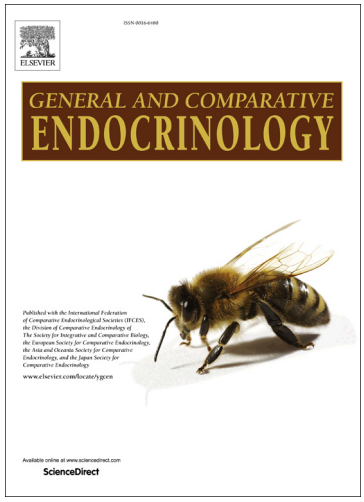

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Different regulation of cortisol and corticosterone in the subterranean rodent *Ctenomys talarum*: responses to dexamethasone, angiotensin II, potassium, and diet

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Abstract

When harmful environmental stimuli occur, glucocorticoids (GCs), cortisol and corticosterone are currently used to evaluate stress status in vertebrates, since their secretions are primarily associated to an increased activity of the hypothalamic-pituitary-adrenal (HPA) axis. To advance in our comprehension about GCs regulation, we evaluated the subterranean rodent *Ctenomys talarum* to assess cortisol and corticosterone response to (1) the negative feedback of the HPA axis using the dexamethasone (DEX) suppression test, (2) angiotensin II (Ang II), (3) potassium (K^+) intake, and (4) different diets (vegetables, grasses, acute fasting). Concomitantly, several indicators of individual condition (body mass, neutrophil to lymphocyte ratio, blood glucose, triglycerides and hematocrit) were measured for diet treatments. Results confirm the effect of DEX on cortisol and corticosterone in recently captured animals in the field but not on corticosterone in captive animals. Data suggest that Ang II is capable of stimulating corticosterone, but not cortisol, secretion. Neither cortisol nor corticosterone were responsive to K^+ intake. Cortisol levels increased in animals fed with grasses in comparison to those fed with vegetables while corticosterone levels were unaffected by diet type. Moreover, only cortisol responded to fasting. Overall, these results confirm that cortisol and corticosterone are not interchangeable hormones in *C. talarum.*

Key words: angiotensin II, corticosterone, cortisol, diet, glucocorticoids, hypothalamic-pituitary-adrenal axis, potassium.

1. Introduction

Glucocorticoids (GCs; cortisol and corticosterone) are currently used to evaluate stress levels in natural and captive populations of vertebrates. It is widely described that harmful environmental stimuli (stressors) stimulate the activity of the hypothalamic-pituitary-adrenal (HPA) axis, triggering increased secretion of GCs from the adrenal glands within a few minutes (Boonstra, 2005). Accordingly, increased GCs in plasma (cortisol or corticosterone depending on which hormone appears in higher levels), faeces, or other matrices have been traditionally considered to indicate stress or poor physiological condition (e.g., Mormède et al., 2007). However, there is evidence suggesting that these hormones show differentiation in their physiological roles and endocrine regulation, depending on the species (zebra finches: Schmidt et al., 2008; Schmidt et al., 2010; Syriam hamsters: Solomon et al., 2011; tuco-tucos: Vera et al., 2011a; Vera et al., 2012). Furthermore, independence between cortisol and corticosterone concentrations in several cortisol-dominant mammal species was found, indicating that even in low concentrations, corticosterone presents potential signaling differentiation (Koren et al., 2012). This is a major issue about GC actions that still remains unclear even though Boswel et al. (1994) and Kenagy and Place (2000) indicated that it is worth of clarification. However, studies reporting both cortisol and corticosterone levels from the same samples are scarce and limited to few species. Moreover, the validity of GC measures as indicators of chronic stress has been recently questioned in wild animals (Dickens and Romero, 2013; Vera et al., 2017). For instance, it is known that GC levels frequently increase due to reasons that do not imply a physiological stress response (e.g., seasonal baseline variations coupled to life-history processes such as reproduction; Romero, 2002; Landys et al., 2006). Moreover, in the existing literature, there is not an empirical consensus on whether chronic stressors produce increased, decreased or do not produce changes in GC levels (Dickens and Romero, 2013). These results, among others, challenge the common assumption that the stressed animals are those with higher GC levels.

In the last two decades, the roles of GCs in mineral-water balance and their regulation by the renin-angiotensin system (RAS) have received very little attention in the field of ecological endocrinology (except for studies in fish, McCormick, 2011; Vera et al., 2017). Nonetheless, evidence from the biomedical literature and studies about domestic animals indicate that cortisol and corticosterone exert mineralotropic actions by acting both systemically and centrally (Agarwal and Mirshahi, 1999; de Kloet and Joëls, 2017; de Kloet et al., 2000; Joëls and de Kloet, 2017; Thunhorst et al., 2007; Shelat et al., 1999; Liu et al.,

2010). For example, GCs can increase the binding of angiotensin and aldosterone to their respective receptors in the brain and potentiate their effects on water drinking and salt appetite (Ganesan and Summers, 1989; Joëls and de Kloet, 2017; Ma et al., 1993; Shelat et al., 1999, Thunhorst et al., 2007). Systemically, GCs increase glomerular filtration rate augmenting urine volume and Na^+ excretion, thus promoting further ingestion of water and Na^+ (Thunhorst et al., 2007). In addition, angiotensin II (the main biologically-active hormone of the renin-angiotensin system, Ang II) has been proved to regulate GC secretion from *zona fasciculata* of the adrenal glands (Rábano et al., 2004; Norris and Carr, 2013). Considering that the above-mentioned issues are poorly represented in current studies about GC dynamics (Vera et al., 2017), this area still represents a knowledge-gap in the study of GC action in wild animals.

GCs are also important hormones regulating energy balance (Sapolsky et al., 2000); cortisol and corticosterone produce mobilization of energy reserves and increased secretion is considered to indicate high energy demands (Boonstra, 2005). Also, GCs have powerful orexigenic actions in mammals and other vertebrates (Dallman et al., 1995; Landys et al., 2004; Pecoraro et al., 2004; Crespi and Denver, 2005; la Fleur, 2006; Uchoa, 2014). This dual effect of GCs occurs because their actions in peripheral tissues are mainly catabolic (Dallman et al. 1995), while they are mostly stimulatory to energy acquisition in the central nervous system (i.e., stimulate foraging behaviour and food intake, Landys et al., 2004; la Fleur 2006; Dallman et al., 2007). The roles of GCs in the regulation of energy balance, including both mobilization of energy reserves and the stimulation of food intake, suggest that the diet could be a strong factor regulating GC concentrations in natural populations (e.g., Reeder et al., 2004). Noteworthy, the above-mentioned actions suggest that quantitative, but also qualitative changes in the diet might trigger variations in circulating GC levels, even in contexts that do not necessarily imply a condition of physiological stress. In other words, changes in the quantity and quality of the diet might fall within the reaction norm of the individuals, but still be able to significantly alter circulating baseline GC levels. All the same, food restriction above certain threshold levels may represent a stressful condition (Garcia-Belenguer et al., 1993). Even moderate food restriction was proved to impact on adrenocortical function – inducing increases in circulating ACTH– according to the timing of food availability (Belda et al., 2005). Indeed, food deprivation for several days induced increases in cortisol levels and changes in immune function (Nakamura et al., 1990).

The subterranean rodent *Ctenomys talarum* (talas tuco-tuco) is an interesting model to study GC regulation since previous studies revealed the following: (1) both cortisol and

corticosterone circulate in the plasma, (2) cortisol is responsive to the factors that typically regulate GC concentrations (acute stressors and adrenocorticotropic hormone, ACTH), but corticosterone is not (Vera et al., 2011a; Vera et al., 2012; Vera et al., 2013). Cortisol and corticosterone differ in their patterns of variation in free-living tuco-tucos, suggesting differences in their endogenous regulation and affectation by environmental stimuli. Both sexes show higher cortisol levels during the reproductive season while corticosterone levels are similar in different seasons (Vera et al., 2011a; Vera et al., 2013). In addition, plasmatic levels of both hormones fluctuate between years -mainly for corticosterone- and as a result, the cortisol: corticosterone ratio in plasma show an important temporal variation (Vera et al., 2011a; Vera et al., 2012; Vera et al., 2013). In field conditions, cortisol levels are considerable higher in females but there are no sexual differences for corticosterone (Vera et al., 2012). Once in the laboratory, cortisol and corticosterone levels decrease considerably. For cortisol, this is more notorious in females, in such a way that sexual differences are sometimes cancel out (Vera et al., 2012), depending on their initial values. Even though this effect of captivity, males respond similarly to acute stressors and both sexes respond to ACTH with increments in cortisol but not in corticosterone. Cortisol, but not corticosterone, responds to acute stressors in both sexes in field conditions (Vera et al., 2011a; Vera et al., 2012; Vera et al. 2013).

The general goal of this study is to advance in our understanding of the mechanisms that underlie the presence of both cortisol and corticosterone in our model. Particularly, we aimed to evaluate whether cortisol and/or corticosterone are (1) responsive to the negative feedback of the HPA axis, (2) regulated by Ang II and (3) responsive to the intake of potassium (K^+). In addition, we aimed to evaluate the responses of cortisol and corticosterone to different diets concomitantly with the determination of other indicators of a general physiological condition to infer to what extent variations in the levels of these hormones reflect changes in the stress status of the animals or represent allostatic responses within the reaction norm of the individuals. We hypothesize that corticosterone presents a differential regulation by the HPA axis in this species and is subjected to positive regulation by Ang II and K^+ (accomplishing a mineralocorticoid role). In addition, we hypothesize that cortisol and corticosterone differentiate in their responses to different diets in captivity. Particularly, we predict that any diet associated with nutritional and/or energetic stress would increase cortisol, but not corticosterone levels. Also, we hypothesize that corticosterone levels could show variation between diets that do not impose a chronic stressor, reflecting physiological adjustments in water and/or ionic balance.

2. Methods

2. 1 Study model

C. talarum is a medium-sized rodent (adults are 120–220 g) that inhabits individual gallery systems parallel to the surface in southern parts of South America (Reig et al., 1990; Antinuchi and Busch, 1992). It is a solitary and highly-territorial species and individuals do not share burrows except in the case of females and their offspring until dispersal and when mating occurs (Busch et al., 1989). The tuco-tucos perform most of their activities inside their burrows, but they venture away short distances to collect vegetation that they later eat belowground. They are generalist herbivores, mainly consuming aerial vegetative parts of almost all the plant species available in the grassland, including grasses, perennials and forbs. In addition, individuals also consume significant amounts of reproductive structures (which are rich in proteins) and, in a lesser extent, below-ground plant parts (Comparatore et al., 1995).

2.2 Study population and capture of animals

The tuco-tucos were trapped in Mar de Cobo (Buenos Aires Province, Argentina; 37º45'S, 57º26'W) during March 2014 (experiment in section 2.3), May-August 2014 (experiment in section 2.6) and April-May 2015 (experiments in sections 2.4 and 2.5) by means of live-capture wire mesh traps used in previous studies (Vera et al., 2011a; Vera et al., 2012). Once in the laboratory, animals were housed in individual plastic cages (25 cm x 32 cm x 42 cm) with wood shavings as bedding and fed *ad libitum* with a diet consisting of lettuce, chicory, sweet potatoes and sunflower seeds, except for the special diet treatments described in section 2.6. *C. talarum* does not drink free water and obtain all the water from the vegetation they consume. Photoperiod and temperature were automatically controlled (12:12 L:D; 24 ± 1°C). The animals remained under these conditions for 10 days before their participation in the negative feedback experiment. Previous studies reported high N: L ratios in individuals of *C. talarum* once they arrived in the laboratory; but after 10 days of acclimation, N: L ratios were similar to those found in the wild (Vera et al. 2008). Individuals used in section 2.6, began with their respective diets immediately after their arrival in the laboratory. In this study, each individual participated in only one experiment (control or treatment group).

The protocols employed in the present study were agreed by the Universidad Nacional de Mar del Plata's committee for the care and use of laboratory animals (CICUAL 2555-03-14). We adhere to the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences (CIOMS) and the International Council for Laboratory Animal Science (ICLAS).

2.3 Responses of cortisol and corticosterone to dexametasone: negative feedback

We performed a dexametasone (DEX) suppression test in a group of captive animals to assess how cortisol and corticosterone respond to the negative feedback of the HPA axis. This test has been extensively used for comparative purposes in free-living vertebrates (e.g., Boonstra and Mccoll, 2000; Bauer et al., 2014, Rich and Romero, 2005; Romero et al., 2008). DEX is a synthetic GC agonist that inhibits the release of ACTH from the pituitary gland and consequently, produces a decrease in endogenous GC levels. Evidence was also provided as regards negative feedback effects of DEX at the hypothalamic level (Feldmand and Weidenberg, 2002). For the present study, the goal of addressing the negative feedback was twofold, as it was also required to optimize the protocols involving the administration of Ang II and K^+ (see sections 2.4 and 2.5). After the acclimation period, the animals were weighed using a precision balance to calculate the required dose of DEX for each individual. Within 3 minutes of handling, a baseline blood sample (~ 300 µL) was obtained from each animal to avoid the influence of the manipulation on hormone levels (Romero, 2002). Subsequently, the individuals were given an intraperitoneal injection of DEX (Decadron®: dexamethasone sodium phosphate, 4 mg/mL). Doses were 1 mg/Kg (n = 9, 8 females, 1 male) or 2 mg/Kg of (n = 9, 6 females, 3 males) according to previous studies (Romero et al., 2008; Bauer et al., 2014). In addition, we assigned 9 individuals (8 females, 1 male) to a control group to be injected with saline solution. We also used saline solution to compensate for the differences in the required volume of DEX (due to the inter-individual variation in body mass), and standardized the total injected volume at 100 µL for all animals. After the injection with DEX, animals were returned to their home-cages for 90 min (Bauer et al., 2014). Subsequently, a second blood sample was obtained (within 3 minutes) to determine post-treatment cortisol (1 and 2 mg/Kg DEX) and corticosterone (1 mg/Kg DEX) levels in the 3 groups of animals. Data from males and females were pooled for analysis. The uneven numbers of males and females are due to a female bias in the field captures at the moment of this experiment.

2.4 Responses of cortisol and corticosterone to Ang II

We performed this experiment in the field because cortisol and corticosterone levels are lower in captive tuco-tucos indicating a depression in the activity of the HPA axis (Vera et al., 2011a; Vera et al., 2012). We implemented a stimulation of the adrenals using human Ang II (Sigma-Aldrich), which is provided as lyophilized powder to be reconstituted with 1 mL of 0.9 % sodium chloride. Considering that there were no data on the effectiveness of human Ang II to stimulate the adrenals of *C. talarum*, we decided to measure plasma aldosterone levels alongside to evaluate whether our protocol was adequate to stimulate the secretion of this mineralocorticoid. Ang II is an important hormone regulating the systemic levels of aldosterone (Peach, 1977). Thus, the quantification of aldosterone enabled us to interpret cortisol and corticosterone responses in a context given by the variations of a hormone that is *a priori* known to be regulated by Ang II. A few traps were placed per trapper and as soon as a capture was detected, animals were taken inside their traps to our van located nearby, where materials for blood extractions were already prepared. Within 5 minutes of capture, blood samples were obtained to determine baseline cortisol, corticosterone and aldosterone levels. For *C. talarum* these samples can be reasonably considered as "baseline samples" because handled individuals did not increase their cortisol levels until up to 7 minutes of manipulation (Vera, 2011). Immediately afterwards, the individuals were weighed using a Pesola® balance and injected with DEX (1 mg/kg) as described in the previous section. DEX was administered due to the potentially confounding influence of the manipulation-induced ACTH secretion on CORT and aldosterone levels (Roesch et al., 2000). Due to this reason, the inhibition of ACTH secretion is desirable for the study of steroid regulation by Ang II, especially under stressful conditions, such as capture and handling (Roesch et al., 2000). Ninety minutes after the administration of DEX, individuals were injected subcutaneously with Ang II (0.8 mg/Kg, n = 7; 3 males and 4 females) or saline solution (controls, n = 7; 6 males and 1 female), and 60 min later a second blood sample was obtained to determine hormone levels in response to the experimental treatments (Dourish et al., 1992). Given the uneven quantities of males and females in Ang II and saline-treated animals, we pooled the data from both sexes to allow a better statistical comparison between treatments. Again, the injected volume was 100 µL for all animals because we used saline solution to compensate for the differences in the required volumes of DEX and Ang II solutions, due to the differences in body mass (Vera et al., 2012). Between the collection of blood samples and the administration of drugs, animals were kept

individually in PVC tubes (10 cm diameter, ~30 cm length), and provided with grasses collected in the place of capture (Vera et al., 2011a). The three authors worked simultaneously to allow rapid handling of the animals and the calculation of the doses to be used. All captures occurred within a narrow range of hours (10 AM-15 PM) to avoid possible circadian variations in hormone levels.

2.5 Responses of cortisol and corticosterone to potassium intake

Considering the possible roles of CORT in the regulation of mineral-water balance, we were interested in the evaluation of CORT responses to K^+ and Na^+ intake. Plasma K^+ levels are potent stimulators of aldosterone secretion and fluctuate in relation to Na^+ levels, which, in turn, inhibit aldosterone secretion and decrease the sensitivity to Ang II (Aguilera et al., 1978; Pratt, 1982). Due to this, we decided to explore whether cortisol and/or corticosterone positively respond to the intake of K^+. Because *C. talarum* does not eat pellets, we perform this evaluation using an acute oral administration of K^+. The protocol was similar to that described in the previous section. After being removed from their traps, animals were weighed and a baseline blood sample was obtained within 5 minutes of capture to determine cortisol, corticosterone and aldosterone levels. Immediately afterwards, animals were treated with DEX, as previously described, and subsequently placed inside PVC tubes provided with grasses for 90 min. After this, they were administered orally with a solution of 15 mEq KCl (894.6 mg /kg, Adam and Campbell, 1984, n = 7, 4 males, 3 females) or saline solution (control group, n = 7, 3 males, 4 females). The volume of KCl solution administered varied between 400 and 679 µL, depending on the body mass of each individual, while for control individuals we fixed a constant volume of 500 µL of saline solution. Briefly, the animals were firmly held from the back and obliged to drink the KCl or saline solutions by placing the tip of a syringe in their mouths and slowly pressing the plunger to allow a gradual influx of the liquid. Finally, 30 min after the administration of the KCl or saline solutions (Adam and Campbell, 1984), we obtained a second blood sample to determine hormone levels in response to the experimental treatments.

2.6 Responses of cortisol and corticosterone to different diets

In order to evaluate the responses of cortisol and corticosterone to different diets a group of animals was transported to the laboratory and randomly assigned to the following

treatments: (a) fed *ad libitum* for 10 days with lettuce, chicory, sweet potatoes and sunflower seeds, which is the usual diet used for the tuco-tucos in captivity (n = 9, 3 males, 6 females), (b) fed *ad libitum* exclusively with the grass *Panicum racemosum*, which is the more abundant grass in the sand dunes inhabited by the study population (vegetative parts, Fanjul et al., 2006, n = 9, 5 males, 4 females), (c) fed *ad libitum* with a diet of mixed grasses (*Bromus sp*, *Lagurus sp*, *Poa sp*. and *Agrostis sp*, vegetative parts), but without including *P. racemosum* (n = 9, 3 males, 6 females) and (d) fed in the same way as group (a), but subjected to fasting for 24 h after 9 days of treatment (n = 10, 5 males, 5 females). The food was daily renewed to assure that animals had constant access to their respective items and that these remained fresh. Also, for diets (b) and (c), grasses were sprayed with tap water before placing them inside the cages.

After 10 days of treatment (9 for group (d)) we obtained a blood sample from each animal for the measurement of plasma cortisol and corticosterone levels, hematocrit, blood glucose levels, triglycerides and neutrophil to lymphocyte ratio (N: L). Individuals were also weighed in a precision balance immediately after arrival in the laboratory and when diet treatments concluded. With this data, the percentage of initial body mass was calculated. The measurement of this set of variables allowed us to interpret cortisol and corticosterone variations, having a broader picture of the physiological condition of the animals. The effect of diet on these variables was tested comparing the groups of animals fed on vegetables *ad libitum*, *P. racemosum*, and mixed grasses (groups a, b and c), while the effect of fasting was tested comparing the same parameters for the animals fed with vegetables and those fed with the same diet but fasted for 24 hours (groups a and d). Once the experiment concluded, the individuals of groups b), c) and d) were fed with vegetables for at least one week in order to regain weight before their return to the field.

2.7 Blood sampling and determination of parameters in blood and plasma

Blood samples (~300 μL) were obtained from the suborbital sinus -after 20-30 s of anesthesia with chloroform- using a syringe fitted with a flexible plastic tube that was connected to a heparinized microcapillary tube (Vera et al., 2008, Vera et al., 2011a). For the determination of the N: L ratio, we prepared blood smears and stained them with May Grunwald-Giemsa solution (Vera et al., 2008; Vera et al. 2011). The N: L ratio has been extensively used as an indicator of stress in many animal models (Davis et al., 2008) and we have previously characterized this indicator in conditions of presence and absence of stress in

C. talarum (Vera et al. 2008, Vera et al., 2011a). Thus, coupled with GC measurements might be a valuable tool for interpreting physiological stress levels. Triglycerides were determined using a colorimetric kit (TG color, Wiener laboratory, Santa Fe, Argentina). Blood glucose was measured using One Touch Ultra® glucometer. The hematocrit was assessed as the proportion of capillary length occupied by packed red blood cells after centrifugation in a micro capillary centrifuge (Giumelli, Buenos Aires, Argentina).

2.8 Validation of hormone assays

Cortisol and aldosterone were measured using DRG® EIA kits (catalog numbers: 1887 and 4128, respectively). The cortisol assay is capable of measuring cortisol levels up to 800 ng/mL and the limit of detection is 2.5 ng/mL, while the aldosterone assay is capable to measuring aldosterone concentrations up to 1000 pg/mL, with a limit of detection of 15 pg/mL. The anti-aldosterone antibody is highly-specific for aldosterone (cross reactivity ≤ 0.017 % with other assayed steroids, as reported by the manufacturer). The anti-cortisol antibody has less than 2% cross reactivity with all assayed steroids, with the exception of progesterone (9 %) and corticosterone (45 %), which would imply that by this assay, cortisol cannot be measured independently from corticosterone using this assay in tuco-tucos. Nonetheless, we were aware that corticosterone EIA kits are not able to detect corticosterone in unextracted plasma samples of *C. talarum* (F. Vera, unpublished data). Therefore, we suspected that this high cross reactivity of the cortisol antibody with corticosterone could be an overestimation for measurements in the plasma of our study species and we decided to evaluate this issue for our own samples. Then, 6 plasma samples were divided in 2 aliquots of 40 µL and spiked with 20 µL of a corticosterone solution containing 83 ng/mL or 20 µL of the cortisol zero calibrator before conducting the cortisol EIA. Cortisol levels measured in aliquots of the same plasma samples did not differ significantly (mean ± SD = 16.95 ± 13.17 ng/mL and 15.86 ± 10.22 ng/mL for plasma sample + corticosterone solution and plasma sample + cortisol zero calibrator respectively; paired *t* test, t = 0.47, p = 0.66, n= 6, Pearson correlation coefficient r= 0.92, p = 0.011), indicating that this assay can be used to measure cortisol in plasma samples of *C. talarum*, without significant cross reaction with corticosterone.

As the cortisol and aldosterone EIAs were used for the first time in the plasma of this species, we subjected them to a previous validation, which included the assessment of (1) parallelism, (2) accuracy and (3) precision (Grotjan and Keel, 1996), as reported previously

for other steroid hormone assays in *C. talarum* (Vera et al., 2011a, b, Vera et al., 2013). Briefly, parallelism was assessed by serially diluting plasma samples with the 0 calibrators, and the resulting hormone measurements were plotted against the standard curves in semi-log graphs. The slopes of the log-transformed dilution curves (regression lines) were compared to that of the standard curve using *t* tests for equal slopes. Also, values from log-transformed dilution curves and standard curves were tested for their correlation. Accuracy was assessed by determining the recovery of the already known quantities of the target hormones that were added to aliquots of plasma samples before the assays. Briefly, plasma samples (40 µL) were spiked using 10 µL aliquots of cortisol or aldosterone calibrators (200 ng/mL and 500 pg/mL, respectively) to compare the observed and the expected concentrations of the target hormones considering values measured in both spiked and unspiked aliquots of each sample. Precision was assessed by calculating the intra and inter-assay coefficients of variation (CV). Intra-assay CVs were calculated from 6 replicated plasma samples (for both assays), while the inter-assays CVs were obtained from another 6 and 4 samples assayed in different trials, for cortisol and aldosterone, respectively.

For the quantification of corticosterone, aliquots of plasma samples were sent to the Laboratory of Molecular Endocrinology of the University of Buenos Aires (UBA) to be measured by a radioimmunoassay (RIA), optimized and used in previous studies (e.g., Grion et al., 2007). The assay has a limit of detection of 2 ng/mL and is able to measure corticosterone levels up to 100 ng/mL. Corticosterone was quantified after an extraction of steroids from the plasma. Briefly, 10 µL of plasma and 40 µL PBS were extracted twice with dichloromethane (300 µL each time) as previously described (Grion et al., 2007). All samples were assayed in duplicate. All samples from an individual were run on the same RIA batch. The inter-assay CV was evaluated using aliquots of 6 samples replicated in two different assays. Intra and inter assay CVs were 12 and 22 %, respectively. To control whether the extraction procedure was able to remove interference from plasma components, we assessed parallelism between a serial dilution of a plasma sample and the standard curve of the assay. Briefly, a plasma sample (40 µL) was spiked with 10 µL of a corticosterone solution containing 83 ng/mL of corticosterone and, subsequently, serial dilutions were prepared using PBS buffer. The sample was spiked due to the relatively low corticosterone levels in the plasma samples (see Vera et al., 2011a).

2.9 Statistical analyses

Parallelism of the serial dilutions of plasma samples in relation to the standard curves was evaluated using *t*-tests for equal slopes and Pearson correlation. Cortisol, corticosterone and aldosterone responses to the administration of DEX, Ang II and K^+ were evaluated using 2-way repeated measures ANOVA. Factors were "treatment" (levels: DEX 1 mg/kg or DEX 2 mg/kg and saline; Ang II and saline; KCl and saline) and sample (pre and post-treatment blood). One-way ANOVA or the non- parametrical Kruskal-Wallis tests were used for comparison among the diets: vegetables *ad libitum*, *P. racemosum* and mixed grasses; for the comparison between vegetables *ad libitum* and fasting for 24 hours, we used a t-test or the non- parametrical Man-Whitney test. Normality and equal variance of data were verified with Shapiro-Wilk and Levene tests. Differences were regarded as significant at a p-value ≤ 0.05.

2.10 Assay validations

The dilution of plasma samples produced curves that were parallel to the standard curves of the assays (*t* tests for equal slopes: cortisol: $t_{obs} = 0.68$, df = 6, p = 0.52; aldosterone: tobs = 0.48, df = 5, p = 0.66). Furthermore, values from log-transformed dilution curves and standard curves were highly correlated (Pearson correlation coefficient: cortisol: r = 1, p<0.001; aldosterone: r = 0.97, p = 0.028). Intra and inter assay CVs were 7.3 and 12 % for cortisol, and 8.4 and 15.4 % for aldosterone. Recovery of cortisol added to plasma samples before the assay was 90.08 % ± 9.01 SD (range: 81.4-105.3). Recovery of aldosterone was 96.18 % ± 30.06 SD (range: 76.51 - 126.62). Taken together, these data indicated that cortisol and aldosterone in plasma samples of *C. talarum* can be directly measured with the DRG® kits.

3. Results

3.1 Responses of cortisol and corticosterone to DEX

The administration of DEX produced a marked decrease in plasma cortisol levels (2-way repeated measures ANOVA, $F_{21,48} = 35.92$, p <0.001, Bonferroni test, p < 0.001, Fig. 1). Both doses (1 and 2 mg/kg) produced similar effect, indicating that the smaller dose is sufficient to produce a maximal inhibition of ACTH release (Fig. 1A). In contrast, corticosterone levels were not affected by the treatment using a dose of 1 mg/kg DEX (2-way repeated measures ANOVA, $F_{15,33} = 0.11$, p = 0.74, Fig 1B). Considering that these results

indicated that a dose of 1 mg/kg of DEX is enough to produce the inhibition of cortisol secretion after 90 min, we decided to use this dose and response time in the experiments in which the inhibition of ACTH release was required (see below sections 3.3 and 3.4).

3.3 Responses of cortisol and corticosterone to angiotensin II

Pre and post-treatment aldosterone levels were not significantly different in animals treated with Ang II, though post-treatment values were significantly lower in individuals treated with saline solution in comparison to pre-treatment values (2-way repeated measures ANOVA, $F_{12,27} = 6.86$, $p = 0.02$, Fig. 2A). In addition, we found a significant interaction between treatments (Ang II-saline solution) and sample (pre-post treatment), indicating a response of aldosterone to the administration of Ang II (2-way repeated measures ANOVA, $F_{12,27} = 15.88$, $p = 0.002$, Fig. 2A). The lower values observed for post-treatment samples in saline-treated animals probably reflect a decrease in aldosterone secretion due to the inhibition of pituitary ACTH release produced by DEX. Thus, the lack of difference between pre and post-treatment values for Ang II-treated individuals actually indicates an increase in aldosterone secretion in this group of animals. Overall, these results corroborate that the protocol was adequate to stimulate the secretion of this mineralocorticoid in our study species.

Ang II and saline-treated animals showed similar variation patterns for cortisol levels: pre-treatment levels were higher than post-treatment levels in both cases and there was not a significant interaction between treatments and sample (2-way repeated measures ANOVA, $F_{12,27} = 63.82$, $p < 0.001$, Fig. 2B). In contrast, for corticosterone we observed a pattern of variation similar to that of aldosterone: a significant decrease in animals treated with saline solution (2-way repeated measures ANOVA, $F_{12,27} = 9.9$, $p = 0.008$, Bonferroni tests, $p < 0.05$, Fig. 2C), but not in animals treated with Ang II ($p > 0.05$, Fig. 2C). The interaction between treatments and sample was marginally statistically-significant ($p = 0.067$), indicating that the effect was less than that observed for aldosterone (Fig. 2C). Overall, these results indicate that under field conditions the administration of DEX produced a marked decrease in cortisol and corticosterone. Cortisol does not respond to the Ang II signal, but a response occurs for corticosterone.

3.4 Responses of cortisol and corticosterone to K intake

Aldosterone levels showed significant increase in post-treatment samples for animals drinking the KCl solution and decreased significantly in post-treatment samples for animals drinking saline solution (2-way repeated measures ANOVA, $F_{12,27}$ = 6.88, p = 0.02, Bonferroni tests, $p < 0.05$, Fig. 3A). There was significant interaction between treatments (KCl - saline solution) and sample (pre-post treatment) (2-way repeated measures ANOVA, $F_{12,27}$ = 12.2, p = 0.004, Fig. 3A). Therefore, these results indicate increased aldosterone secretion in animals drinking the KCl solution and confirm that the protocol was capable of stimulating the secretion of this mineralocorticoid in *C. talarum* under the conditions present during the experiment. In contrast, we did not find any evidence that cortisol or corticosterone respond to K^+ intake. Post-treatment cortisol and corticosterone levels were lower than pre-treatment levels in both animals drinking the KCl or saline solutions (2-way repeated measures ANOVAs, cortisol: $F_{12,27}$ = 26.96, $p < 0.001$; corticosterone: $F_{12,27}$ = 16.54, p = 0.002, Bonferroni tests, $p < 0.05$, Figs. 3B and 3C) and there was no interaction between treatments and sample (cortisol: p = 0.92, corticosterone: p = 0.36, Figs. 3B and 3C). The decrease in hormone levels in post-treatment samples might be explained by the suppression of ACTH secretion by DEX for cortisol and corticosterone.

3.4.2 Responses of cortisol and corticosterone to different diets

Cortisol levels were lower in animals fed with vegetables but only statistically different to those individuals fed with mixed grasses (Kruskal-Wallis, H = 11.07, p = 0.004, multiple comparisons: Dunn's Method, $p < 0.05$, Table 1). Animals under fasting conditions for 24 hours after a diet with vegetables *ad libitum* showed higher cortisol levels than individuals maintained on the same diet but without fasting (Mann-Whitney, U = 16, n = 9, 10, p = 0.02, Table 1). In contrast, corticosterone levels did not differ among treatment groups (One-way ANOVA, $F_{24,26}$ = 0.74, p = 0.48, Table 1) and no effect of fasting was detected (Mann-Whitney, U = 34, n = 9, 10, p = 0.39, Table 1). The N: L ratios were lower in animals fed with vegetables and *P. racemosum* and statistically different to those individuals fed with mixed grasses (Kruskal-Wallis, H = 10.86, p = 0.004, multiple comparisons: Dunn's Method, $p < 0.05$, Table 1). Animals fasting for 24 hours showed higher N: L ratios than individuals maintained on the same diet but without fasting, but this difference was not significant (Mann-Whitney, U = 23, n = 8, 10, p = 0.14, Table 1). Triglycerides tended to be higher in animals fed with vegetables than those fed with other diets or fasted, but differences were not significant (Kruskal-Wallis, H = 4.78, p = 0.09 and t-test, t = 1.83, d. f. = 17, p = 0.085,

respectively, Table 1). Glucose levels were affected by diet treatment but differences were not detected between groups (Kruskal-Wallis, H = 6.78, p = 0.034, multiple comparisons: Tuckey test, p > 0.05, Table 1). Fasted individuals showed lower glucose levels though this difference was not significant either (Mann-Whitney, U= 112, n= 10, 9, p= 0.08). Individuals fed with mixed grasses showed higher hematocrit levels than those fed with vegetables or mixed grasses (One -way ANOVA, $F_{23,25}$ = 4.87, p = 0.017, multiple comparisons: Holm-Sidak test, p < 0.05). Moreover, hematocrit values were higher for fasted individuals (Mann-Whitney, U= 20, n= 10, 9, p= 0.044, Table 1).

Individuals fed with vegetables maintained their body mass at the end of the treatment while those fed with *P. racemosum* or mixed grasses suffered body mass loss (One- way ANOVA, $F_{25,27}$ = 14.71, p < 0.001, multiple comparisons: Holm-Sidak test, p<0.05, Table 1). Tuco-tucos fasted during 24 h lost some body mass, but this magnitude was not statistically significant (t-test, t = -1.74, d. f. = 17, p = 0.099, Table 1).

Discussion

The differential response of cortisol and corticosterone to DEX found in *C. talarum* maintained for a ten-day captivity period is remarkable considering that both hormones responded when the same ACTH suppression test was performed with recently captured animals in the wild for the Ang II and K experiments. This result, and contrary to that expected according to the pattern of response to acute stressors and ACTH exhibited by these hormones in field and captive conditions (Vera et al., 2011a; Vera et al., 2012), confirm that cortisol and corticosterone in *C. talarum* are subjected to the same endocrine regulation, but corticosterone presents a differential physiological role from cortisol. Given the contrasting results obtained in field and captivity, we cannot discard a difference in corticosterone negative feedback efficacy between these conditions. We previously found that the levels of both GCs diminish in captivity, a fact that can be considered a chronic stress condition. This is noticeable for corticosterone, while the decrease in cortisol depends on initial levels. Besides this, cortisol still responds to ACTH and acute stressors while corticosterone does not (Vera, 2011; Vera et al., 2011a, Vera et al., 2012). Furthermore, even though a clear pattern of endocrine response to chronic stress is difficult to identify, a decrease in negative feedback seems to be the most robust response (Dickens and Romero, 2013). To better understand the results obtained, several characteristics of the present study must be considered. In this sense, one possibility to explore in the future is whether deficiency in water availability in captive

conditions may account for such differences. Nonetheless, hematocrit levels found in animals maintained with the diet of fresh vegetables offered daily (this study) do not indicate signs of dehydration. Also, even though efforts were made to keep the sexes balanced in each experimental group, this was difficult to achieve in *C. talarum*, where the sex ratio is skewed towards the females. Although this was quite accomplished in most of the cases, corticosterone levels following the ACTH suppression test in captivity was only measured in females. Even though we have not yet found differential glucocorticoid responses by sex, other studies should surely focus on deepening sexual differences to rule out this source of variation. Finally, it must be considered that due to protocol differences between the DEX test vs the Ang II y K^+ tests, cortisol and corticosterone were measured 90 min after DEX injection in DEX tests but 150 and 120 min after DEX injection in Ang II and K^+ tests.

In wild animals there exist substantial differences with regard to the variation patterns exhibited by cortisol and corticosterone. In some species, both hormones exhibit similar responses to acute stressors, ACTH and seasonal variation patterns (e.g., deers *Cervus elaphus*: Smith and Bubenik, 1990, ferrets *Mustela putorius*: Rosenthal et al., 1993; chipmunks *Tamias amoenus*, Kennagy and Place, 2000, Romero et al., 2008), while in other species significant differences were reported (e.g., squirrels *Spermophilus saturatus*: Boswell et al., 1994; zebra finches *Taeniopygia guttata*: Schmidt et al., 2008 ; tuco-tucos *C. talarum:* Vera et al., 2011a; Vera et al., 2012). This is a major issue that still deserves clarification and more studies are needed to evaluate whether cortisol and corticosterone vary in similar or contrasting patterns in each study model. Up to the present, there seems to be a remarkable variation, even in closely-related species as regards the relative amounts of cortisol and corticosterone. For instance, in the social tuco-tuco *Ctenomys sociabilis* corticosterone levels are higher than cortisol levels (Woodruff et al., 2010), while in the degu *Octodon degus* cortisol is the predominant GC and corticosterone circulates at very low concentrations, or is undetectable (Kenagy et al., 1999; Soto-Gamboa et al., 2005).

Though in our study the observed effect of Ang II on corticosterone levels was modest at first glance, it is a meaningful result for 2 reasons. The first one is a variation in corticosterone levels of a few nanograms per mL that would probably be overlooked, or considered as trivial if we are thinking about this hormone as a typical glucocorticoid, even in tuco-tucos that have low GC levels in relation to other mammals (Vera et al., 2011a). This is because variations in GC levels during the response to acute stressors or energetically-demanding life-history stages are generally much larger (i.e., dozens to hundreds of ng/mL, but could be much more, depending on the study model, e.g., Kennagy and Place, 2000;

Schradin, 2007; Romero et al., 2008, Bauer et al., 2014). However, in a scenario in which corticosterone is suspected to function mainly as a mineralocorticoid, even a change of fractions of a nanogram could be meaningful, considering that corticosterone levels are much higher than aldosterone levels (given in nanograms per milliliter and picograms per milliliter, respectively). In this study, the different responses of Ang II and saline-treated animals imply a difference of ~ 3-4 ng/mL (3000-4000 pg/mL, Fig. 3C). Therefore, assuming a similar binding of aldosterone and corticosterone to the type I receptor (also called the *mineralocorticoid receptor*) in certain target tissues, such as the hippocampus (de Kloet et al., 2000), we recognize that this apparently small difference in corticosterone levels should not be assumed as trivial and deserves further research. The second reason refers to the roles of cortisol and corticosterone in mineral-water balance which need to be considered in GC dynamics of wild vertebrates (Vera et al., 2017). The roles of cortisol and corticosterone in regulation of body fluids and the regulation by Ang II have been widely studied in the biomedical area (Agarwal and Mirshani, 1999; Joëls and de Kloet, 2017; de Kloet et al., 2000; Thunhorst et al., 2007; Shelat et al., 1999; Liu et al., 2010). For example, GCs have been shown to increase the binding of angiotensin and aldosterone to their respective receptors and potentiate their effects on water drinking and salt appetite (Ganesan and Summers, 1989; Ma et al., 1993; Shelat et al., 1999, Thunhorst et al., 2007).

Since cortisol and corticosterone are subjected to the same endocrine regulation -but negative feedback efficacy differ according to field or captive conditions- and different physiological roles in *C. talarum*, it is interesting to propose that factors might exist that differentially regulate their binding to the type I and/or type II receptors (Gomez-Sanchez, 2010; Uchoa et al., 2014). Indeed, because plasma cortisol levels were more than one order of magnitude higher than corticosterone levels, differential effects of corticosterone through the type I receptor would require that the accessibility of both hormones to the receptor is differentially regulated, at least in certain target tissues (Schmidt et al., 2008; Schmidt et al., 2010). On the other hand, access of GCs to the type I receptor is restricted in certain tissues due to the co-expresion of the enzyme 11b-hydroxysteroid dehydrogenase, which converts GC into inactive forms, conferring specificity for aldosterone binding (reviewed by de Kloet, 2000; Gomez-Sanchez, 2010). Furthermore, it is important to take into account both that the type II receptor is ubiquitiously expressed in virtually all tissues while the type I receptor has a more limited distribution (de Kloet et al., 2000; Gomez-Sanchez, 2004) and, also, that the dominant GC can be organ-specific (Schmidt et al., 2008). Hence, the fact that cortisol (or corticosterone) is predominant in the plasma does not necessarily imply that this mirrors their

concentrations in target tissues (Schmidt et al., 2008). In consequence, attention should be also drawn to the GC that circulates at lower concentrations, instead of measuring solely the hormone that circulates at higher levels (Koren et al. 2012).

K^+ had no effect on CORT secretion in spite of the observed effect of Ang II. This is likely related to the fact that K^+ effects in steroid mineralocorticoid secretion occur particularly at the *zona glomerulosa* of the adrenal glands, which produce aldosterone (Randall et al., 2002). The stimulation of aldosterone secretion by increments in extracellular potassium concentration is associated with increases in the volume of the adrenal glomerulosa cell (Randall, 2002). On the other hand, corticosterone and cortisol are produced in the *zona fasciculata*, which contains receptors for both Ang II and ACTH, though they are not usually responsive to changes in electrolyte levels (Bornstein et al., 1999). Considering that the protocol employed in this study was adequate to increase the secretion of aldosterone, we can reasonably conclude that GCs are not responsive to acute changes in K^+ intake in *C. talarum*. An important factor influencing K^+ and Ang II-induced aldosterone secretion is the plasmatic concentration of Na^+ (Wotus and Engeland, 2003). Low levels of Na^+ increase the responses to Ang II and potassium, while relatively higher levels produce the opposite effect (Pratt, 1982). For *C. talarum* no significant differences in plasma and urine levels of Na^+ were found throughout the reproductive cycle, suggesting that intake of Na^+ does not significantly vary across the year (Baldo, 2016). This was also true for K^+ and chlorure levels and hematocrite, indicating that the hydric status of the animal is relatively stable during the year (Baldo, 2016).

When, different diets were provided to tuco-tucos, changes in body mass indicate that the animals fed with *P. racemosum* and mixed grasses were in negative energy balance, while the group fed with vegetables was able to maintain their body mass. It is interesting to note that this non-natural diet allows the animals to better cope with captivity. This is probably related to the combination of high water content and high energetic content of the provided items (sweet potatoes, sunflower seeds, lettuce and chicory). Water is critical because *C. talarum* obtain all the water from the vegetation they consume. As it was previously shown (Martino et al., 2007), a decrease in body mass of individuals eating grasses indicate that these diets are not optimal for the maintenance of animals in captivity. Although not significant, the tendency of higher levels of triglycerides and glucose in animals fed with vegetables are congruent with higher availability of energy and better general body condition in this experimental group. Fasted animals showed some lower levels of triglycerides and glucose than individuals fed with vegetables but these differences were not significant.

Increased levels of the hematocrit in animals fed with mixed grasses and fasted for 24 hours suggests that these tuco-tucos might have suffered some dehydration.

Decreases in the body mass of animals fed with mixed grasses matched with increases in cortisol levels, suggesting that these individuals were indeed experiencing higher energetic and/or nutritional stress. The effect of the 24 h fasting was not severe in terms of body mass loss but an increment in cortisol levels was detected. In contrast, we did not observe effects of these treatments on corticosterone levels, adding more evidence on the fact that patterns of corticosterone and cortisol are differently affected in this species. These data confirm that corticosterone is not indicative of allostatic load -or chronic stress- in *C. talarum*. The variation pattern of N: L ratio accompanied -although not tightly- that of cortisol. Individuals fed on mixed grasses showed increments in cortisol and N: L ratio. When individuals were fasted, some incremente in N: L was detected but difference was not significant. Increased N: L ratios are recognized to indicate a condition of stress (Davis et al., 2008). Nonetheless, GCs and N: L ratios might show different sensitivities in their responses to stressors and also the direction of change might vary depending on the duration of a given stressor (i.e., acute or chronic). An increase in N: L ratios, but not in cortisol levels, was reported in *C. talarum* subjected to a chronic restriction in food intake (though these animals were fed with limited amounts of vegetables, instead of grasses) producing a 25% decrease (Schleich et al., 2015) or 13% decrease in body mass (Merlo et al., 2016). In the last case, decreased levels of glucose, triglycerides and an inflammatory response to phytohemagglutinin also accompanied food restriction (Merlo et al. 2016). Results of the present and previous study illustrate that single indicators of stress or physiological condition (including GCs and N: L ratios) are not always capable of detecting the effect of stressors or other challenging conditions. Thus, single indicators of stress status or allostatic load should not always be assumed as reliable, but contrasted with the measurement of other indicators. The idea of a "stress profile" is emerging as a valuable alternative: for a given set of conditions, it might be possible to identify significant deviations in the biological parameters that, as a whole, would indicate the stress status of each individual (Milot et al., 2014).

In conclusion, obtained results corroborate the inhibitory effect of DEX on cortisol and corticosterone in field conditions, but not on corticosterone in captivity. On the other hand, Ang II is capable of stimulating corticosterone, but not cortisol secretion. In addition, the present data does not support a role of K^+ in the control of cortisol and corticosterone secretion. Finally, cortisol and corticosterone differentiated in their responses to diet type in captivity. Taken together, these results extend previous findings showing differential

regulation of cortisol and corticosterone in tuco-tucos (Vera et al., 2011a; Vera et al., 2012). Overall, these results confirm that in *C. talarum* (1) cortisol and corticosterone are not interchangeable hormones, strongly suggesting differentiated physiological roles and (2) regulation by the RAS should be considered in studies about GC dynamics of wild vertebrates. From our point of view, the field of GC actions in wild vertebrates is currently in a transition period. The more traditional view of GCs as "stress hormones" and regulated solely by ACTH is changing into a renewed view that encompasses other less-considered roles of GCs – e.g. orexigenic and mineralotropic actions - and mechanisms of endocrine regulation (see Vera et al., 2017). Moreover, it is important to consider both GCs, cortisol and corticosterone, to explore their participation in the regulation of the physiological processes evaluated. This emergent view further balances these actions during normal day-to-day and life-history related activities with their roles during stress responses.

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Figure captions

Figure 1. Plasma cortisol (A) and corticosterone (B) levels (mean ± SE) before and after (90 min) administration of dexametasone (DEX: 1 mg/Kg, n = 9 or 2 mg/Kg, n = 9) or saline solution (controls, n = 9) in the subterranean rodent *C. talarum*. Statistically-significant differences are indicated with * ($p < 0.05$).

Figure 2. Pre and post-treatment (60 min) aldosterone (A), cortisol (B) and corticosterone (C) levels in individuals of *Ctenomys talarum* treated with angiotensin II (Ang II, n = 7) or saline solution (controls, n = 7) in the field at the moment of their captures. Both groups were treated with dexametasone (DEX) 90 minutes before Ang II/saline injection to inhibit the

secretion of adrenocorticotropic hormone (ACTH). Statistically-significant differences are indicated with * ($p < 0.05$).

Figure 3. Pre and post-treatment (30 min) aldosterone (A), cortisol (B) and corticosterone (C) levels in individuals of *Ctenomys talarum* treated with KCl (n = 7) or saline solutions (controls, n = 7) in the field at the moment of their captures. Both groups were treated with dexametasone (DEX) 90 minutes before potassium/ saline intake to inhibit the secretion of adrenocorticotropic hormone (ACTH). Statistically-significant differences are indicated with * ($p < 0.05$).

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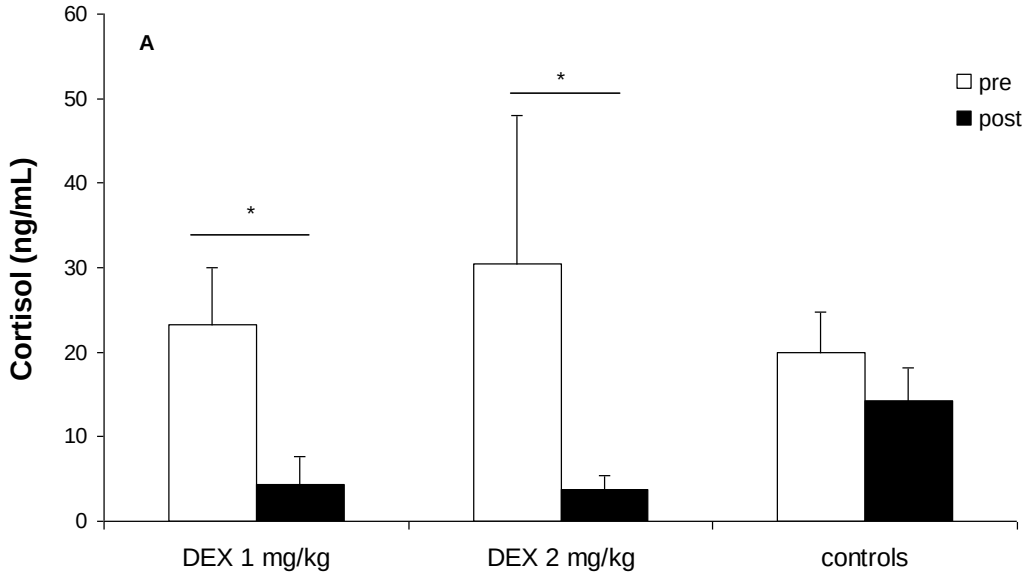

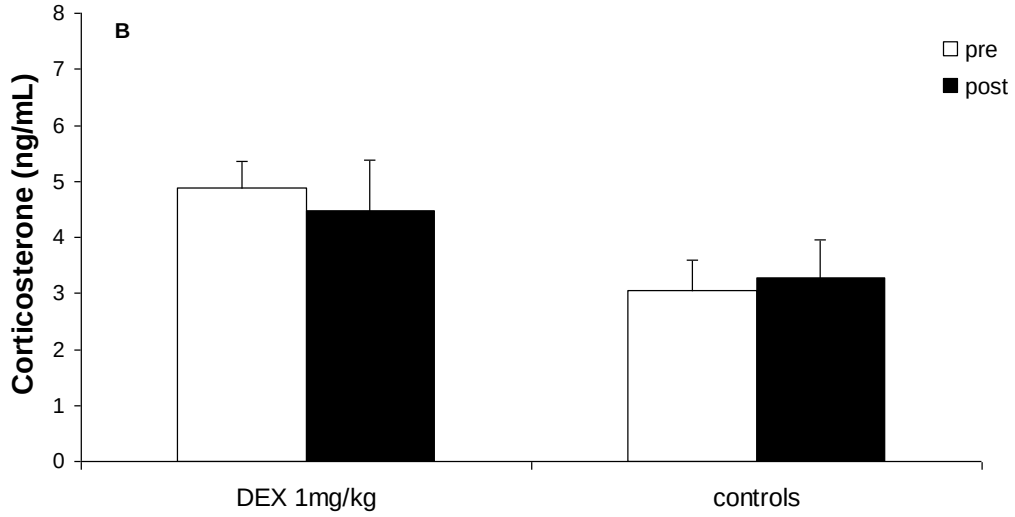

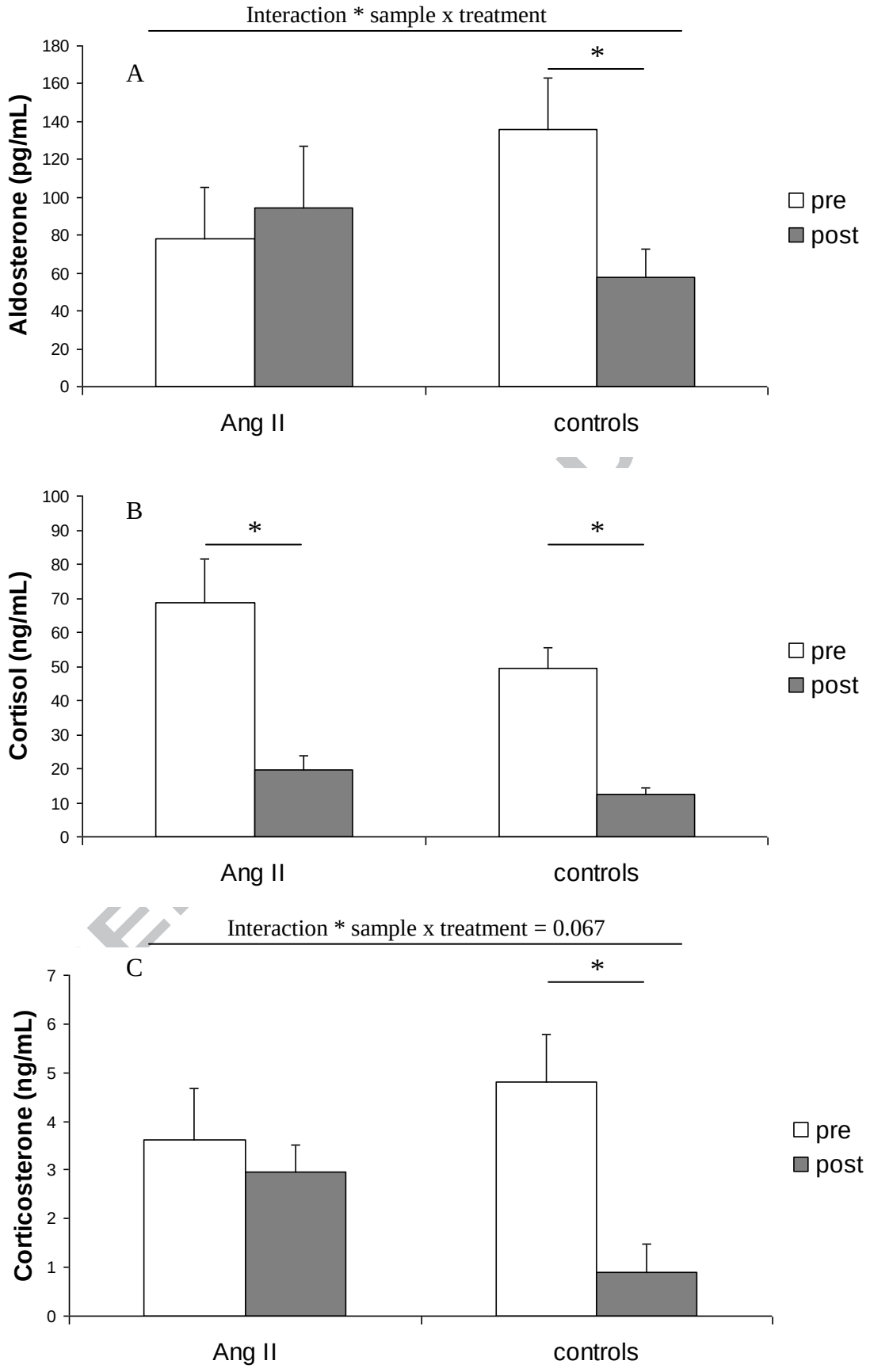

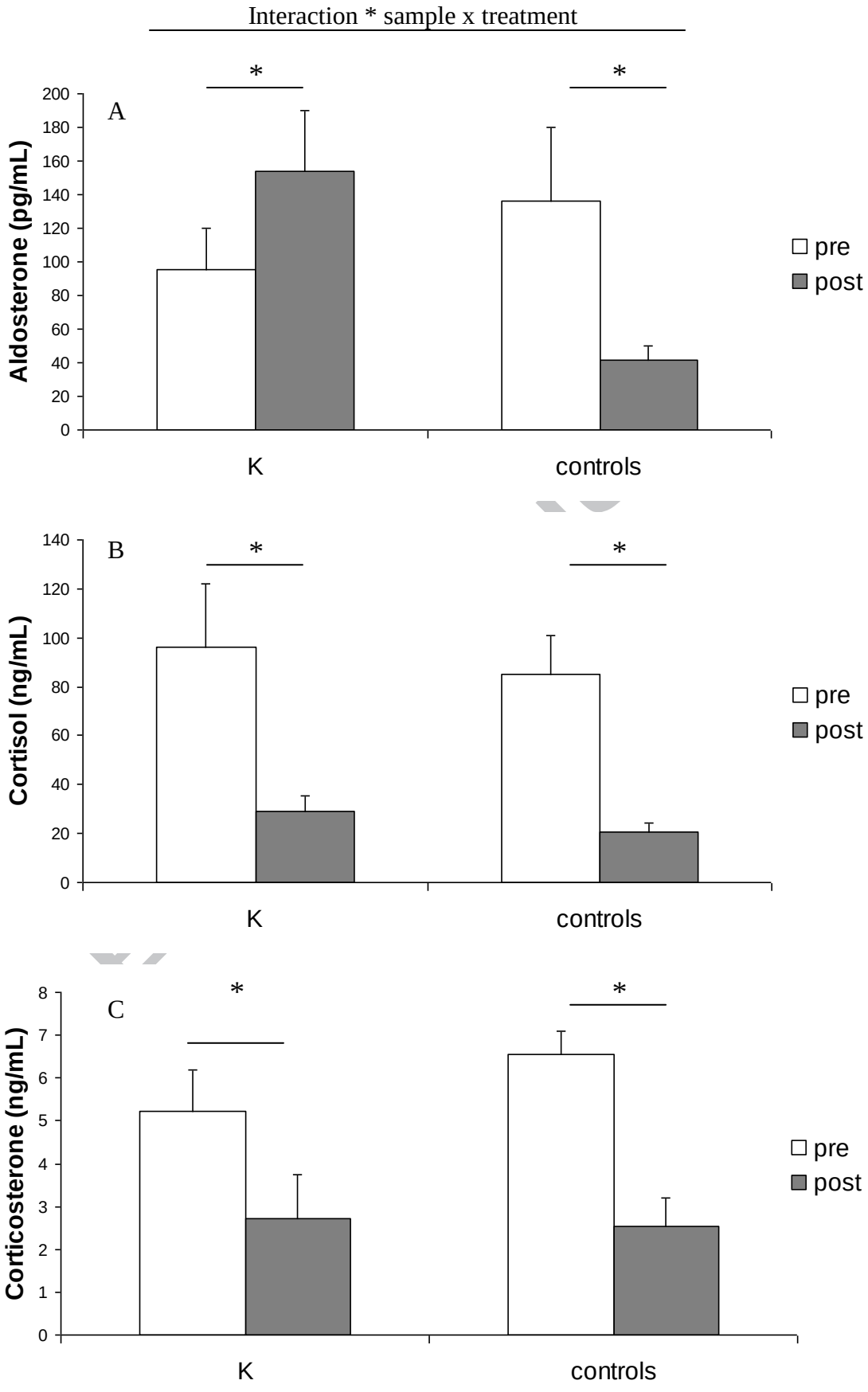

Highlights

- Differences in the physiological roles of cortisol and corticosterone are evaluated in *C. talarum*
- Cortisol and Corticosterone respond to DEX suppression test.
- Ang II is capable of stimulating Corticosterone.
- Cortisol responds to diet as a chronic and acute stressor.

Table 1. Cortisol, corticosterone, triglycerides, glucose, hematocrit, N: L ratio, and % of initial body mass (mean ± SE) in individuals of *Ctenomys talarum* fed with different diets at the laboratory for ten days (9 days for the fast. 24 h). Abbreviations: veg. *ad libitum* = vegetables *ad libitum* (lettuce, chicory, sweet potatoes, sun flower seeds); *P. racemosum*: fed exclusively with the grass *Panicum racemosum*; mixed grasses: fed with a variety of species, but excluding *P. racemosum* (see methods); fast. 24 h: fasting during 24 hours. Comparisons among diets: veg. *ad libitum* (n = 9), *P. racemosum* (n = 9) and mixed grasess (n = 9); statistically-significant differences are indicated with different letters ($p < 0.05$). Fasting effect: veg. *ad libitum* (n = 9) and fast. 24 h (n = 10); statistically-significant differences are indicated with * ($p < 0.05$).

Group/ parameter	Cortisol (ng/mL)	Corticosterone (ng/mL)	N: L ratio	Triglycerides (g/L)	Glucose (mg/dL)	Hematocrit	% initial body mass
Veg. *ad libitum*	11.39 ± 1.52[a]	5.83 ± 1.18	0.77 ± 0.33[a]	1.52 ± 0.38	117.78 ± 35.3	45,11 ± 1.15a	102.93 ± 2.29[a]
P. racemosum	33.01 ± 9.28[a,b]	4.81 ± 1.31	0.63 ± 0.09[a]	0.62 ± 0.06	62.88 ± 3.28	45.5 ± 0.86a	87.65 ± 2.71[b]
Mixed grasses	31.34 ± 5.92[b]	3.98 ± 0.56	1.65 ± 0.19[b]	0.87 ± 0.33	63.55 ± 3.48	49.11 ± 0.96b	87.89 ± 1.87[b]
Fast. 24 h	19.85 ± 3.16[*]	4.79 ± 0.87	1.61 ± 0.47	0.78 ± 0.18	67.6 ± 7.62	48.7 ± 1.15*	95.51 ± 3.69