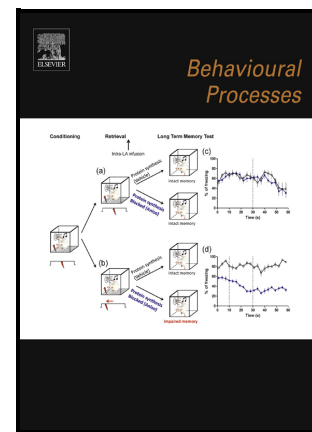


Individual differences in behaviour are related to metabolism, stress response, testosterone, and immunity in the subterranean rodent *Ctenomys talarum*

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Individual differences in behaviour are related to metabolism, stress response, testosterone, and immunity in the subterranean rodent *Ctenomys talarum*

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Abstract

The growth of personality research has led to the integration of consistent variation of individual behaviour in multidimensional approaches including physiological variables, which are required to continue building a more comprehensive theory about coping strategies. In this study, we used wild-caught males of *Ctenomys talarum* (tuco-tucos), a solitary subterranean rodent, to assess the relationships among personality traits and several physiological variables, namely stress response, testosterone, immunity, and energy metabolism. Subjects (n=21) were used in experimental tests assessing behaviour, energy metabolism, testosterone levels, inflammatory cell-mediated and humoral immunity, and stress response to a simulated predator attack. The structural equation model explained a

moderate portion of the variance of personality behaviours related to activity (52 %), boldness (35 %), and socioaversion (30%). More active and bold individuals showed higher oxygen consumption. While those subjects had lower baseline cortisol levels, there was no relationship between cortisol levels of the stress-induced response. Cell-mediated immune response was related to activity levels. Finally, testosterone only affected boldness. Despite some of these relationships diverge in direction to predicted ones, overall they support the existence of coping styles in male *C. talarum*; and are discussed in the light of current hypotheses and particular behavioural and ecological traits of tuco-tucos.

Keywords: *Ctenomys*, subterranean rodents, personality, coping styles, immunity, stress, energy metabolism

1. Introduction

Individual behavioural traits are considered highly variable in response to environmental change (West-Eberhard, 2003). But flexibility of such behavioural responses is limited, and consequently, it is possible to distinguish personalities (also known as temperament or behavioural types), that is, individuals are consistent in behavioural traits over time and in different ecological contexts (Réale et al., 2007). Variations in personalities and observed correlations between behavioural traits (i.e. behavioural syndromes), were associated with similar patterns of variation in underlying state-variables (Sih et al., 2004a). Indeed, many models explain individual differences in behaviour based on differences in the “state” of individuals, which implies an adaptive integration of both traits, possibly mediated by shared control mechanisms (Houston and McNamara, 1999; Sih et al., 2015). However, later Niemelä and Dingemanse (2018) pointed out that many studies fail to prove that the intrinsic state of individuals explains much individual variation in behaviour. These state-

variables involve characteristics such as physiological traits (e.g., metabolic rate, stress, and immune responses; Sih et al., 2015), as well as the requirements to display a behaviour, including energy reserves or skill sets (Sih et al., 2004b; Sih and Bell, 2008; Wolf and Weissing, 2012). Variation in behaviour and physiological responses has been related to the way individuals cope with stressful situations, described as “coping styles” along a proactive- reactive continuum (Koolhaas et al., 1999). However, the evidence supporting relationships between a consistent set of behavioural and physiological characteristics is mixed (Koolhaas et al., 1999; Niemelä and Dingemanse, 2018; Royauté et al., 2018) and focuses primarily on a limited number of variables. Links have been reported between the personality of individuals and different physiological aspects, such as metabolism (Biro and Stamps, 2010; Careau et al., 2008), stress response (Afshar et al., 2015; Boulton et al., 2015; Grace and Anderson, 2014; Mell et al., 2016), testosterone levels, and immune performance (Koolhaas, 2008; Monceau et al., 2017; van Oers et al., 2011; Zylberberg et al., 2014). Recently, McMahon et al. (2022) compiled empiric evidence on physiological processes linked to personality with, among others, the goal of establishing personality-specific physiological profiles. The authors found that only a few studies, out of 145 reported, included multiple measures, thus emphasizing the need to characterize a more comprehensive physiological profile including multiple behavioural and physiological measures.

In a stressful situation, while some individuals avoid the stressor or respond aggressively to it, others may remain passive and unresponsive, showing different “coping styles” (Koolhaas et al., 1999). Then, the behavioural response to a stressful situation like the presence of a predator is not fixed and may depend not only on the individual's personality, but also on the magnitude and responsiveness of glucocorticoid (GC) levels (Boonstra, 2005), which are increased in vertebrates in response to stress, via the

hypothalamus-pituitary-adrenal (HPA) axis. The “proactive” strategy refers to a more aggressive and active reaction and is expected in individuals living in stable environments, while the “reactive” strategy is related to avoidance behaviour and less activity, which individuals under environmental changes (Koolhaas et al., 1999). Concomitantly earlier models proposed that proactive individuals show low HPA-axis reactivity (low GCs response to a stressor but high sympathetic reactivity), while reactive individuals show both higher HPA-axis and parasympathetic reactivity (unidimensional model; Koolhaas et al., 1999). However, such patterns seems to be more diverse than previously thought (see Westrick et al., 2019). Glucocorticoids maintain homeostasis (Sapolsky et al., 2000) and can modulate the behavioural response by regulating the availability of energy (Boonstra, 2005). Supporting a stress response, as well as other energy-consuming behavioural traits of an active and/or bold lifestyle, would require an adjustment of the metabolic machinery to meet those requirements, which in turn may lead to the coevolution of these traits (Biro and Stamps, 2010; Careau et al., 2008). If this is the case, according to the performance model, bolder/more active individuals are also expected to have larger energy-consuming organs which will result in higher energy expenditure, even at rest (Careau et al., 2008; Réale et al., 2007). However, evidence has supported both the performance and the other proposed models: the allocation model, which proposes that an animal has a limited amount of energy to spend, so a higher RMR will result in less energy available for activity (Careau et al., 2008), and the independent model, which assumes that RMR and physical activity are independent (Careau and Garland, 2012).

Defence against pathogens is an important selective force (Ezenwa et al., 2016). Behaviour can be the first line of defence, reducing an individual’s exposure to pathogens. Thus, individuals with different personalities are expected to face different challenges; for example, more exploratory and active individuals (i.e., proactive) should be more exposed

to pathogens (Ezenwa et al., 2016; Zylberberg et al., 2013). The immune system is the next line of defence against pathogens. Since differential exposure to pathogens is associated with variation in behavioural types, investment in immunity is expected to be related as well (Zylberberg et al., 2014, 2013). Under this hypothesis, proactive individuals are expected to have more robust immune defences (Lee, 2006; Réale et al., 2010), since these animals are more exposed to novel pathogens (Novikov et al., 2010; Snoeijs et al., 2004; Voigt et al., 2020). At the other extreme, “reactive” individuals, being more shy, cautious, and less exploratory, would invest less in immunity (Lee, 2006; Réale et al., 2010). Particularly, most evidence seems to indicate that immune activity in exploratory individuals favour increased cell-mediated responses, devoting few resources to fast-acting nonspecific innate immunity or to slower, longer-lasting humoral immunity. Thus, it is proposed that the cell-mediated response would benefit animals facing new challenges during their exploration. Moreover, bolder and aggressive individuals are also expected to use the rapid innate immune response. While in the case of more social organisms, the humoral response would be valuable due to the probability of being exposed to the same infections repeatedly during interactions (see review by McMahon et al., 2022).

Variation in circulating testosterone is associated with variation in several personality traits, and it has been hypothesised to be a marker of personality in humans and rodents (de Ruiter et al., 1993; Newman and Josephs, 2009). In addition, it is known that the immune system is affected by testosterone (see review by Braude et al., 1999; Dhabhar, 1998), as well as the stress response mediated by glucocorticoids (Cain and Cidlowski, 2017; Dhabhar, 1998; Franco et al., 2019; Morrow-Tesch et al., 1994). The link between testosterone and immunity in males has been a topic of great interest because females are generally more immunocompetent than males (Folstad and Karter, 1992). Although this sex bias is unclear, the most accepted explanation proposed is that testosterone acts as an

immunosuppressant (Folstad and Karter, 1992). While correlational evidence related high testosterone levels with increased vulnerability to pathogens (Roberts et al., 2004), a recent meta-analysis showed that testosterone had a moderate immunosuppressive effect across species (Foo et al., 2017). Furthermore, LaVere et al. (2021) emphasize that testosterone-immunity relationships are variable for different immune measures and are also affected by intrinsic factors, indicating the complexity of these interactions. Furthermore, direct effects of testosterone levels on behaviour must also be considered. Indeed, variations in testosterone have been linked to aspects such as social interactions (Soto-Gamboa et al., 2005), or increased mate searching and mobility (Mills et al., 2009).

For all that was described above, additional assessments using different behaviours and physiological responses would contribute to building a more comprehensive hypothesis about coping strategies (Réale et al., 2010), as well as improving our comprehension of the adaptive capacity and vulnerability to stress of animals under diverse conditions (Koolhaas et al., 1999). In this work, we assessed the relationship between personality traits and physiological parameters: metabolism, cortisol-mediated stress response, testosterone, and immunity in wild-caught males of *Ctenomys talarum* (“Talas tuco-tuco”, thereafter tuco-tucos; Thomas, 1898), a solitary subterranean rodent. Male tuco-tucos represent an excellent opportunity to assess these relationships because they exhibit behavioural types differentiated by their activity levels, degree of socioaversion, and boldness (Fanjul and Zenuto, 2020). Individual variability in this species was also detected in endocrine, immune, and metabolic responses (Antinuchi et al., 2007; Merlo et al., 2018a; Vera et al., 2011a, 2013), all of which may possibly be related to personality variation as well. Studying males is particularly interesting since they face specific selective pressures imposed by reproduction (Hämäläinen et al., 2018; Tarka et al., 2018). For male tuco-tucos, aggression and territorial dominance play a key role in the polygynous mating system of this species

(Zenuto et al., 1999). Despite both sexes being territorial, agonistic encounters among males are more frequent and severe, leading to the establishment of dominance hierarchies where the dominant male monopolises multiple females (Zenuto et al., 2002). In this reproductive system, male territorial exclusiveness and dominance are important qualities for mate choice by females (Fanjul et al., 2018; Fanjul and Zenuto, 2017). As described for *C. talarum* and many other species, polygyny imposes high intrasexual competition during mating season, which results in a high variance of reproductive outcomes among males. Following this stressful period, plasmatic cortisol levels in male tuco-tucos vary seasonally and among individuals (Vera et al., 2011a, 2013). Inflammatory (cell-mediated) and humoral immune responses also show important inter-individual variance (Cutrera et al., 2010; Merlo et al., 2014a, 2014b, 2016, 2018a). To evaluate the relationships among personality and multiple physiological traits, we designed a series of experimental procedures using wild-caught individuals. We employed a combined approach that included behavioural tests and the measurement of physiological parameters: baseline cortisol levels and, in response to acute stress, testosterone levels, energy metabolism, and the inflammatory (cell-mediated response to phytohemagglutinin - PHA) and humoral immune response (in response to sheep blood cells -SRBC- as antigen). We expect covariation between behavioural and physiological traits in wild-caught males of *C. talarum*. Particularly, we expect bolder and/or active individuals to show: a) both higher energy metabolism and immune response; b) low baseline and reactivity of cortisol levels when faced with a stressful situation such as a simulated predator attack event; c) high testosterone levels, which can also affect immune response.

2. Materials and methods

2.1 Animal capture and housing conditions

We captured adult *C. talarum* males (body mass range: 120 -130g) using live-traps at the

south-eastern coastal grasslands of Argentina (37° 18' 26" S 57° 02' 30" W). We transported all animals to our laboratory using PVC tubes conditioned with bedding and grasses during an 80-min trip. There, each individual was housed in a plastic cage (42 x 34 x 26 cm) conditioned with wood shavings for bedding, a half terracotta flowerpot as shelter, and a wire-mesh top. Food was continuously available for individuals in their own home cage; an *ad libitum* diet consisting of fresh vegetables (sweet potatoes, catalogna chicory, corn, and mixed grasses) was provided daily, also securing water provision since *C. talarum* does not drink free water (as guidelines for maintenance of animals in research recommended; Cox et al., 2019). All individuals were maintained in the same room at $25 \pm 1^\circ\text{C}$ and natural photoperiod. .

2.2 Experimental design and general considerations

Animals were allowed to acclimatize to captive conditions for 7 days before the experimental tests began. The time schedule detailed in Table 1 provides the sequence of behavioural and physiological assessments. All subjects were used in all experimental procedures/tests to assess behaviours (activity, socioaversion, and boldness) and physiological parameters, i.e., metabolic rate, testosterone levels, cell-mediated and humoral immune responses, and stress response to a simulated predator attack. The time lapse between physiological samplings was long enough to avoid interfering with the next measure (Merlo et al., 2018b, 2014b; Schleich et al., 2015).

The experiments were carried out during the breeding season, from May to January (Busch et al., 1989), from 9 a.m. to 2 p.m since they present no regular activity pattern in field (Cutrera et al., 2006) or in captivity (Luna et al., 2000). Food provisioning and cage maintenance were carried out daily after testing procedures. As a result, the potential impacts of unpredictable feeding schedules on response variables are avoided (e.g., Ulyan et al., 2006). During physiological data recording and experimental trials, we used disposable

gloves. To ensure that no trace odours from previous trials remained, all equipment used during the study was washed with odourless glassware cleaner and tap water, wiped with 95% ethanol, and allowed to air dry. We used a total of 21 mature males that were subjected to only one test a day throughout the study. At the end of the experiments, all the animals were released to their sites of capture.

2.3 Behavioural measurements

The complete experimental design for personality assessment consisted of three tests: Open Field, Social Encounter test, and Open Field with predator odour (see details in (Fanjul and Zenuto, 2020), and 27 behaviours were recorded and classified according to the context of the test-novelty, risk, and presence of a conspecific, into four behavioural traits: boldness, exploration, activity and socioaversion (Réale et al., 2007). Males of *C. talarum* showed high variability in the expression of behaviours, with temporal (15 out of 27 behaviours; 55.55%) and context (3 out of 3 behaviours; 100%) consistency (Fanjul and Zenuto, 2020). For the present study, a subset of behaviours was selected, being the most informative of the observed personality traits. From the video recordings of each test, behaviours were manually documented using a hand stopwatch (Martin and Bateson, 1993). Between behavioural tests, animals were given at least a 1-day rest. An hour prior to each behavioural test, each subject was taken from its own home cage to a dark test Plexiglas cage (containing some soiled shavings from its own home cage; 45x 30x 30 cm), where it was allowed to habituate before the door was opened and the test began (Zenuto and Fanjul, 2002). Subjects were allowed to enter the test device at will, with a maximum waiting time of 30 min.

2.3.1 Activity

Activity was assessed by giving the animal the free choice to explore an Open Field (Réale et al., 2007). Each individual was introduced into the experimental home-cage that was

connected by a 10 cm tunnel to the Open Field measuring 1 m². The test was recorded from the first entry of the subject for 20 minutes by an overhead camera. We recorded the total distance that the subject travelled in the Open Field (quantified as the number of lines that the subject crossed; Brachetta et al., 2016, 2015) as a measure of activity (Réale et al., 2007).

2.3.2 *Socioaversion*

As tuco- tucos are solitary, the degree of social avoidance was measured by the number of times the subject investigated (sniffing, watching, or scratching the mesh) the presence of a same-sex conspecific in a Social Interaction test. Animals were habituated to an experimental home-cage as previously described. When the test began, the subjects were allowed to enter the experimental set up consisting of a neutral clear Perspex box in which a conspecific of the same sex is present in an adjoining chamber (20 x 10 x 10 cm) separated by a wire mesh. The test was recorded from the first entry of the subject and lasted 7 minutes by using a digital HD camera (Sony HDR-XR100).

2.3.3 *Boldness*

We employed an Open Field test with a predator stimulus. The odour of a domestic male cat was used as a scent source, as reported in previous physiological and behavioural studies involving antipredatory responses in *C. talarum* (Brachetta et al., 2015, 2014; Fanjul and Zenuto, 2020). A piece (6 x 6 cm) of cloth impregnated with fur odour obtained allowing a cat to rest on it for one week was placed in a Petri dish and covered with a wire mesh. Boldness was measured as the total time spent in the Open Field with the presence of predator odour sample. Each test was recorded, as mentioned earlier, and lasted 10 min.

2.4 *Metabolic rate*

Oxygen consumption was determined on day 14 by a computerized positive pressure open-flow respirometry system (Sable Systems, Las Vegas, USA). Subjects were individually placed in a chamber (460 ml) that received air at 600ml/min from a flow meter

(Side-Trak Sierra model 830/840, Sierra Instruments, USA). Before entering the chamber, air passed through a CO²-absorbent (self-indicating IQB®, IQB Laboratories, Argentina) and water scrubber (Drierite®, Hammond Drierite Co. Ltd., USA). Excurrent air from the chamber was sub-sampled at 110 ± 10 ml/ min and was passed through IQB® and Drierite®, before being analysed by an O₂ analyser (model FC-1B, Sable Systems, USA) every 1 s by Expedata PC program (Sable Systems, USA). Rates of oxygen consumption were calculated using the equation 4a of Withers (1977), $V_{\dot{O}_2} = FR(FiO_2 - FeO_2 / 1 - FiO_2)$, where FR is the flow rate through the system, FiO₂ and FeO₂ are the fractional O₂ concentration in the incurrent and the excurrent air, respectively (FiO₂ was 0.2095). Three measures of metabolic rate were used to analyse oxygen consumption: the resting metabolic rate (RMR, as the lowest 5-minute steady-state of the 90-minute trial), the average metabolic rate during the 90-minute trial (O₂Total), and the average metabolic rate during the first 10 min in the metabolic chamber (O₂First; Martins et al., 2011). Body mass (M) was measured using an electronic scale (model FX-3000, ± 0.01 g, A&D Company Limited, USA), whereas T_b was measured as rectal temperature with a YSI probe (model 93k73545-402) connected to a Cole-Parmer thermistor meter (model 8402-10 ± 0.1°C, Cole-Parmer Instrument Company, USA) after each measurement.

2.5 Testosterone dosages (T)

2.5.1 Blood sample collection and storage.

Blood samples (nearly 250 µl) were obtained within 3 min from the suborbital sinus after 20 seconds of halothane anaesthesia, using a syringe fitted with a flexible plastic tube which was connected to a heparinized micro capillary tube (Vera et al., 2008, 2011b, 2013, 2019). Subsequently, blood samples were centrifuged 15 min at 660 g, plasma was separated from cells and stored at -20 °C until analysis.

2.5.2 Testosterone levels

On day 16, a blood sample was obtained from each individual to measure plasmatic levels of testosterone (blood sample A, Table 1). Blood was sampled and plasma stored as detailed above (Section 2.5.1). Previous to testosterone quantification using the Testosterone Elisa kit (EIA-1559; DRG® International, Inc., USA), plasma samples were heated (56°C, 30 min) and diluted with zero standard (1:10) to eliminate the interference of plasma components and measure testosterone levels near the optimal range of the evaluation (i.e. 50% binding). Calculated intra assay CV% were (5.44 % for 15.29 ± 0.833 ng/mL, n=6 and 7.75 % for 70.24 ± 5.44 ng/mL, n=7) and inter assay CV% were (5.77 % for 14.947 ± 0.862 ng/mL, n=7 and 5.05 % for 2.61 ± 0.13 ng/mL n= 6). The testosterone EIA was used for the first time in the plasma of *C. talarum*, then we subjected them to a validation that included parallelism, accuracy and precision (see Appendix A for details) as reported previously for other steroid hormone assays in this species (Vera et al., 2019, 2013, 2011c).

2.6 Humoral immune response

Sheep red blood cells (SRBC) are used as a non-pathogenic antigen that triggers a T- and B-lymphocyte-dependent immune response (Bacon, 1992). The strength of the immune response against SRBC is considered indicative of resistance to extracellular infections as bacteria or macro-parasites (see Deerenberg et al., 1997). On day 16, subjects were weighed and injected intra-peritoneally (IP) with 10% suspension of SRBC (Sigma R3378, Sigma Chemical Co., St Louis, MO, USA, 1.5 µl per gram of animal mass) in PBS. Seven days after the first SRBC injection, and following the protocol of Cutrera and collaborators (2010), all subjects were weighed and injected again with the antigen as described above (booster injection). Fourteen days after the first injection, on day 29 of our experimental schedule, animals were weighted and we collected blood (200 µl) and the plasmatic fraction was obtained as explained above (Section 2.5.1). Before stored, plasma was heated at 56°C

for 30 min to inactivate the complement. Then, plasma was stored as explained above (Section 2.5.1), until used in the hemagglutination assay. Antibody production against SRBC, a measure of induced immunity, was assessed by titration using a hemagglutination assay as described in Cutrera et al. (2010).

2.7 Cortisol levels and stress response

On day 30, we obtained the plasmatic level of cortisol before (baseline cortisol levels, named as “Cb”), and after a simulated event of predation through immobilization, as performed in previous studies in the same species (e.g. Brachetta et al., 2014; Mastrangelo et al., 2009). Immobilization of an animal was carried out for 1 min by holding it tightly by hand, using a leather glove. Blood collection was done 24 h previous to the immobilization and an hour after it was finished, to record baseline and response plasmatic cortisol levels. The difference between these two measures of plasmatic cortisol is a measure of the plasmatic cortisol responsiveness to acute stress and we named it “Cr”.

Blood was sampled and plasma stored as detailed above (Section 2.5.1). Blood sampling did not take more than 3 min to guarantee that cortisol levels were affected by the blood collection process itself. Cortisol determinations were made using a DRG™ kit (Cortisol ELISA EIA-1887, solid-phase enzyme-linked immunosorbent assay). This cortisol assay is capable of measuring cortisol levels up to 800 ng/ml, the limit of detection is 2.5 ng/ml and intra- and inter-assay CVs were 7.3 and 12% (see validation in Vera et al., 2019).

2.8 Cell-mediated immune response

On day 35, a phytohemagglutinin (PHA) skin test was performed on all individuals (n=21) following the protocol used previously for *C. talarum* (Merlo et al., 2018a, 2018b, 2016, 2014a) as a measure of cell-mediated immunity. Briefly, animals were injected subcutaneously in the instep of the right hind foot with PHA (*Phaseolus vulgaris* PHA-Sigma L-8754, 3 mg/ml in PBS solution; 0.3 µl/g of body mass) and on the left hind foot

with sterile PBS as a control (0.3 $\mu\text{l/g}$ of body mass) using a 30G needle. Swelling measurement pre and 24 h post-injection was performed by the same researcher using a digital micrometer (Insize[®], Sao Paulo, Brazil). Swelling response in PHA or PBS-injected foot was calculated as the difference between pre-and post-injection thickness divided by initial foot thickness (Gouÿ de Bellocq et al., 2006; Merlo et al., 2014a).

2.9 Data analyses

We assessed the normality and homoscedasticity of each variable measured. When these assumptions were not met, we applied Box-Cox transformations as follows: Baseline Cortisol levels (theta= 0.001), Testosterone levels (theta= -1), SRBC (theta= 0.999), O₂Total (theta= -1.2), and O₂First (theta= 0.05). Every behavioural trait was tested for a) consistence using bivariate Spearman's rank cross-correlations (Sigmaplot 14; Systat Software Inc., Chicago, IL, USA) and b) possible effects of habituation, for which differences in each behaviour were assessed using mixed-effect model (fixed factor "Time" and the random factor "Individual"; "nlme" package (Linear and Nonlinear Mixed Effects Models, (Pihneiro et al., 2019) of the "R" software (R Core Team, 2019)). Temporal consistency of behaviours was assessed during a second test (day 43). All behaviours selected showed temporal consistency (activity: Spearman Correlation $r=0.56$, $p=0.008$; socioaversion $r= 0.533$, $p= 0.012$, boldness $r= 0.442$, $p= 0.044$;) and no habituation effect (Mixed Models $F= 0.391$, $p= 0.845$; $F= 2.52900$, $p= 0.127$, $F= 1.3099$, $p= 0.266$) (Fanjul and Zenuto, 2020). In the analysis, the mean values of recorded behaviours during both tests were used. Then, for each behaviour, the average of both measurements (day 7 and day 43) was used as the value for each individual.

We conducted a preliminary analysis to examine the relationships between personality and physiological traits using Pearson correlations (Zar, 2010). Structural Equation Modeling (SEM; Grace, 2006) was employed to identify direct and indirect effects

of physiological variables on three personality traits of *C. talarum* (activity, socioaversion and boldness), which were considered as “endogenous” variables. We utilized a correlation matrix and estimated parameters through Maximum Likelihood. The overall model, examined the associations between each physiological variable and the behavioural variables, following a logical and biologically (background) or experimentally (correlation finding) based arrangement following (Boyer et al., 2010), for testing cause-effect relationships (Scheiner et al., 2000; Shipley, 2004). Physiological variables with correlation values ≥ 0.20 , as well as predicted relationships based on existing knowledge were included in the model. To ensure stability of path coefficients variables with collinearity above 0.8 were excluded. As the variables involved had different units, standardized coefficients were used for comparison. Model fit was assessed using a Chi-square test, Comparative Fit Index (CFI, Hu and Bentler, 1999), the Tucker Lewis Index (TLI; Tucker and Lewis, 1973), and the Root Mean Square Errors of Approximation (RMSEA) (Browne and Cudeck, 1992). Multiple indexes were employed for a more accurated assessment (Lambert et al., 2013). CFI and TLI values closer to 1 indicate better fit with acceptable values being above 0.9. RMSEA and SRMR values closer to 0 indicate better fit, with values above 0.8 considered acceptable (Hu and Bentler, 1999). Parameter estimations and model fit tests were performed with the *lavaan* libraries (Rosseel, 2012) using R (R Core Team, 2013) inside the RStudio (RStudio Team, 2015).

Given our small sample size in relation to the number of paths in a model, we evaluated model stability using a bootstrapping procedure (Ievers-Landis et al., 2011). Then, if bootstrapped data sample weights did not differ from the mean of the weights of actual data, the model was considered as unbiased (Ievers-Landis et al., 2011). To achieve that, we compared each regression path in the model and the pairwise differences for each bootstrap interaction with the original model path. When zero fell inside the 95% confidence interval

of the distribution of resampled differences, it was considered that the estimator did not differ between bootstrapped and original models (Canepuccia et al., 2018; Manly, 2018). The bootstrap produced 7500 samples for each regression path of the model. Bootstrapping procedures and iterations were performed the package ‘*asbio*’ (Aho, 2014) using R (R Core Team, 2013) inside RStudio (RStudio Team, 2015).

2.10 Ethical note

Animals’ welfare was ensured in all instances, as we followed: ASAB/ABS (“Guidelines for the treatment of animals in behavioural research and teaching,” 2020), and ASM guidelines (Sikes and the Animal Care and Use Committee of the American Society of Mammalogists, 2016). All procedures were approved by the IACUC (CICUAL, FCEyN, UNMdP; Protocol approval number: RD 467-17).

3. Results

Personality traits correlated to a few of the measured physiological variables, as shown in Table 2. Activity was not significantly correlated to any of the variables but showed a slightly negative association with oxygen consumption (O_2 Total: $p= 0.078$; O_2 F: $p= 0.08$). Socioaversion showed a non-significant higher r with the O_2 First ($p= 0.091$). Boldness, on the other hand, showed a significant positive association with baseline cortisol levels ($p= 0.009$), a negative association with cortisol reactivity to a stressful situation (Cr, $p= 0.037$), but no association with oxygen consumption ($p= 0.087$). Relationships among physiological variables were also found. The plasmatic baseline cortisol levels (Cb) increased as cortisol reactivity decreased (Cr $p= 0.006$) and the O_2 T increased ($p= 0.007$). Finally, as expected, O_2 T correlated with RMR ($p= 0.003$) and O_2 F ($p= 0.029$). Despite not being significant ($p= 0.117$), there is a slight relationship between testosterone (T) and the humoral immune response (SRBC).

After 7500 iterations all paths were found to be stable in the comparison with the parameter of the tested model. The overall model proposed was accepted (Maximum Likelihood $\chi^2_{21}=4.651$, $p=0.864$) as the correlation matrix did not differ from that expected. The index of fit RMSEA indicated a good fit of the model (RMSEA<0.0001). CFI and TLI values were above 0.9, being 1 and 1.335, respectively, also indicating a good fit of the model. Moreover, there were no differences among standardized regression coefficients from the original model and the bootstrapped ones, suggesting that model parameters are stable. The accepted model (Figure 1, see Appendix B for the whole model) explained about 51.9% of the variance of activity, 30.3% of socioaversion, and 34.7% of boldness score. Activity increased together with O₂ Total ($p=0.002$) but showed a negative association with the cell-mediated immune response ($p=0.029$) and with baseline cortisol levels ($p=0.055$). Likewise, a non-significant trend of increase was detected together with humoral immunity ($p=0.124$, $\beta=0.280$), which is significantly associated with testosterone levels ($p=0.015$). Socioaversion did not show any significant path. Only a slightly negative association with baseline cortisol level ($p=0.145$, $\beta=-0.369$) should be noted. Boldness was positively associated with testosterone levels ($p=0.051$) and oxygen consumption (O₂Total, $p=0.033$). Also, baseline cortisol levels decreased as bold behaviour increased ($p=0.073$). In addition, is a non-significant trend was detected, increasing boldness along with humoral immunity ($p=0.138$, $\beta=0.310$). Furthermore, given the association of testosterone with humoral immunity, an indirect and negative effect can be observed on both activity levels and boldness. Cell-mediated immunity increases together with oxygen consumption (O₂ Total, $p=0.000$). Finally, as expected, measures of oxygen consumption were related (O₂ Total to RMR, $p=0.002$, and O₂ First ($p=0.000$). Baseline cortisol levels were found to be negatively associated with cortisol reactivity ($p=0.024$).

4. Discussion

Our results support the existence of relationships between behavioural traits and physiological variables in male *C. talarum*. The obtained model explained a modest (about 50 %) portion of the variance of personality variables, mainly activity and boldness, as found in other studies assessing the same state variables and personality traits (Niemelä and Dingemanse, 2018). More active and bolder males showed higher total oxygen consumption (O_2 Total); specifically, both RMR, a measure related to organ size and consumption, and O_2 First, a measure related to the coping style of an individual in a given situation, were elevated in more active males. Also, while more active and bolder subjects presented lower baseline cortisol levels, there was no relation to stress-induced responses. Immune responses were related to activity levels. This work constitutes one of the few contributions involving several measures of behaviour reflecting the main dimensions of personality, such as activity, sociaversion, and boldness (Fanjul and Zenuto, 2020) and a comprehensive range of physiological measures of the individual state and health in mammals.

Given that average oxygen consumption (O_2 Total) was higher for more active and bolder individuals, our results support performance model predictions of metabolic rate and activity levels (Biro and Stamps, 2010; Careau et al., 2008; Réale et al., 2010). Research supporting such association, however, is still mixed (Niemelä and Dingemanse, 2018) and there is no clear consensus about its causes (Careau et al., 2008; Réale et al., 2010). For tuco-tucos, and in accordance with those results, the boldness of males was positively correlated to oxygen consumption. Locomotor activity is an energy-consuming behaviour (Schmitz, 2005), that is related to territory patrolling and defence (Biro and Stamps, 2010; Finerty et al., 2009; Ros et al., 2006) and also to the monopolisation of the resources within these territories. For a subterranean species like *C. talarum*, the permanent activity of digging and patrolling is key to defending and retaining a territory (Fanjul and Zenuto, 2020; Luna and

Antinuchi, 2006). Thus, relationships between metabolism and these personality traits, point out the importance of activity and the associated supporting metabolic machinery for male tuco-tucos (Fanjul et al., 2018; Fanjul and Zenuto, 2017) allowing them to maintain and defend the territory, obtain food, avoid predators, and monopolise reproductive females. All these activities contribute significantly to survival and differential reproductive output.

Glucocorticoid levels were directly related to the boldness and activity measures of personality, with baseline cortisol levels being lower in bolder and more active individuals. Despite initial examination of our data showed that stress-induced cortisol levels (cortisol reactivity, Cr) were negatively related to boldness, path analyses revealed no relationship, indicating that the first correlation was in fact an indirect effect through baseline cortisol level, now cleared. A proactive coping style, characterised by bold, active individuals, with low HPA-axis activity (both in baseline and stress-induced GC levels), is expected in animals living in stable environments (Koolhaas et al., 2011, 1999; Koolhaas and Van Reenen, 2016; Korte et al., 2005). The subterranean habitat provides environmental stability (e.g., reduced thermal fluctuations) and protection against predators (Nevo, 1999). Tuco-tucos occupy these stable and protected habitats, but are vulnerable to aerial and terrestrial predators while foraging (owls, foxes, wild cats, and domestic dogs and cats in suburban areas (Antinuchi and Busch, 1992; Busch et al., 2000; Canepuccia, 2005; Vassallo et al., 1994; C.E. Schleich, personal communication). Hence, leaving the burrow to obtain food (i.e., aerial plant parts) would require a certain amount of boldness (Fanjul and Zenuto, 2020). Perceived predation risk influences foraging decisions, with animals preferring to feed in safer areas where there is no predator odours and increasing time lapses between feeding excursions after a predatory experience event (Brachetta et al., 2018). Bold and active individuals face greater risks moving on the surface, but avoid the high energy costs of digging new tunnels or extending others (Luna et al., 2002) using plant cues present in

the soil to direct digging towards plant patches of high nutritional quality (Schleich and Zenuto, 2010). The experimental exposure to predatory stress triggered increases in plasma cortisol and the appearance of anxiety states. Nonetheless, these physiological and behavioural responses, of moderate magnitude, were in accordance with the predation pressure alleviated by the use of the subterranean environment (Brachetta et al., 2020).

Links between behaviour and physiological stress responses were predicted in earlier studies, mostly based on artificial selection experiments (unidimensional model; Koolhaas et al., 1999). Later studies conducted in wild populations revealed mixed results and weak support for that model (Raulo and Dantzer, 2018; Vobrúbová et al., 2021; Westrick et al., 2019). These discrepancies led to the development of a “two-tier model” that instead, predicts the independence of both variables (Koolhaas et al., 2010; Van Reenen et al., 2005). Our study found a negative correlation between behaviour and baseline cortisol levels as predicted by (Koolhaas et al., 1999), but there was no association with stress-induced cortisol levels. Similar relationships have been found for other species of mammals, such as the Belding’s ground squirrels, the Plateau pikas, the wild eastern chipmunks (only females), and the bank voles (Clary et al., 2014; Mazza et al., 2019; Montiglio et al., 2012; Qu et al., 2018). Among the research conducted to date, relationships between behaviour and HPA reactivity are diverse, with some studies supporting the unidimensional model, some others showing a correlational association but with a different direction from that predicted, and some showing no relation (see Raulo and Dantzer, 2018; Westrick et al., 2019). For example, in the North American red squirrel, behavioural and physiological responses would be independent and uncorrelated traits, supporting the idea that hormonal pleiotropy is not the basis of a proactive-reactive continuum of coping styles in this species (Westrick et al., 2019). Processes associated with stress response, often involving the increase of GC levels, have been suggested as one of the endocrine pathways not only affecting personality

variation but also immune response (Dosmann et al., 2015; Ellis et al., 2006). However, in our study, we found no relation between the magnitude of the immune response and either baseline or stress response levels of cortisol. Androgens, and more specifically, testosterone, are the other endocrine path proposed as an endocrine effector involved in the variation of personalities and immune responses (Ezenwa et al., 2012; Monceau et al., 2017; O'Brien et al., 2018). In this study, testosterone levels in male tuco-tucos increased with boldness and more marginally with activity levels, although this later relationship was not significant. High variability in testosterone levels may be related to aggression and dominance in tuco-tucos (Vera et al., 2013; Zenuto et al., 2002). Our data show that the link between the personality components of male tuco-tucos, testosterone, and immune response is complex. We found that testosterone affected boldness and, indirectly, activity through the humoral immune response. A negative path denoted that testosterone was higher in males that showed a lower humoral response. Several hypotheses have been proposed to explain how this suppressive link works. First, the immunocompetence handicap hypothesis (ICHH) proposed that testosterone may suppress the immune response, imposing a health cost, so that males that still achieve healthy and desirable characteristics or performances would show an honest signal of their quality as mates (Folstad and Karter, 1992). A second hypothesis agrees with the ICHH that there is a trade-off between immunocompetence and sexual signalling but suggests it is rooted in the effect of immune activation on testosterone (Boonekamp et al., 2008). This could be the result of specific chemical signals with a suppressive effect on the HPG (Hypothalamic-Pituitary-Gonadal) axis or limitations in resource allocation, i.e., the energetic costs of mounting an immune response prevent the production of testosterone (Burness et al., 2010; Demas and Nelson, 2012). A third alternative hypothesis is that testosterone and immunity do not affect each other directly but through an immunosuppressive mediator, such as GCs (stress-linked ICHH; Evans et al.,

2000). Although our experimental design did not allow us to discern between these hypotheses, we can suggest that cortisol does not seem to be involved in either testosterone or the immune responses measured here.

Our results indicate that, contrary to our expectations, the cell-mediated immune response of male tuco-tucos is directly and negatively related to activity levels while it is positively related to mean oxygen consumption. Although the relationship between humoral response and behaviour was not significant, the trend was slightly higher in more active and bold males. According to Niemelä and Dingemanse (2018), relationships between personality and immunity are expected. In this regard, the pathogen defence optimisation hypothesis (PDOH; Zylberberg et al., 2013) suggested that individuals may benefit from trading off investment on costly behaviours (such as modulating activity or exploration levels) and immune defences. Consistent with the theoretical background, more active and bolder individuals would rely on a fast and low-cost immune response such as the inflammatory immune arm. Contrary to these expectations, we find that more active tuco-tucos show a lower cell-mediated response, although this immune response was higher in individuals with higher oxygen consumption. The local inflammatory response is important for these rodents as wounds could be caused while digging underground and, particularly in males, during aggressive territorial contests (Zenuto et al., 2002). On the contrary, a tendency was detected to relate humoral immunity to the more active and bolder individuals. However, we found no relationship between the magnitude of this response and the oxygen consumption of individuals. Considering the perspective of life-history theory, proposed by (Lee, 2006), slow-living species are expected to rely more on adaptive immune responses since they provide memory, which is important for animals that suffer repeated infections with the same pathogen throughout their lives. Tuco-tucos, compared to other rodent species, show a slow pace of life (longevity: 2 years, late maturity condition: 6 and 9 months

for females and males, respectively; (Busch et al., 1989; Malizia and Busch, 1997) and face low levels of parasite exposure, characteristic of the subterranean habitat (Rossin and Malizia, 2002). High prevalence but low diversity of pathogen infections may facilitate innate immunity to reduce investment towards more expensive adaptive immunity, as has been proposed in subterranean rodents in general (Novikov et al., 2016) and *C. talarum* in particular (Cutrera et al., 2022). However, parasite burden as well as the probability of encountering novel pathogens may vary spatially and among individuals (Novikov et al., 2016; Voigt et al., 2020). For example, dispersed individuals of red-backed voles showed more robust immune responses than territorial animals (Novikov et al., 2010), which goes in line with our findings that humoral immunity seems to be stronger in more active and bolder tuco-tucos. Relationships between behaviour and immune response may differ depending on the environment (Demas and Carlton, 2015). This dependency should be considered when wild-caught animals are kept captive for behavioural and physiological assessments. Previous studies have reported that proactive individuals showed a stronger immune response when the effects of GCs were experimentally eliminated (Belding's ground squirrels and pigs, (Dosmann et al., 2015; Hessing et al., 1995), supporting the idea that proactive individuals are more prone to chronic stress responses leading to immune suppression (Koolhaas, 2008). However, in our study, in which tuco-tucos were held captive for 45 days, there was no evidence that stress responses related to coping styles explained differences in immunity among individuals. Another explanation for our findings may be related to captive conditions, where animals are maintained in plastic boxes (where no digging is possible, thermoneutrality is secured, and *ad libitum* feeding is provided), which may lead to an energy surplus that can be allocated to immune function. Although an energy-based trade-off between behaviour, endocrine (glucocorticoids or testosterone), and immune responses is proposed (Burness et al., 2010; Hõrak et al., 2003), the patterns are far from

being simple or unidimensional, requiring further research to understand them in the light of life history, the ecology of the species and the experimental conditions.

In conclusion, the present study supports some of the relationships predicted by coping styles strategies (Koolhaas et al., 1999). The polygynous mating system imposes particular behavioural and physiological responses that allow for maximising reproductive success (Hämäläinen et al., 2018). In this context, a high variance is expected among males, where a few of them, showing better performance, monopolise reproductive activity. In the case of male tuco-tucos, this would imply achieving territorial dominance, securing food resources, shelter from predators, and access to females. So, the interrelationships of behaviour and physiological traits seem to support variability in this direction. As we found in this study, some relationships follow the selective pressures of the species, specifically, those related to the occupation of the underground environment, while others may be related to sex-specific selective pressures.

CRedit authorship contribution statement

Maria Sol Fanjul, MS: Conceptualization, Methodology, Investigation, Formal analysis, Funding acquisition, Writing - original draft; **Ana P. Cutrera:** Investigation, Funding acquisition, Writing - review & editing. **Facundo Luna:** Investigation, Funding acquisition, Writing - review & editing. **Roxana R Zenuto:** Methodology, Investigation, Funding acquisition; Project administration; Supervision; Biochemical test validation; Writing - review & editing.

Declarations of interest

None

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Table 1. Timing of experimental procedures conducted in male *Ctenomys talarum* (n= 21) from day of capture to their release in the field. First week (Week 0) corresponds to the acclimatization period. Abbrev.: resting metabolic rate (RMR), sheep red blood cells (SRBC), phytohemagglutinin (PHA), and phosphate buffer solution (PBS).

Time		Trait measured	Experimental procedure
Week	Day		
1	8	Activity	Time spent in motion (e.g. running or walking) in the Open field test
	9	Socioaversion	Times exposing their back to another male in the test of Social interaction with a conspecific
	10	Boldness	Time spent investigating predator odour in a Open field with predator odour cues test
2	14	Metabolism	RMR and metabolic rate variability
	16	Testosterone levels	Blood collection (A) to measure testosterone
		Humoral immunity	Injection of SRBC first dose
3	22	Humoral immunity	Injection of SRBC second dose
4	29	Humoral immunity	Blood collection (B) to measure the titer of SRBC antibodies and baseline cortisol level
	30	Stress response	Immobilization of individuals resembling a predator attack event
		Stress response	An hour later, blood collection (C) to measure the level of cortisol post treatment.
	35	Cell-mediated immunity	Foot thick measurement. PHA and PBS (control) injection.
	36	Cell-mediated immunity	Foot thick measurement post treatment
6	43	Activity	Time spent walking in the Open field
	44	Socioaversion	Times exposing their back in the Social Interaction test with a conspecific
	45	Boldness	Time spent investigating the predator odour in the Open field with predator odour test

Table 2. Pearson's correlation matrix showing r values (above) and P values (below) of personality traits (Activity levels, Socioaversion, and Boldness) and physiological variables of *Ctenomys talarum* males (Cb, baseline cortisol; Cr cortisol response after acute stress; T, testosterone; PHA, cell-mediated immune response; SRBC, Humoral response; RMR resting metabolic rate; O₂Total, total oxygen consumption; O₂First, oxygen consumption during the first ten minutes of measurement). See method section for further details.

	Activity	Socioaversion	Boldness	C _b	C _r	T	PHA	SRBC	RMR	O ₂ Total	O ₂ First
Activity		0.519 (0.015)	0.753 (0.000)	-0.089 (0.702)	0.246 (0.283)	0.110 (0.636)	0.243 (0.288)	-0.206 (0.371)	-0.165 (0.474)	-0.392 (0.078)	-0.387 (0.088)
Socioaversion			0.455 (0.038)	-0.197 (0.392)	0.034 (0.882)	-0.182 (0.429)	-0.046 (0.841)	0.192 (0.403)	-0.024 (0.915)	0.159 (0.492)	0.377 (0.091)
Boldness				0.549 (0.009)	-0.457 (0.037)	-0.218 (0.343)	0.250 (0.275)	-0.023 (0.920)	0.228 (0.320)	0.382 (0.087)	-0.047 (0.840)
C_b					0.578 (0.006)	-0.005 (0.983)	-0.028 (0.906)	-0.181 (0.433)	0.369 (0.100)	0.564 (0.007)	0.173 (0.454)
C_r						0.285 (0.210)	0.175 (0.447)	-0.056 (0.808)	-0.265 (0.245)	-0.387 (0.087)	-0.196 (0.394)

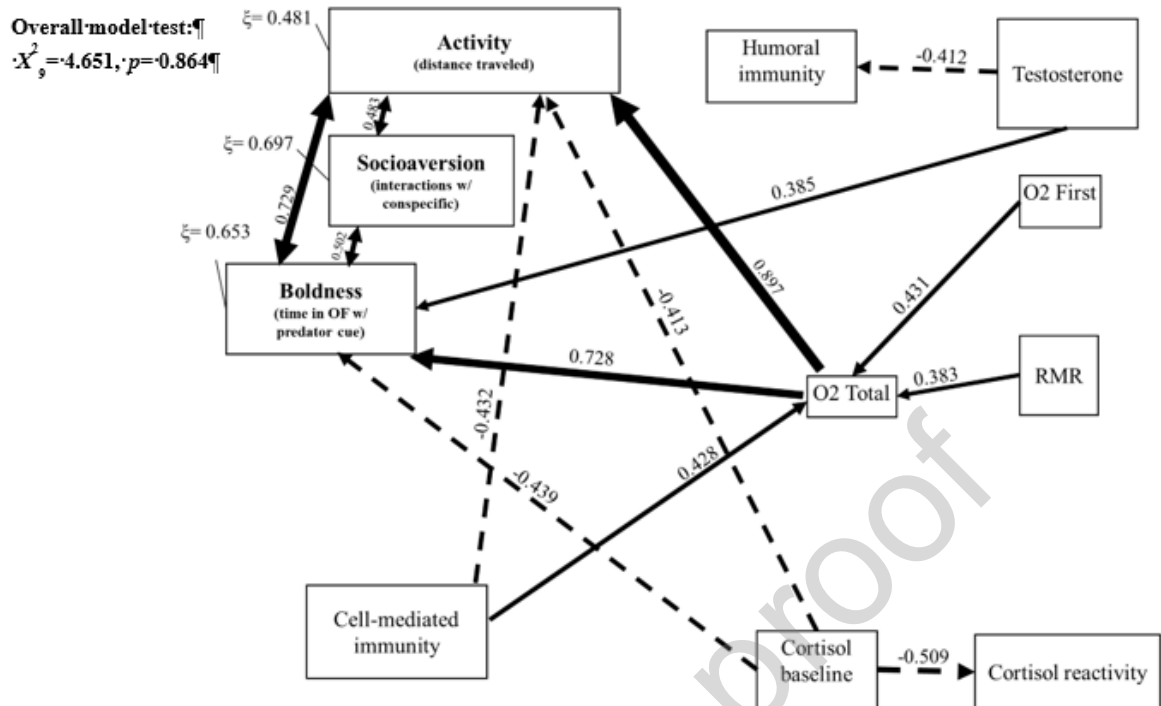


Figure 1. Path diagram explaining the behavioural traits of personality in male *Ctenomys talarum*: activity, socioaversion, and boldness and its potential direct or indirect relationships in the cause-effect linkages between physiological variables. All potential causal effects (regression) are shown. Above the arrows, standardized path coefficients are provided for each causal relationship. The error terms are provided [$\xi = 1 - (\beta_1^2 + \dots + \beta_j^2)^{1/2}$] for the exogenous variables. The thickness of the arrow lines was pondered to denote the strength of the relationship. The presented relationships correspond to $p \leq 0.1$, while whole model is presented in Appendix B. Full lines indicate positive effects while dashed ones indicate negative effects. Abbrev.: Total consumption of oxygen (O2 Total), First 10 minutes oxygen consumption (O2 First), and resting metabolic rate (RMR).

Highlights

- Assessed the links between personality and a diverse set of physiological variables
- More active individuals showed higher oxygen consumption.
- Bold and active males had lower baseline cortisol but no stress-induced cortisol.
- Cell-mediated immune response was related to activity levels.
- Testosterone was only related to boldness.