$, $p = 0.439$; sex \times treatment: $F_{2,65} = 0.2$, $p = 0.81$). *Post hoc* analysis (Tukey test) revealed no differences in latency between the two groups subjected to predatory cues (treatment 1 vs. treatment 2, $p > 0.05$) or between sexes ($p > 0.05$) for each different trials (Fig. 2a, b).

The number of errors made during learning trials was also higher in individuals exposed to predatory cues than in controls even though differences were only marginally significant (GLM, $F_{2,65} = 3.09$, $p = 0.052$). No differences were detected in the number of errors made by males or females (GLM, $F_{1,65} = 0.977$, $p = 0.327$; Fig. 3a, b). No interaction between factors was observed (GLM, $F_{2,65} = 0.14$, $p = 0.86$).

Physiological Stress Measures

The N/L ratio did not differ between stress-exposed treatments and the control groups (two-way ANOVA, $F_{2,65} = 0.68$, $p = 0.506$), although higher values were observed in males with respect to females, being this difference only marginally significant (two-way ANOVA, $F_{1,65} = 3.86$, $p = 0.054$). The interaction between treatment and sex was not significant (ANOVA, $F_{2,65} = 0.446$, $p = 0.630$) (Fig. 4a).

Significant differences were observed in hematocrit levels between sexes (ANOVA, $F_{2,65} = 24.001$, $p < 0.001$) with higher values observed in males. No differences were detected between stress-exposed treatments and control groups (two-way ANOVA, $F_{2,65} = 1.564$, $p = 0.218$). The interaction between treatment and sex was not significant (two-way ANOVA, $F_{2,65} = 0.708$, $p = 0.497$). Tests *a posteriori* (Tukey tests) showed significant differences between males of the control group and females under treatment 1 ($p = 0.002$), between control males and females under treatment 2 ($p = 0.009$), between males and females under treatment 1 ($p = 0.004$), and between

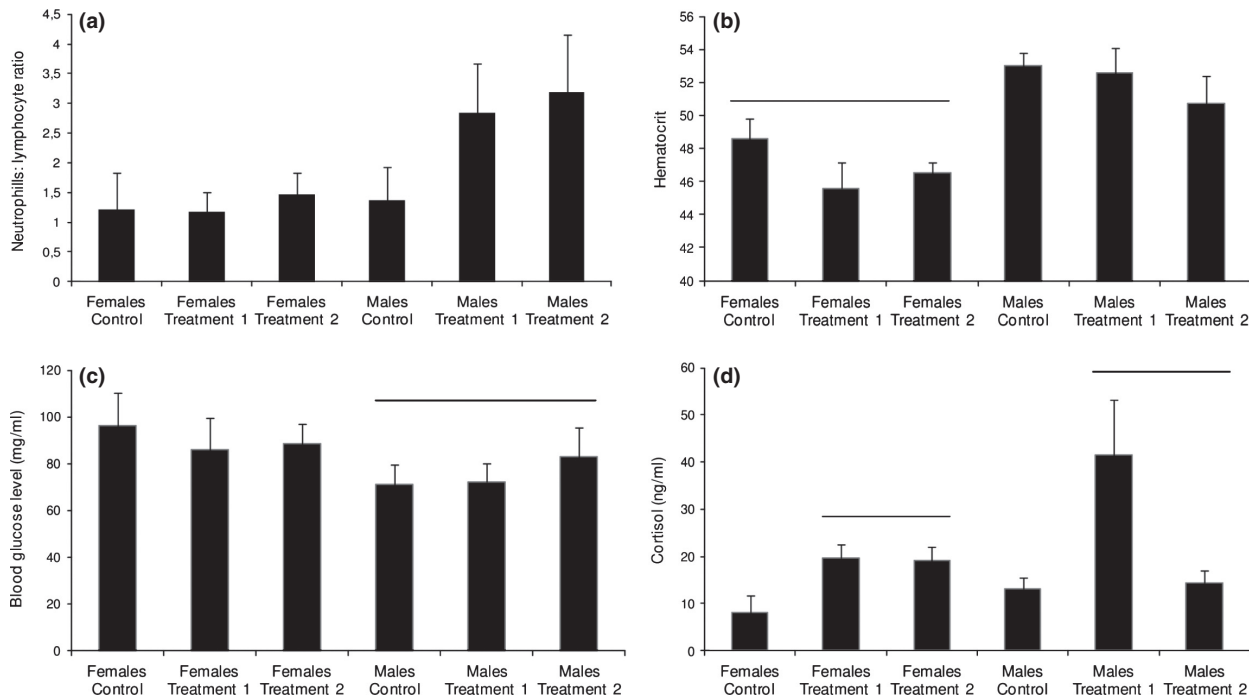


Fig. 4: (a) Neutrophils: lymphocyte ratio ($\bar{x} \pm SD$), (b) hematocrit ($\bar{x} \pm SD$), (c) blood glucose ($\bar{x} \pm SD$), and (d) plasma cortisol levels ($\bar{x} \pm SD$) in males and females of control groups and the groups subjected to single stress after finished spatial learning trials. The bars indicate significant difference ($p < 0.05$) levels between males and females in hematocrit levels, and in plasma cortisol levels between control and acute treatment groups.

males and females under treatment 1 and treatment 2, respectively ($p = 0.018$) (Fig. 4b).

Blood glucose differed between sexes (ANOVA, $F_{1,65} = 46.77$, $p = 0.035$), being higher in females. No differences were observed between treatments and control groups (ANOVA, $F_{2,65} = 0.781$, $p = 0.463$). Type of treatment and sex interaction was not significant (ANOVA, $F_{2,65} = 0.27$, $p = 0.765$) (Fig. 4c).

An overall effect of acute stress on cortisol levels was observed (ANOVA, $F_{2,65} = 5261$, $p = 0.008$), whereas no differences were observed between sexes in these values (ANOVA, $F_{1,65} = 0.132$, $p = 0.717$). The interaction between treatment and sex was not significant (ANOVA, $F_{2,65} = 1.195$, $p = 0.31$) (Fig. 4d).

Effects of Chronic Exposure to Predatory Cues on Spatial Learning Performance

Similar to that observed in the previous experiment, control and chronically stressed individuals also showed improvement in their spatial learning performance, which was reflected in the lower number of errors (Tukey test, trial 1 vs. trial 10, control females: $p = 0.001$, chronic females: $p = 0.19$, control males: $p < 0.001$, chronic males: $p < 0.001$) and the time to reach the goal of the longitudinal labyrinth as the

number of trials increased (Tukey test, trial 1 vs. trial 10, control females: $p < 0.001$, chronic females: $p = 0.008$, control males: $p < 0.001$, chronic males: $p < 0.001$).

Furthermore, the analysis also revealed differences between the control and the chronically stressed groups in both studied parameters (latency: GLM, $F_{1,43} = 30.2$, $p < 0.01$; errors: GLM, $F_{1,43} = 25.84$, $p < 0.01$). However, no differences were detected between sexes as regards in the number of errors and latency to reach the goal (latency: GLM, $F_{1,43} = 0$, $p = 0.82$; errors: GLM, $F_{1,43} = 0.02$, $p = 0.86$; Fig. 5a, b) or interaction between factors (sex \times treatment: latency, $F_{1,43} = 0$, $p = 0.83$; errors, $F_{1,43} = 0.03$, $p = 0.86$).

Physiological Stress Measures

No differences were observed in the neutrophil/lymphocyte ratio neither between control and chronic stressed groups (ANOVA, $F_{1,43} = 1.508$, $p = 0.226$) nor between males and females (ANOVA, $F_{1,43} = 1.539$, $p = 0.222$). Type of treatment and sex interaction was not significant (ANOVA, $n = 43$, $df = 1$, $F = 0.364$, $p = 0.55$) (Fig. 6a).

Differences were observed in hematocrit levels between treatments (ANOVA, $F_{1,43} = 5.086$,

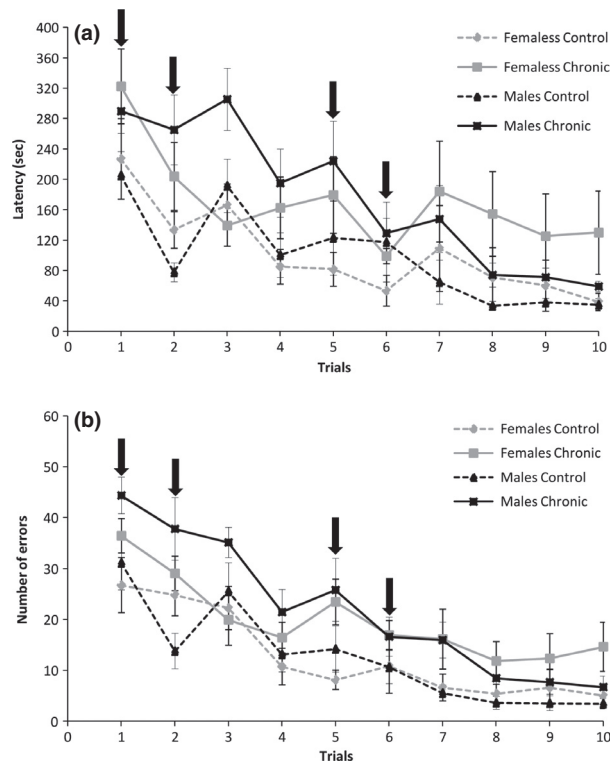


Fig. 5: Mean (\pm SD) of latency (a) and the number of errors (b) to achieve the goal during each trial of spatial learning in the longitudinal maze. The arrow indicates the trial that followed the exposition to predatory cues.

$p = 0.029$) and between sexes (ANOVA, $F_{1,43} = 22.31$, $p < 0.001$). The treatment and sex interaction was not significant (ANOVA, $n = 43$, $df = 1$, $F_{1,43} = 0.267$, $p = 0.608$) (Fig. 6b). Tests *a posteriori* (Tukey) revealed that values for males of control and chronic stressed groups were higher than those observed in stressed females (test de Tukey, $p < 0.001$ y $p = 0.002$, respectively). Similarly, values for control males were higher than those for control females (test de Tukey, $p = 0.02$).

Blood glucose levels differed between sexes (ANOVA, $F_{1,43} = 0.436$, $p = 0.043$), being higher in females than in males. No differences were observed between chronically stressed groups and control groups (ANOVA, $n = 43$, $df = 1$, $F_{1,43} = 0.198$, $p = 0.658$). Type of treatment and sex interaction was not significant (ANOVA, $F_{1,43} = 0.068$, $p = 0.796$) (Fig. 6c).

Cortisol levels did not differ neither between control and chronically stressed groups (ANOVA, $F_{1,43} = 0.078$, $p = 0.781$) nor between sexes (ANOVA, $F_{1,43} = 0.117$, $p = 0.734$). The treatment and sex interaction was not significant (ANOVA, $F_{1,43} = 0.194$, $p = 0.662$) (Fig. 6d).

Single vs. Chronic Exposure to Predatory Cues

When comparing spatial learning performance in the longitudinal labyrinth under the application of two different modalities of predatory exposure, we found statistical differences between the control, the acute (T1 and T2 pooled), and the chronically stressed groups with respect to latency (GLM, $F_{2,86} = 0.976$, $p < 0.001$) and number of errors (GLM, $n = 86$, $df = 2$, $F_{2,86} = 10.16$, $p < 0.001$). *Post hoc* analysis (Tukey test) revealed differences between these groups as regards time until reaching the goal of the labyrinth. Individuals exposed to acute and chronic predatory cues showed higher latencies than control groups, although in the case of acute exposure, the difference was marginally significant (control vs. acute: $p = 0.066$, control vs. chronic: $p < 0.001$). Chronically stressed individuals required more time to reach the goal of the maze than those under acute exposure (acute vs. chronic: $p = 0.015$) (Fig. 7a).

Regarding the number of errors made to complete the labyrinth, individuals undergoing acute and chronic treatments committed more errors than tuco-tucos in the control group (control vs. acute: $p = 0.026$, control vs. chronic: $p = 0.0002$). Moreover, chronically stressed animals made more errors than those subjected to a single exposure event (acute vs. chronic: $p = 0.03$) (Fig. 7b). No differences in learning performance between males and females were found for both parameters (latency: GLM, $F_{1,86} = 0.756$, $p = 0.757$; errors: GLM, $F_{2,86} = 0.113$, $p = 0.737$).

Discussion

Predators can induce a state of stress in preys not only by direct physical attack but also by the presence of predatory cues in the environment (Fletcher & Boonstra 2006). In response, prey species develop antipredatory behaviors that allow them to recognize, avoid, and defend from predators. In many cases, these antipredator defenses include increased sensitivity to predator odors like those originated in the fur, urine, feces, and anal gland secretions (Apfelbach et al. 2005). Studies both in field and laboratory conditions showed that predator odors have different behavioral effects on the prey, such as inhibition of the activity, suppression of non-defensive behaviors (feeding, reproduction, and growth), and displacements to safer places, where these odors are not present (Blanchard & Blanchard 1989; Apfelbach et al. 2005). In vertebrates, the adaptive response to stressors, including predatory risk, includes an increase in sensory and

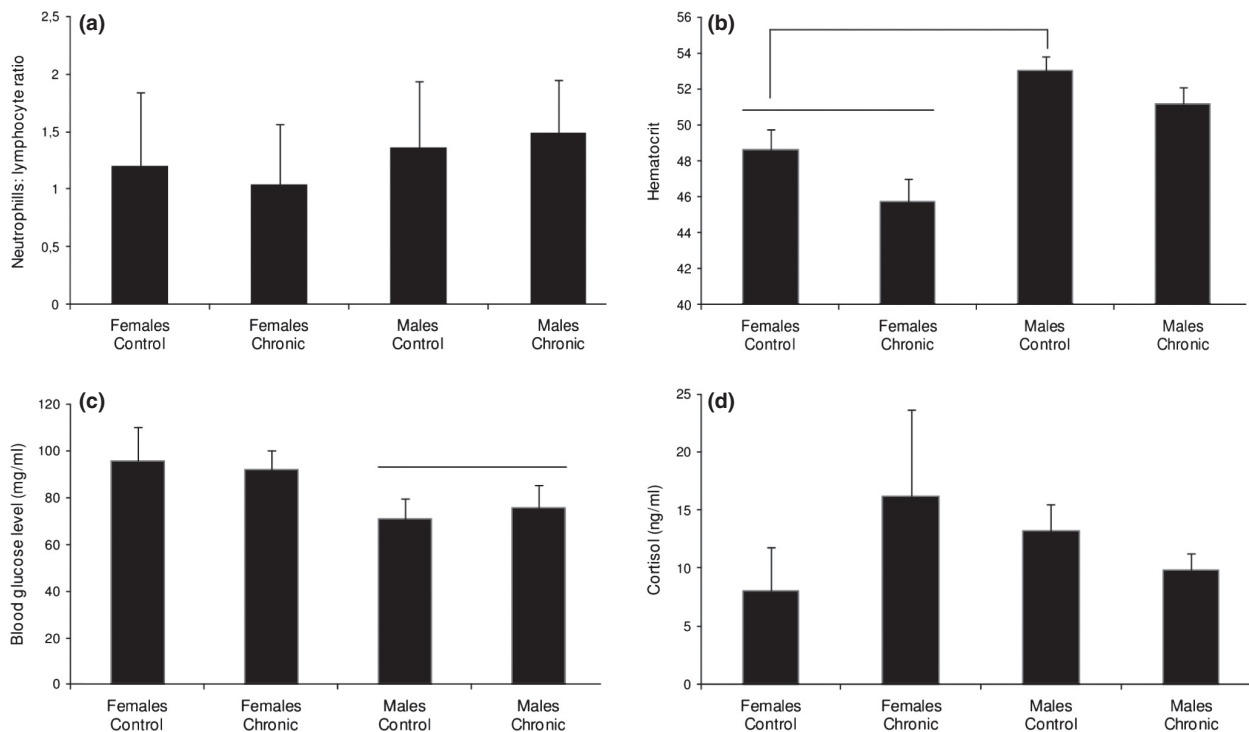


Fig. 6: a) Neutrophils: lymphocyte ratio ($\bar{x} \pm SD$), (b) hematocrit ($\bar{x} \pm SD$), (c) blood glucose ($\bar{x} \pm SD$), and (d) plasma cortisol levels ($\bar{x} \pm SD$) in males and females of the control groups and the groups subjected to chronic stress after finished spatial learning trials. The bars indicate significant differences ($p < 0.05$) in hematocrit levels between males and females and between control and chronic treatment groups, and in glucose levels between males and females.

mnemonic functions (Sapolsky et al. 2000; Boonstra 2005). However, under severe or prolonged stress conditions, adverse or deleterious effects on several aspects of cognition are expected (Armario 2006). A long-term exposure to stress factors or an HPA axis hyperactivity, which produces an excess of glucocorticoids, can cause morphological and physiological modifications of the hippocampus, such as dendritic atrophy, reduced neurogenesis, differential response to neurotransmitters and decreased hippocampal volume (Endo et al. 1996; Lupien & Lepage 2001; Joëls et al. 2006).

The differential effect of stressors on learning (deleterious or beneficial) depends not only on its intensity and duration, but also on the moment in which the stressors occur (Adlard et al. 2011). Previous studies have shown that if an individual is exposed to a single stress factor before or during the codification process, learning can be altered and the subsequent memory enhanced or diminished (Schwabe et al. 2011) revealing the complexity of the relationship between stress and learning. In the present work, we exposed tuco-tucos to predatory cues in two different modalities: acute or chronic. The stress factor was applied only once in the acute exposition, but in two different

moments in relation to the spatial learning process: at the beginning or in the middle of the learning period, when individuals display a clear improvement in their spatial performance (Mastrangelo et al. 2010). Under the chronic modality, predatory cues were alternated in an unpredictable sequence to avoid the progressive reduction in the behavioral response due to stimulus repetition (De Boer et al. 1990). Results showed that an acute exposure to the combination of direct and indirect predatory cues negatively affected spatial learning performance in *C. talarum*. Specifically, this was clearly observed in latency and, to a lesser extent, in the number of errors made to reach the goal of the maze, when differences were marginally significant. These results are consistent with those obtained by other authors who analyzed the effects of an acute exposure to predatory factors on spatial learning capabilities. For example, one single exposure of individuals of *Mus musculus* to a predator (cat) between days 16 and 22 before starting spatial trials resulted in impaired learning in the radial maze (El-Hage & Belzung 2002; El-Hage et al. 2006). Similarly, Park et al. (2008) showed that Sprague-Dawley rats exposed to a predator (cat) for 30 min before the start of learning trials committed more errors during the acquisition

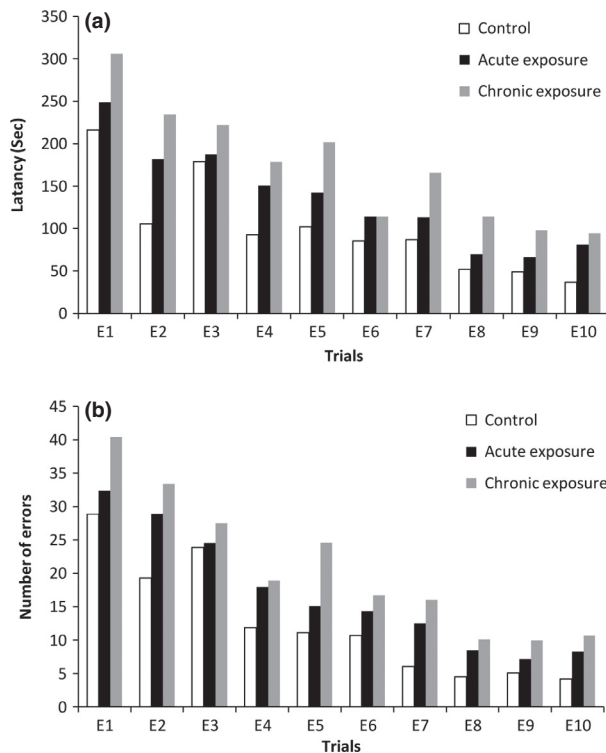


Fig. 7: Mean of latency (a) and the number of errors (b) to achieve the goal for each spatial learning trial in the maze longitudinal in the control groups and the groups subjected to single and chronic stress.

phase in the water radial maze than control subjects. Using rats (Sprague-Dawley) as predators of mice (strain CD-1), Bonsignore et al. (2008) found that individuals under predatory stress before or during the learning period travelled greater distances to reach a hidden platform in the Morris water maze when compared to unstressed mice. This result also coincides with those obtained by Grootendorst et al. (2001). Although some studies have suggested that an acute exposition to a stress factor within the learning context may have a positive role in learning and memory formation (Joëls et al. 2006), the results obtained in *C. talarum* as well as in other rodent species seem to provide support for a negative effect of acute predatory stress on these cognitive processes.

While short-term exposure to stressors was considered to produce both positive and negative variations on associative and non-associative learning, the effects of chronic exposure were often assumed to exert negative effects on learning (Conrad 2010). For example, rats repeatedly exposed to immobilization for 6 hours during 21 d made more errors during learning of a T-maze than control rats (Sunanda et al. 2000). In another series of experiments, male rats subjected to a chronic stress factor (ice water) during

12 weeks, displayed deficient spatial learning when evaluated in a radial maze (Nishimura et al. 1999). In our experiments, tuco-tucos exposed chronically and unpredictably to predatory cues also showed severe deficiencies during spatial learning in the longitudinal maze, committing more errors and requiring more time to reach the goal. Chronic exposure also affects spatial learning ability of *C. talarum* to a greater extent than acute exposition to predatory cues. Although the effects of single and chronic predatory stress on spatial learning abilities in other rodent species have not been compared yet, the result of this work is consistent with the general view, suggesting that chronic stress usually causes a higher negative impact on most physiological systems compared to acute stress (McEwen 2002), with effects that are not often predictable.

Several studies have demonstrated that sex is an important factor in understanding the effects of stress on learning and memory (Conrad et al. 2004). This is the case in adult male rats, in which exposure to stress by immobilization for 21 d caused learning deficits in the radial maze and in the T-maze, while females subjected to the same stressor showed a better performance in these labyrinths (McFadden et al. 2011). Although males are more susceptible to predation than females due to their longer aboveground exposition (Malizia et al. 1995; Zenuto et al. 1999, 2002), the absence of differences between males and females of *C. talarum* in their spatial performance after exposure to acute or chronic stress shows that the cognitive impairment caused by predatory stress affects both sexes in a similar manner. Future studies should evaluate the effect of stress on female learning during the reproductive period as it has been observed that different levels of gonadal hormones affect spatial abilities (Galea et al. 1995, 1996).

The estimation and quantification of the stress response in vertebrates principally involves measuring the concentration of leukocytes (N/L), glucocorticoids, parameters of immune response, and the analysis of volume of erythrocytes, glucose concentration, stress proteins, and oxidative stress (Epel et al. 2004; Armario 2006; Herring & Gawlik 2007; Johnstone et al. 2012). *Ctenomys talarum* shows an N/L ratio increment in response to chronic stress caused by captive conditions and food restriction, but also in response to stress factors such as immobilization both in the field and in captive conditions (Vera et al. 2011). In this study, N/L values in control individuals were similar to those observed in tuco-tucos kept in captivity (Vera et al. 2011), so neither food restriction necessary to reach the appetitive condition nor animal manipulations during trials affected stress levels

caused by captivity. Besides treatments, males subjected to immobilization and the odor of cat urine presented higher levels in the N/L ratio, although the high variance in the data did not allow us to detect a significant interaction between treatment and sex factors. In the case of chronic exposure, no effects of the treatment or N/L ratio differences between sexes were observed. Nonetheless, the possibility that *C. talarum* N/L ratio response to predation stress exposure could no longer be evident when a suite of stressors are applied must also be considered.

In this study, we found that the hematocrit was lower in tuco-tucos chronically subjected to predatory cues. Furthermore, and regardless of the treatment applied, males presented higher hematocrit levels than females. As all individuals were fed in the same manner and *C. talarum* do not drink free water (conditions that can affect hematocrit levels), these differences cannot be attributable to differential hydration. Regarding glucose levels, no differences between control, acute, or chronic stressed groups were observed, although overall females displayed higher values than males. The use of blood glucose as stress parameter seems to be limited in the case of acute and chronic stressors, particularly when the evaluation of glycemia is not made almost immediately after the application of the stressor and under controlled dietary conditions (Vera et al. 2008; Mastrangelo et al. 2009). Finally, previous works in *C. talarum* showed that cortisol levels increased significantly within 30–60 min after immobilization both in recently caught individuals and in tuco-tucos maintained in captivity for 10 d (Vera et al. 2011). Similar increments were also detected after treatment with ACTH (corticotrophic hormone, Vera et al. 2012). However, under captivity, a condition of chronic stress, tuco-tucos presented low levels of cortisol (20 ng/ml) causing that differences between sexes found in the wild (where female exceeds male levels) could no longer be detected (Vera et al. 2011). Regarding cortisol levels, in this study, there was no difference between sexes, a result consistent with the mentioned antecedents. However, the response to acute predatory stress was different compared to the chronic treatment. In the first case, there was a notable increase in plasmatic cortisol in males and females subjected to immobilization in the presence of odor of cat urine, not considering whether this was done at the beginning or in the middle of the learning period. Concerning the lower response of cortisol to chronic predatory stress, it may be possible that the application of a chronic stressor on individuals under a chronic stress state due to captivity can lead to an inhibition in the secretion of

glucocorticoids, resulting in chronically stressed individuals but with lower levels of cortisol compared to unstressed animals (Vera et al. 2011). This can be interpreted as a physiological response to avoid the harmful effect of a high activation of HPA axis (Nadal & Armario 2010).

Finally, and even though predator-induced stress has been typically used to exemplify the stress concept, research using predatory-induced stress in wildlife is scarce. In recent times, this model has been increasingly considered, changing the older vision that a predatory attack will only produce an acute and transitory effect on the prey, and emphasizing that sustained psychological stress in nature (comparable to chronic stress in humans) affects cognitive and emotional aspects having an impact in demographic parameters (Clinchy et al. 2013). In our study, we proved that exposure to predatory cues has a strong impact on the spatial learning performance of *C. talarum*. The negative effect on spatial abilities, which affected both sexes equally, was stronger in animals exposed to the chronic variable stress modality in comparison with the acute one. These results strongly suggest that the magnitude of the cognitive impairment caused by the exposure to predatory cues is significantly associated with the frequency and mode of exposure to the stress factor. While at first only the wounds and injuries caused to the preys were considered as the detrimental effects of a predatory aggression, now it is clear that also a failed attack might cause strong effects on prey fitness. In *C. talarum*, for which most of the vital activities like food searching or mate localization depend on proper spatial orientation, the effects of a predatory attack may also have serious consequences for survival and reproduction, as both acute and chronic exposure to predatory cues affected the spatial capabilities of this species of subterranean rodent.

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