General and Comparative Endocrinology 170 (2011) 550–557

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Cortisol and corticosterone exhibit different seasonal variation and responses to acute stress and captivity in tuco-tucos (*Ctenomys talarum*)

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ARTICLE INFO

Article history:
Received 18 February 2009
Revised 8 November 2010
Accepted 17 November 2010
Available online 21 November 2010

Keywords:
Captivity
Corticosterone
Cortisol
Ctenomys
HPA axis
Stress
Seasonality

ABSTRACT

In this work we aimed to evaluate variations in plasma glucocorticoids (GCs, cortisol and corticosterone) levels throughout an annual cycle in free-living male tuco-tucos (*Ctenomys talarum*) and compare their responses to acute and chronic stressors (trapping, manipulation, immobilization, confinement in a novel environment, transference to captivity). In addition, we used leukocyte profiles to allow discrimination between basal and stress-induced seasonal changes in GC concentrations. Our results showed that cortisol and corticosterone are differently affected by environmental stimuli in *C. talarum*. Both hormones showed different patterns of variation in the field and responses to captivity. Moreover, only cortisol was responsive to acute stressors. Leukocyte profiles indicated that animals were unstressed in the field and therefore, that we were able to measure basal, stress-independent, fluctuations in GC levels. GC concentrations were low in comparison to values frequently reported for other mammals. Our results suggest differentiated physiological roles for cortisol and corticosterone in our study species and further emphazise the complexity of GC physiology in wild mammals.

1. Introduction

Glucocorticoids (GCs, cortisol and/or corticosterone, depending on the species) are important hormones that serve many adaptive functions in vertebrates. GCs are secreted by the adrenal gland and regulate the availability of energy by influencing glucogenesis, glucose use and protein metabolism [7]. In addition, adverse environmental conditions (i.e., stressors) are known to induce the hypothalamic–pituitary–adrenal (HPA) axis to secrete GCs above basal levels, which seems critical to maintain or restore homeostasis during or after the challenge [41]. However, chronically-elevated GCs have a variety of deleterious effects upon health [40] and can suppress the hypothalamic–pituitary–gonadal (HPG) axis, affecting reproduction [44]. It has been demonstrated in free-living animals that GC concentration can be negatively related with survival [33,37], though the relationship between GC levels and fitness is not always clear [6,12]. An additional important finding of recent field studies is that free-living animals can seasonally modulate GC secretion, leading to differences in both baseline and stress-induced GC concentrations, though in mammals the pattern of GC release varies from species to species [35].

One unresolved question related to HPA axis physiology is why some mammals have both cortisol and corticosterone in detectable amounts in plasma (e.g., chipmunks, ground squirrels [10,31,38]). Since Kenagy and Place [24] pointed that the physiological meaning of two different plasma GC hormones should be studied little was done in order to shed light on this matter. From the scarce available information, it is widely assumed that cortisol and corticosterone share their physiological roles and that their relative importance depends on their concentrations in plasma [9,31,38]. One initial approach to evaluate if cortisol and corticosterone indeed accomplish the same functions in a given species may be to compare their responses to experimental treatments (including both acute and chronic stressors) and their patterns of variation under natural conditions. While similarities would confirm the accepted view of shared physiological functions, differences would suggest that both hormones do not completely overlap in their roles and exhibit some differentiation in function.

Another issue that deserves further clarification is the physiological meaning of the seasonal fluctuations in baseline GC levels [35]. Though baseline samples have been collected in a number of studies (defined as blood samples taken within 3 min of capture, see Section 2), the fact that the story of animals prior to capture can't be usually known prevents from assessing if these variations represent changes in strictly *basal* GC levels or are influenced by naturally-occurring stressors operating before the captures. This problem may be overcome by coupling data on GC levels with the determination of leukocyte profiles (or other confident stress indicators), if one has characterized such profiles during stress and unstressed conditions in the study species (see [16]). Specially,

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doi:10.1016/j.ygcen.2010.11.012

the neutrophil: lymphocyte (N:L) ratio is rapidly increased by stress and can be directly related to stress hormone levels [16]. Thus, whilst parallel seasonal increases in N:L ratios and GC levels would indicate increased stress levels, variation in GC levels concurrently with relatively low and constant N:L ratios may indicate that strictly basal GC concentrations are being modulated on a seasonal basis .

The subterranean rodent *Ctenomys talarum* (talas tuco-tucos, see [23] and below *Species characteristics*) constitutes an interesting model to explore the ecophysiology of the HPA axis in mammals. Both cortisol and corticosterone are present in plasma of other hystricomorph rodents such as guinea pigs [15] and chinchillas [32], though in the colonial tuco-tuco *Ctenomys sociabilis* only corticosterone was present in significant amounts [46]. The species show high levels of intraspecific aggression [50] suggesting important roles for GCs in the mobilization of energy reserves during aggressive interactions, especially in the case of males for territory defense and access to females during the reproductive season. On the other hand, their subterranean niche reduces seasonal environmental fluctuations (e.g., temperature, photoperiod; [5,14]), imposing a situation that contrasts with that of other studied free-living mammals inhabiting above-ground environments. In addition, this species can be successfully maintained in captivity in our laboratory where the responses of the HPA can be monitored during the acclimation process and the effects of chronic and acute stress can be studied under controlled conditions.

Here, we report the results of samplings and experiments performed in the field and the laboratory aimed at contributing to the abovementioned knowledge gaps with regard to the ecophysiology of HPA axis in wild animals and *Ctenomys* in particular. The specific goals were (i) to evaluate seasonal variations in cortisol and corticosterone in free-living male tuco-tucos throughout the breeding cycle, (ii) compare the responses of cortisol and corticosterone to acute stressors under field and laboratory conditions (iii) evaluate the responses of both GCs to captivity and (iv) assess if seasonal changes in GC levels in free-living animals indicate changes in stress levels or reflect fluctuations of basal concentrations with the use of leukocyte profiles.

2. Materials and methods

2.1. Species characteristics

A total of 97 adult males of *C. talarum* were live-trapped in Mar de Cobo (Buenos Aires Province, Argentina; 37°45′S, 57°26′W). *C. talarum* is a medium-sized rodent (adults are 120–220 g) that inhabits individual galleries systems parallel to the surface in southern parts of South America [2,34]. This species is solitary and highly territorial; individuals do not share burrows, except in the case of females and their offspring until dispersal and when mating occurs [13]. It is a highly-aggressive species as evidenced by the presence of scars in captured individuals and the wounds produced in aggressive encounters can be very severe [50]. Tuco-tucos perform most of their activities in their burrows, but they venture away short distances from burrow openings to collect aerial portions of vegetation that they later consume belowground. The species presents a polygynous mating system in which some males monopolize the access to multiple females [48,50]. The natural breeding season extends over nine months starting in late autumn (June, [13,19,28]) and, since pregnancy extends over 95 days [49], most births occur during spring. Their high level of polygyny and high male–male aggression levels represents a scenario for high local mating competition where the spatial location of males represents an important advantage to obtain matings [48]. Both evidences from studies in the wild (skewed adult sex ratio towards females, spatial pattern of sexes, presence of wounds resulted from fights) and captive conditions [13,50] allow us to propose that male–male competition is an important component for mate acquisition.

2.2. Cortisol and corticosterone variations in the field

To assess GC variations in the field throughout the year we captured individuals in three different stages during 2007: (1) non-reproductive season (NRS: April–May, $n = 12$), (2) beginning of the reproductive season (BRS: June–July, $n = 14$) and (3) peak of the reproductive season (PRS: October, $n = 13$, [13,19,28]). The distinction among seasons was made according to the reproductive cycle of females, since males after attaining reproductive maturity do not undergo regression of their testes and contain sperm in their epididymes year round [28]. All samplings were conducted between 10:00 AM and 15:30 PM so that blood samples were obtained within a restricted range of hours of the GC circadian rhythm [4]. However, it is important to note that *C. talarum* individuals showed arrhythmic activity patterns during day and night [27]. Also, in each sampling date we visited a different area of our study population to avoid sampling an individual more than one time. Animals were caught using plastic, tube-shaped live traps (10 cm diameter, 35 cm length) set at fresh surface mounds. These traps have a false floor that triggers the closing of the entrance when an animal steps on it. Holes (20–40 cm depth) were dug in order to access the galleries and the traps were placed as a prolongation of the tunnels. Because traps were monitored closely, animals did not remain in them for more than 20 min (range <1–20 min). After we detected that an individual has been captured (a wire that is tied to the traps' door is not seen anymore from outside when the door has closed), we took the animal in its trap to our van located nearby where blood extractions were performed. Blood samples (500–900 μL) were obtained from the suborbital sinus after 20–30 s of anesthesia with chloroform, using a syringe fitted with a flexible plastic tube which was connected to a heparinized micro-capillary tube. Blood sampling (including previous anesthetization) did not take more than 3 min to guarantee that GC levels were not affected by the extraction procedure. For most species studied, GCs start to increase 3 min after the perception of a stressor, so that samples collected within this period are considered to reflect pre-stress concentrations [35]. In addition, usually 1–2 min more passed between the detection of captures and beginning of blood sampling. However, no correlation was observed between cortisol levels and manipulation time in tuco-tucos that were handled for 1–8 min, indicating that these animals do not consistently increase cortisol in response to handling within this time window (Vera, unpublished data). In other caviomorph rodents (such as guinea pig and yellow-toothed cavy) GC concentrations did not change within a time span of 5 min in response to a stressor [25]. Blood samples were transported to the laboratory in a refrigerated cooler and centrifuged 15 min at 660*g*. The plasma was separated from cells and stored at −20 °C until analysis. All samples were analyzed for cortisol and the ones with enough residual volume ($n = 7$, 8 and 11 for NRS, BRS and PRS, respectively) were further analyzed for corticosterone.

It is known that trapping can elicit increases in plasma GC concentration in a variety of mammals [9,31,38], though this is not always the case [30]. To assess the effects of trapping we conducted an additional sampling during April–May 2008 (i.e., NRS 2008) using a modified trapping technique in which traps were constantly monitored to obtain GC baseline levels (within 3 min) and compare them with delayed samples. To guarantee that blood samples were obtained within 3 min of capture, we set only 6–8 traps per trapper (the three authors and a lab member) and stayed in their proximities to allow a constant monitoring of the traps. As

soon as one animal was captured, a chronometer was started and we proceeded as described above as rapidly as possible. Thus, this procedure requires more effort but it assures that samples are obtained within 3 min. We managed to achieve this objective in 10 captured individuals. As inter-annual variations in hormone levels could not be ruled-out, it was not appropriate to compare these data with samples obtained during NRS 2007. Hence, in this later sampling we also obtained blood samples from another two groups of individuals that stayed inside the traps for 5 ($n = 5$) or 20 ($n = 8$) min prior to blood extraction to perform adequate comparisons with baseline samples. To mimic our sampling procedure of 2007, these traps were not handled until the above-mentioned times have passed.

During the sampling conducted in 2007 the neutrophil/lymphocyte ratio (hereafter N:L ratio) was also determined during the different stages. Leukocyte profiles are considered particularly useful in the field of conservation physiology because they can be directly related to stress hormone levels [16]. In this sense, stress causes increases in the N:L ratio that are proportional to GC release [16]. In *C. talarum* N:L ratios attain values between 0.1 and 1.5 in undisturbed animals kept in captivity, once they have acclimated to laboratory conditions. When animals are subjected to acute stress N:L ratios increase up to 2.5–3, reaching values up to 5 during chronic stress (e.g., acclimation to captivity, food restriction, repeated blood sampling). When chronically-stressed animals are subjected to an acute stress treatment (immobilization) N:L ratios may reach values up to 10 [45] and Vera, unpublished data). Thus, the calculation of N:L ratios may be used as an approach to discern if seasonal variations in GC concentrations mirror variations in stress levels or merely reflect changes in basal levels of these hormones. Briefly, blood smears were prepared in the field after taking the blood sample and fixed in methanol 70% for 10 min. After arriving at the laboratory they were stained with May-Grunwald Giemsa solution and examined at magnification 450×. All cell types (lymphocytes, neutrophils, eosinophils, basophils and monocytes) were counted until the cumulative total was 200 cells and the N:L ratio was then calculated. Eosinophils, basophils and monocytes were rare, representing only 0.5–7.85% of the cells in the smears.

2.3. Cortisol and corticosterone responses to acute stress and captivity

To evaluate the role of cortisol and corticosterone in the response to acute stress a group of individuals was taken to the laboratory and subjected to immobilization after 20 days of captivity. The animals were housed in individual plastic cages (25 cm × 32 cm × 42 cm) with wood shavings as bedding and fed *ad libitum* with a diet consisting of lettuce, carrots, sweet potatoes, and mixed grasses. Since *C. talarum* does not drink free water, this source was not provided to the animals. Photoperiod and temperature were automatically controlled (12:12 L:D; 25 ± 1 °C). For the stress treatment animals were firmly held for 2 min in a restraint apparatus that consisted of two parallel wire grids spaced by 3.5 cm that precluded them from making almost any kind of movement. Blood samples for cortisol and corticosterone determination were obtained within 3 min from individuals assigned to 2 treatment groups 30 or 60 min ($n = 7$ and 6, respectively) after the finalization of the stressor and to a control group ($n = 6$) that was not disturbed prior to blood extraction. After the immobilization treatment animals were returned to their home cages until blood sampling.

In addition, another group of animals captured during NRS 2008 was subjected to 2 min of immobilization in the field immediately after capture. Blood samples were obtained for cortisol and corticosterone determination from two treatment groups 30 or 60 min after the immobilization ($n = 8$ each group). These animals remained in individual plastic cages (25 cm × 32 cm × 42 cm) between the immobilization and blood sampling (i.e., for 30 or 60 min), therefore GC levels could also be influenced by their confinement in the cages after the manipulation. In this sense, the cages represent an unfamiliar environment for field animals, which unsuccessfully tried to climb their walls or dig in order to leave. The 10 baseline samples (collected within 3 min) were used as cortisol and corticosterone control values.

To assess a finer response of cortisol to captivity a group of males captured during NRS 2008 ($n = 12$) was taken to the laboratory and kept there for 30 days. Blood samples were obtained in the field immediately after capture and after 10, 20 and 30 days in captivity for the determination of cortisol concentrations. As another index of condition, animals were weighed the same days when blood extractions were performed.

2.4. Hormone analysis

Plasma samples were taken to Laboratory of Clinical Assays Dr. Daniel Samaruga for cortisol and corticosterone determination. Cortisol was measured using the Coat-A-Count procedure (Siemens Medical Solutions Diagnostics), which is a solid-phase radioimmunoassay (RIA, catalog numbers: TKCO1) in which ^{125}I-labeled cortisol compete with cortisol in the samples for antibody sites. The assay is capable of measuring cortisol concentrations up to 200 ng/mL. Detection limit is 2 ng/mL, as provided by the manufacturer. Intra- and inter-assay coefficients of variation were 4.8% and 5.2%, respectively. Corticosterone was measured using a rat corticosterone EIA kit (DSL-10-81100) provided by Diagnostic Systems Laboratories, Webster, Texas, USA. The detection limit of this assay is 1.6 ng/mL and can be used to measure corticosterone concentrations up to 2000 ng/mL. Samples with corticosterone levels below the limit of detection (1.6 ng/mL) were assigned this nominal value for statistical treatment. The intra and inter-assay coefficients of variation were 2.3% and 6.1%, respectively. All samples were assayed in duplicate. Cross-reactivities of the antibodies used in both assays with other structurally-similar molecules are very low, as reported by the manufacturers.

2.5. Validation of assays

2.5.1. Cortisol

We examined parallelism between the standard curve of the RIA and serially diluted plasma samples. Considering the relatively low cortisol concentrations in tuco-tucos (see Section 3) plasma samples were previously dosed and thereafter diluted with PBS buffer (PH 7) considering the measured concentration to avoid being near the detection limit of the assay. We also determined recovery of known quantities of cortisol that were added to plasma samples before RIA. Briefly, cortisol (4 mg, Steraloids Inc.) was diluted in 4 mL absolute ethanol and then serial dilutions were made with buffer PBS to obtain two solutions of 61 and 127 ng/mL, respectively. Then, 20 μL of these solutions (containing 1.22 or 2.54 ng of cortisol) were added to 40 μL of plasma samples and recovery was calculated considering cortisol concentrations measured in unspiked aliquots of these samples.

2.5.2. Corticosterone

Due to the low corticosterone levels in relation to the kit standards (see Section 3), two plasma samples of 90 μL were first spiked with 20 μL of the 2000 ng/mL kit calibrator (equivalent to 40 ng of corticosterone) and thereafter serially diluted with PBS buffer to examine parallelism. Recovery of exogenous corticosterone was also evaluated by adding 30 μL of the 200 ng/mL kit calibrator (i.e., 6 ng of corticosterone) to four plasma samples (30 μL) and considering corticosterone levels measured in unspiked aliquots of these samples.

2.6. Statistical analyses

Data were analyzed using one-way ANOVA or the Kruskal–Wallis test when assumptions of parametric tests were not met, followed by Tukey or Dunns' tests when significant differences were detected. Student *t* test or Mann–Whitney rank sum test were used to compare hormone levels between NRS of 2007 and 2008. Cortisol and testosterone levels throughout the 30 day period in captivity were compared using repeated measures ANOVA. Finally, we used *t* test for equal slopes to determine if log-transformed curves of serially-diluted plasma were parallel to log-transformed standard curves [47]. Data are presented as mean ± standard error (SE).

2.7. Ethical note

The animals were cared for in accordance with the Guidelines for the use of animals in research and teaching [3]. None of the animals died during or directly after blood sampling. Stress factor used in this study (immobilization during 2 min) is reversed in short time (cortisol levels decline two hours since stressor exposure, Vera, unpublished data). The animals that were taken to the laboratory were released at the site of capture after their use in the experiments.

3. Results

3.1. Validation of assays

Serial dilutions of plasma samples were parallel to the standard curves of the cortisol RIA (*t* test for equal slopes, low-cortisol male $t = 0.75$, $df = 6$, $p = 0.47$, high-cortisol male: $t = 1.63$, $df = 5$, $p = 0.16$, female: $t = 0.35$, $df = 6$, $p = 0.74$, Fig. 1A) and corticosterone EIA (*t* test for equal slopes, male plasma: $t = 0.90$; $df = 4$; $p = 0.42$, female plasma: $t = 0.75$, $df = 4$, $p = 0.49$, Fig. 1B). In addition, mean recovery percentages were 101.12 ± 3.76% for cortisol and 94.6 ± 5.2% for corticosterone (Tables 1 and 2). Taken together, these results show that cortisol and corticosterone can be measured directly in plasma samples of *C. talarum* without an extraction step.

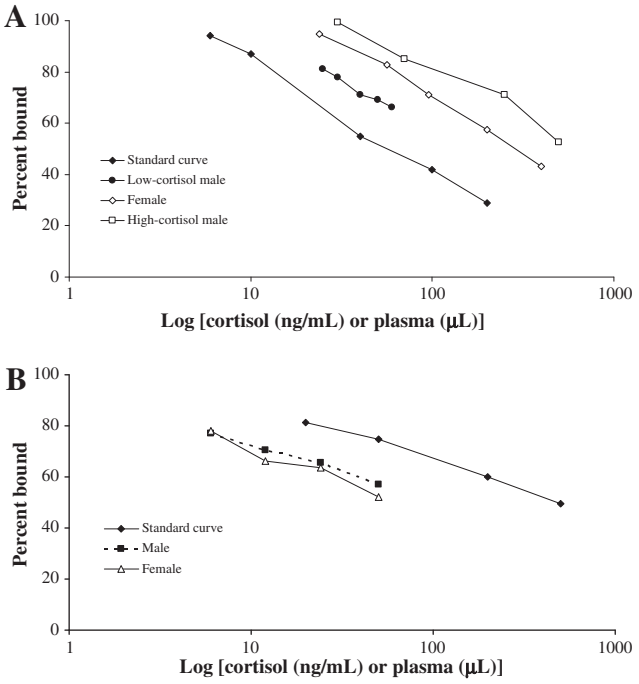

Fig. 1. Parallelism of the cortisol RIA (A) and corticosterone EIA (B).

Table 1
Recovery percentages of exogenous cortisol added to plasma samples of *C. talarum* before RIA. Samples 1–4 were spiked with a solution containing 61 ng/mL of cortisol while samples 5–8 with a solution containing 127 ng/mL of cortisol.

Sample	Cortisol (ng/mL)	Expected (ng/mL)	Observed (ng/mL)	Recovery (%)
1	21	34.3	34	99
2	16	31	31	100
3	20.4	31.2	34.8	111
4	17.3	28.9	32.3	112
5	42.5	70.6	69.5	98
6	17.5	54	61.5	114
7	21	56.3	51	91
8	24	58.3	49	84

Table 2
Recovery of exogenous corticosterone added to plasma samples of *C. talarum* before EIA.

Sample	Corticosterone (ng/mL)	Expected (ng/mL)	Observed (ng/mL)	Recovery (%)
1	30.2	115.1	105.1	91
2	19.8	109.9	120.7	110
3	30.8	115.4	105.1	91
4	17.6	108.8	93.6	86

3.2. Cortisol and corticosterone variations in the field and captivity

No significant differences in cortisol concentrations were found among blood samples collected within three min of capture and those taken from animals that stayed for 5 or 20 min inside the traps prior to blood extraction (cortisol: within 3 min = 20.15 ± 7.36 ng/mL; 5 min = 15.40 ± 0.92 ng/mL; 20 min = 18.97 ± 3.51 ng/mL; Kruskal–Wallis, $H = 0.85$, $p = 0.65$). This indicated our trapping technique did not elicit measurable changes in cortisol concentrations that could mask seasonal variations.

Cortisol and corticosterone were found in similar amounts in the field throughout the breeding cycle, though corticosterone concentration was below detectable limits in 5 out of 26 samples analyzed (1 for NRS and BRS and 3 for the PRS) and cortisol was above detection limits in all samples. Cortisol concentrations were significantly lower during BRS in comparison with NRS and PRS (Kruskal–Wallis, $H = 13.07$, $p = 0.001$, Dunn's test: $p < 0.05$, Fig. 2). In contrast, corticosterone did not show statistical significant variations throughout the year (One-way ANOVA, $F_{23,25} = 2.22$, $p = 0.13$, Fig. 2). Cortisol/corticosterone ratios calculated from

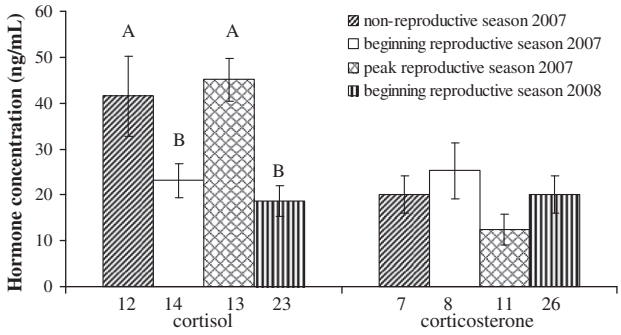

Fig. 2. Seasonal changes in plasma cortisol and corticosterone concentrations (mean ± SE) in *C. talarum* males. Different letters indicate significant differences for cortisol ($p < 0.05$), while no differences were registered for corticosterone. Sample size is provided at the base of each bar.

mean seasonal concentrations were 2.06, 0.91, 3.62 for NRS, BRS and PRS, respectively (Fig. 2). The N:L ratio remained consistently low throughout the breeding cycle in all captured individuals, attaining typical values of unstressed animals and no significant differences were found among the different seasons (One-way ANOVA, $F_{31,33} = 0.61$, $p = 0.55$, Table 3). Cortisol concentrations were significantly lower during NRS 2008 than NRS 2007 (NRS 2007 = 41.5 ± 8.78 ng/mL; NRS 2008 = 18.69 ± 3.37 ng/mL, pooled samples, $n = 23$, $U = 28.5$, $p < 0.001$, Fig. 2). However, corticosterone levels were not different between NRS of 2007 and 2008 (NRS 2007 = 20.13 ± 4.08 ng/mL, NRS 2008 = 17.04 ± 1.78 ng/mL, pooled samples, $n = 26$, $t = 0.78$, $p = 0.44$, Fig. 2).

Cortisol concentrations did not vary significantly throughout the 30-day period in captivity (repeated measures ANOVA on ranks, Chi-square = 2.75, $df = 3$, $p = 0.43$, Fig. 3). Body weigh significantly decreased at days 10 and 20 in relation to field values but increased again at day 30 of captivity (repeated measures ANOVA, $F_{32,46} < 0.05$, multiple comparisons: Holm–Sidak method, Fig. 3).

3.3. Cortisol and corticosterone responses to acute stress

As expected, cortisol concentrations significantly increased at 30 min in animals subjected to immobilization in captivity (One-way ANOVA, $F_{15,17} = 3.69$, $p = 0.05$, Tukey test, $p < 0.05$) and in the field (Kruskall–Wallis, $H = 15.24$, $p < 0.001$, Dunn's test, $p < 0.05$, Fig. 4A). In contrast, corticosterone levels were unaffected in animals that were subjected to this stressor in the field (One-way ANOVA, $F_{21,23} = 0.54$, $p = 0.59$, Fig. 4B). Surprisingly, in captive individuals corticosterone was below detectable levels in 14 of the 19 samples analyzed, including both control and stressed individuals. Assigning these samples a concentration of 1.6 ng/mL (detection limit of EIA) resulted in mean corticosterone levels lower than 3 ng/mL (Fig. 4B). Therefore, this result indicated an additional effect of captivity on corticosterone levels.

4. Discussion

4.1. GC variations in the field and the significance of leukocyte profiles

Free-living male tuco-tucos showed seasonal variations in plasma cortisol, but not corticosterone concentrations throughout the breeding cycle. On the other hand, the evaluation of GC levels in captive animals indicated strong suppressing effects of captivity on corticosterone, but no effect was observed on cortisol (though the lack of effect on the later might reflect acclimatization at the moment of sampling, see below). Finally, acute stress increased cortisol levels but did not affect plasma corticosterone. Thus, both GCs showed markedly different variation patterns in all aspects that were tested in our study species.

The fact that cortisol and corticosterone differentiated in their patterns of variation in free-living individuals suggests that, under natural conditions, both hormones are differently affected by environmental stimuli or that they are subjected to different endogenous regulation of their seasonal secretion. Contrasting seasonal variation for both GCs have been reported for golden-mantled ground squirrels [10], though in yellow-pine chipmunks cortisol and corticosterone showed similar seasonal variation [24,31]. Thus, not only the period with highest GC levels is variable among species of mammals [35], but the seasonal variations of cortisol and corticosterone may coincide or show opposite or different trends depending on the species.

To interpret what the seasonal changes in hormone concentrations represent, two important results are (1) the lack of effect of trapping on hormone levels and (2) the low N:L ratio values recorded during all seasons. We found no differences in cortisol levels among blood samples taken within 3 min of capture and those collected after 5 or 20 min of confinement in the traps, indicating that we measured hormone concentrations characteristic of undisturbed animals (commonly referred as baseline levels). The word "baseline" is frequently used because the activity of the animals prior to capture is unknown and, therefore, it can not be ruled-out that natural stressors may account for fluctuations in GC concentrations [36]. However, our data on N:L ratios indicate that

Table 3
N:L ratio values (mean ± SE) in free-living male tuco-tucos during different stages of their reproductive cycle. Sample size is provided within brackets. There are no statistical differences among seasons.

	N:L ratio
Non-reproductive season	0.51 ± 0.13 (8)
Beginning of reproductive season	0.53 ± 0.05 (13)
Peak of reproductive season	0.40 ± 0.11 (13)

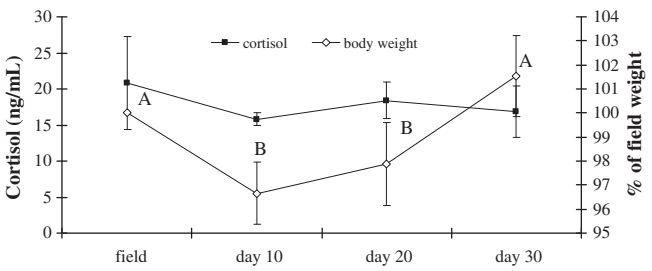

Fig. 3. Changes in plasma cortisol concentrations and body weight (mean ± SE) during the first 30 days in captivity in relation to field values. Different letter indicate statistical significant differences. While body weight decreased significantly at days 10 and 20, no significant changes were observed in cortisol levels, $n = 12$.

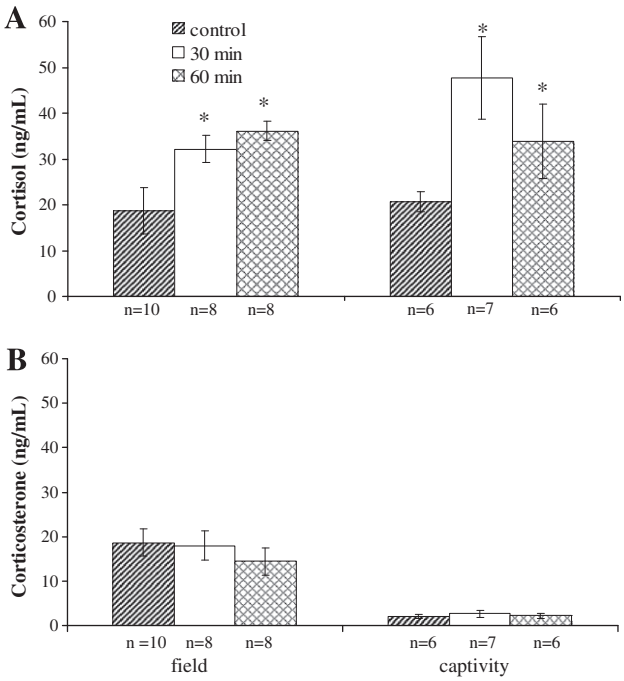

Fig. 4. Differential responses of cortisol (A) and corticosterone (B) to acute stress and captivity conditions in males of *Ctenomys talarum*. Individuals were subjected to 2 min of immobilization in the laboratory or in the field, immediately after capture. Blood samples were obtained within 3, 30 or 60 min after the finalization of the stressor. Control animals were not subjected to immobilization and represent baseline levels. Prior to blood sampling, field animals were also subjected to capture and confinement in plastic cages (see Section 2). Values indicate mean ± ES. * indicate statistical significant differences in immobilized animals relative to their respective controls ($p < 0.05$). Sample size is provided at the base of each bar.

free-living animals were unstressed, emphasizing that the subterranean niche indeed provides protection from physical and biological stressors. Such consistently low N:L ratio values can not be attained in tuco-tucos under even moderate stress [45] and Vera unpublished data). Also, although cortisol levels did not differ between day 10 in captivity and in the field (Fig. 3), N:L ratios remained higher in captive animals at day 10 in relation to field values [45], indicating that leukocyte counts are very sensitive stress indicators in the species. Hence, these data indicate that we were able to measure stress-independent fluctuations in GC concentrations throughout the breeding cycle in *C. talarum* males (i.e., strictly basal levels). Increases in the N:L ratio after exposure to a stressor occur rapidly in *C. talarum* (30 min after immobilization, unpublished data). Thus, for a given stressor to affect an individuals' cortisol levels, but not the N:L ratio, it must have occurred just a few minutes before capture, which is highly unlikely. To our knowledge, this is the first study that measures coupled seasonal fluctuations of GC levels and leukocytes proportions in free-living animals to discriminate between basal and stress-induced variations in GC concentrations. Though immune function may also vary seasonally [22] (which is not the case of our N:L ratio data), the counts of monocytes and eosinophils may help to distinguish leukocyte responses to stress from those caused by infection [16].

Our data also show that inter-annual variations in total cortisol levels exist, as individuals captured during NRS 2008 had lower cortisol concentrations when compared with individuals captured the previous year in the same season. Indeed, cortisol levels in field animals subjected to immobilization during NRS 2008 were similar to basal levels recorded during NRS 2007 (Figs. 2 and 4), which means that stress concentrations also differed between these seasons. Seasonal changes in basal as well as in stress-induced GC levels have been reported previously in other vertebrate species [35]. The evaluation of seasonal modulation of corticosteroid-binding globulin levels (CBG, which regulate free, biologically-active GC concentrations [11,39]) and GC receptor numbers might clarify temporal differences in stress-induced cortisol levels in future studies. However, our data on N:L ratios indicate that if any changes in CBG levels occurred, they would only represent fluctuations in free cortisol levels within a range of basal concentrations and not related to changes in the stress status of the individuals.

4.2. Cortisol and corticosterone responses to acute stress and captivity

Our immobilization experiments in the field and laboratory show that only cortisol is responsive to acute stress in our study species. The lack of response of corticosterone in animals that were immobilized in the field strongly argues against a typical GC role of this hormone in the response to acute stress since these individuals were subjected to multiple stressors that could affect their corticosterone levels after 30 and 60 min. These include removal from their tunnels after capture with the subsequent manipulation, immobilization treatment and confinement in plastic cages (see Section 2). We think that not having responded to this sum of stressors reasonably rules out the possibility that corticosterone would positively respond to any given natural stressor, such as intraspecific aggression or a predator attack. This is the first study to report such a contrasting response to acute stress of both GCs. In yellow-pine chipmunks, both GCs positively responded to acute stress [24,31,38], though in golden-mantled ground squirrels corticosterone was less responsive to stress than cortisol [38]. Overall, our results indicate that it should not be assumed that both GC, if present, are stress-responsive. On the contrary, this should be experimentally tested before interpreting GC data in free-living species. From a physiological point of view, these results suggest that cortisol may have a greater sensitivity to adrenocorticotropic hormone (ACTH) than corticosterone in this species. Most likely, corticosterone may not be responsive at all to ACTH within physiological levels in *C. talarum*, which would explicate the lack of response to acute stressors.

In the laboratory, the complete depletion in corticosterone levels was robust, as most samples were below the detection limit of the assay, including both control and stressed animals (Fig. 4B). The causes of this result are worth of future research and may shed light on the role of this hormone. One interesting possibility is the role of corticosterone in the regulation of food intake. Previous work demonstrates a link between central feeding systems and the regulation of corticosterone, indicating that this hormone is necessary for the stimulation of food intake [21,29]. Given that the animals were fed *ad libitum* during their stay at the laboratory (thus, we assume that they were satiated), we hypothesize that corticosterone secretion was inhibited to moderate food consumption. An additional possibility is that corticosterone functions mainly as a complement for aldosterone in mineral–water balance (i.e., functions as a mineralocorticoid, see [1]). Future studies will address these possibilities.

The lack of effect of captivity on cortisol levels probably reflects acclimation at the moment of sampling (i.e., day 10 in captivity). Previous results showed that animals arrive at the laboratory with 5-fold increased N:L ratio values, indicating a strong stress response to transportation, but then this values gradually decline till day 10, as they acclimate to the laboratory conditions [45]. Given the direct relationship between GCs and N:L ratio [16], these data suggest that cortisol may show a similar variation pattern during this period, remaining then stable from this moment onwards (Fig. 3). In coincidence, animals also showed a significant decrease in body weight at day 10 (~3.4%), but weight increased again at days 20 and 30 (see Fig. 3). Taken together, these data indicate that chronic stress is important during the first days of captivity and by day 10 animals acclimate to laboratory conditions. Repeated blood sampling at shorter time intervals was not performed to assure the animals' recuperation and avoid the effects of sampling on body condition and hormonal data.

4.3. Plasma GC concentrations in tuco-tucos in relation to other rodents

It is remarkable that plasma GC concentrations of tuco-tucos were low in comparison to other previously-assayed rodents such as Arctic ground squirrels [9], red squirrels [8], yellow-pine chipmunks [24,31], guinea pigs [25], the Degu [43], stripped mice [42], lemmings, golden-mantled ground squirrels [38], but similar to rat-like hamsters [26] and ground squirrels *Spermophilus saturatus* [10] and *Spermophilus beldingi* [30]. Lower plasma GC levels were reported for juvenile golden hamsters [18] and more recently for females of the colonial tuco-tuco *C. sociabilis* born in captivity [46]. There is a very large degree of variation in GC levels across rodent species [38] and the two studies in *Ctenomys* are at the lower extreme of this range ([46], this study). It is possible that other traits like CBG capacity and the numbers of GC receptors (i.e., sensitivity of target tissues) differ among species and compensate, at least in part, for variations in GC concentrations. Interestingly, cortisol and corticosterone were produced in similar amounts in the field, which also contrasts with previous studies in other rodent species showing marked differences in the amounts of both GCs [9,24,31,38], which include the colonial tuco-tuco *C. sociabilis* [46]. At present, the reasons for the enormous range of GC concentrations that exits among species remain poorly understood [38].

4.4. No effect of trapping

The lack of effect of trapping on hormone levels is a somewhat unexpected, but also interesting, result. From the recent studies

performed in wild mammals it is emerging that the effect of live-trapping on GC levels varies among species and depending on the particularities of the trapping technique employed. For instance, while Arctic ground squirrels responded to capture stress merely by increasing cortisol production [9], closely related Richardson's ground squirrels showed a rapid decrease in CBG levels which allowed a much more pronounced cortisol response [17]. Furthermore, in golden-mantled ground squirrels females increased GC concentrations in response to live-trapping but males did not [30]. Surprisingly, Fletcher and Boonstra [20] found no relationship between corticosterone levels and the amount of time spent by voles in a live trap (from 2 up to 16 h), though live-trapped animals had higher values than snap-trapped individuals. In our case, traps were located below the soil surface and were connected to the animals' tunnels, which could be expected to reduce the perceived risk of being caught in a trap. Likely, permanence within the traps for longer times (2 h or more) may trigger a measurable response of the HPA axis in tuco-tucos.

5. Conclusions

(1) To date, it is widely assumed that cortisol and corticosterone share the same physiological roles in free-living mammals and that their relative importance depends merely on their concentrations. However, our results show that both hormones are differently affected by environmental stimuli in *C. talarum*, showing distinct patterns of variation in the field and captivity and a differential response to acute stress, strongly suggesting differentiation in their roles. Thus, this study situates tuco-tucos as an interesting model for the study of HPA axis physiology and sets the basis for future research addressing the physiological foundations of these differences. (2) We recorded for the first time coupled seasonal fluctuations of GC levels and leukocytes proportions to allow discrimination between basal and stress-related seasonal variations in GC concentrations. Though the reasons underlying the seasonal changes in GC levels are difficult to address [35], our present data contributes to this issue showing that strictly basal-as opposed to baseline- GC levels can show seasonal variation in natural populations. (3) These data further highlights the important degree of variation and complexity of GC physiology in mammals pointed by Romero and colleagues [41], which indicates that this stimulating field has yet to be explored vastly.

Acknowledgments

We thank the members of Laboratorio Ecofisiología for helping us in the field samplings and laboratory work. We are indebted to Dr. Gustavo Somoza for providing cortisol for the validation assays. In addition, we want to express our gratitude to two anonymous reviewers whose suggestions significantly improved the manuscript. This study was supported by PIP 2287 granted to R. Zenuto and C.D. Antenucci and PICT-06 2102 granted to C.D. Antenucci. F. Vera was supported by a doctoral fellowship from CONICET.

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