

Do Cortisol and Corticosterone Play the Same Role in Coping With Stressors? Measuring Glucocorticoid Serum in Free-Ranging Guanacos (*Lama guanicoe*)



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ABSTRACT

Habitat can constrain and shape successful ecological and physiological strategies, thus providing the context for the evolution of life-history traits. However, unpredictable challenges, such as storms, natural disasters, and human activities can also have great effects on stress. Glucocorticoids (GCs) are adrenal steroid hormones that play an important role in how vertebrates cope with these predictable and unpredictable environmental challenges. Although assessing GCs levels can have many applications in the study of wildlife and/or captive animals, with or without capturing individuals, it requires a species-specific complete validation (analytical and biological) before its use. In this work, our aim was to: (a) validate a radioimmunoassay (RIA) for measuring GCs levels in *L. guanicoe* serum; (b) assess cortisol and corticosterone levels (if present) in serum of wild *L. guanicoe* individuals; and (c) compare the response to acute stressors (handling, shearing, and release). Our results successfully: (a) validated RIA for assess GCs levels in wild ungulates; (b) confirmed the presence for cortisol and corticosterone and showed that both GCs are differently affected by environmental stimuli in *L. guanicoe*; and (c) showed that GCs exhibit different patterns in the field and in response to acute stressors, making these camelids an interesting endocrinological model when seeking the adaptive functions of a given variation and further emphasizing the complexity of GC physiology in wild mammals. *J. Exp. Zool.* 319A:539–547, 2013. © 2013 Wiley Periodicals, Inc.

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Glucocorticoids (GCs, cortisol, and/or corticosterone depending on the species) are adrenal steroid hormones that play an important role in maintaining homeostasis and serve many adaptive functions in vertebrates (Wingfield, '97; Jacobs and Wingfield, 2000; Wingfield and Sapolsky, 2003; Goymann and Wingfield, 2004). GCs are secreted by the adrenal gland and regulate the availability of energy by influencing gluconeogenesis, glucose use and protein metabolism (Boonstra, 2005). Animals facing immediate environmental challenges (i.e., stressors) such as predation, aggression from conspecifics, food scarcity, high density, or others derived by human activities such as tourism (Creel et al., 2002) and management (DeNicola and Swihart, '97; Vilá and Lichtenstein, 2006; Carmanchahi et al., 2011), respond with the hypothalamic–pituitary–adrenal (HPA) mechanism. They react by increasing GCs above basal levels, which seems critical when coping with a challenge (Bartolomucci et al., 2001; Creel, 2001; Romero, 2004; Raouf et al., 2006). For this reason, a good measure of animal response to stress has been the plasma concentration of GCs (Sapolsky et al., 2000; Wingfield and Kitaysky, 2002; Wingfield and Sapolsky, 2003; Romero, 2004; Palme, 2005).

Conservation biologists are often concerned with mitigating the effects of environmental conditions and human-induced disturbances on wildlife (e.g., Foley et al., 2001; Millsaugh et al., 2001; Tempel and Gutierrez, 2003). Determination of GCs levels is important in physiological ecology (in the field and/or captivity) studies when assessing the interplays between the physiology mechanism (and their adaptive function) that individuals use to cope with social environment, climate change, behavior, and reproductive challenges. Moreover, and considering that chronic stressors usually produce negative effects on the HPA axis (Sapolsky, '92; Tilbrook et al., 2000; Wingfield and Sapolsky, 2003), the evaluation of GCs may be a valuable complement in conservation studies aimed at monitoring animals' health and fitness. For animals in zoos, the effects of an animal's surroundings, such as space, food availability, and social conditions (e.g., Wielebnowski et al., 2002) are often of interest.

Thus, the effects of stress are considered as having important conservation and economic implications.

The most important and biologically relevant GCs are cortisol and corticosterone. 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; Boswell et al., '94; Kenagy and Place, 2000). Since Kenagy and Place (2000) pointed out that the physiological meaning of the two different plasma GC hormones should be studied, little progress has been gained in shedding 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 (Kenagy and Place, 2000; Romero et al., 2008). One experimental approach to evaluate if cortisol and corticosterone indeed accomplish the same functions in a given species may be to compare their responses to management activities (e.g., handling, shearing, and release) and their patterns of variation under natural conditions. While similarities would confirm the accepted view of shared physiological functions, differences would suggest that the hormones do not completely overlap in their roles and exhibit some differences in their functions.

Stress is generally viewed as a negative consequence of captivity (Balcombe et al., 2004; Morgan and Tromborg, 2007). However, field studies tend to view stress as a natural process of life and focus on the physiological mechanism and their adaptive functions (Creel, 2001; Reeder and Kramer, 2005; Ovejero and Carmanchahi, 2012). Although prolonged activation of the HPA axis can negatively impact reproduction, immune function and growth (De Kloet et al., 2004; Goymann and Wingfield, 2004), short-term activation of this system can be beneficial, resulting in mobilization of energy reserves needed to respond to acute environmental challenges (Sapolsky et al., 2000; Romero, 2004; Bonier et al., 2009).

Given the complexity of these responses and the context-dependent manner in which they have been interpreted, data from both captive and free-ranging conspecifics can enhance our understanding of how environmental conditions affect physiological stress. A key issue is to validate the assay methodology to insure that the species-specific levels of hormones are detectable (analytical validation) and that the level detected is indicative of the level of adrenal activity (biological validation) in the species studied (Palme, 2005). This can be accomplished pharmacologically by administering ACTH (the most widely used challenge test) to stimulate adrenal hormone production, or administering dexamethasone to suppress adrenal function (Palme, 2005). However, we suggest other alternative methods to stimulate adrenal responses.

Guanacos (*Lama guanicoe*) are the largest wild-endemic artiodactyls of South America and have a resource-defense polygyny mating system (Raedeke, '79; Franklin, '83). In this

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territorial system, males compete for access to resources required by females. The territorial defense effort has been assessed in family groups, bachelors and solitary males of guanacos (Young and Franklin, 2004; Marino and Baldi, 2008), however systematic comparisons of GCs levels between different disturbances and their intensity are lacking. Thus, the measurement of GCs hormones would allow us a further integration and grasp between different levels of biological analysis. By understanding individual physiological mechanisms (e.g., stress response) triggered by a given biological process, we can predict the adaptive significance of that mechanism and how it is related with survival or reproductive success.

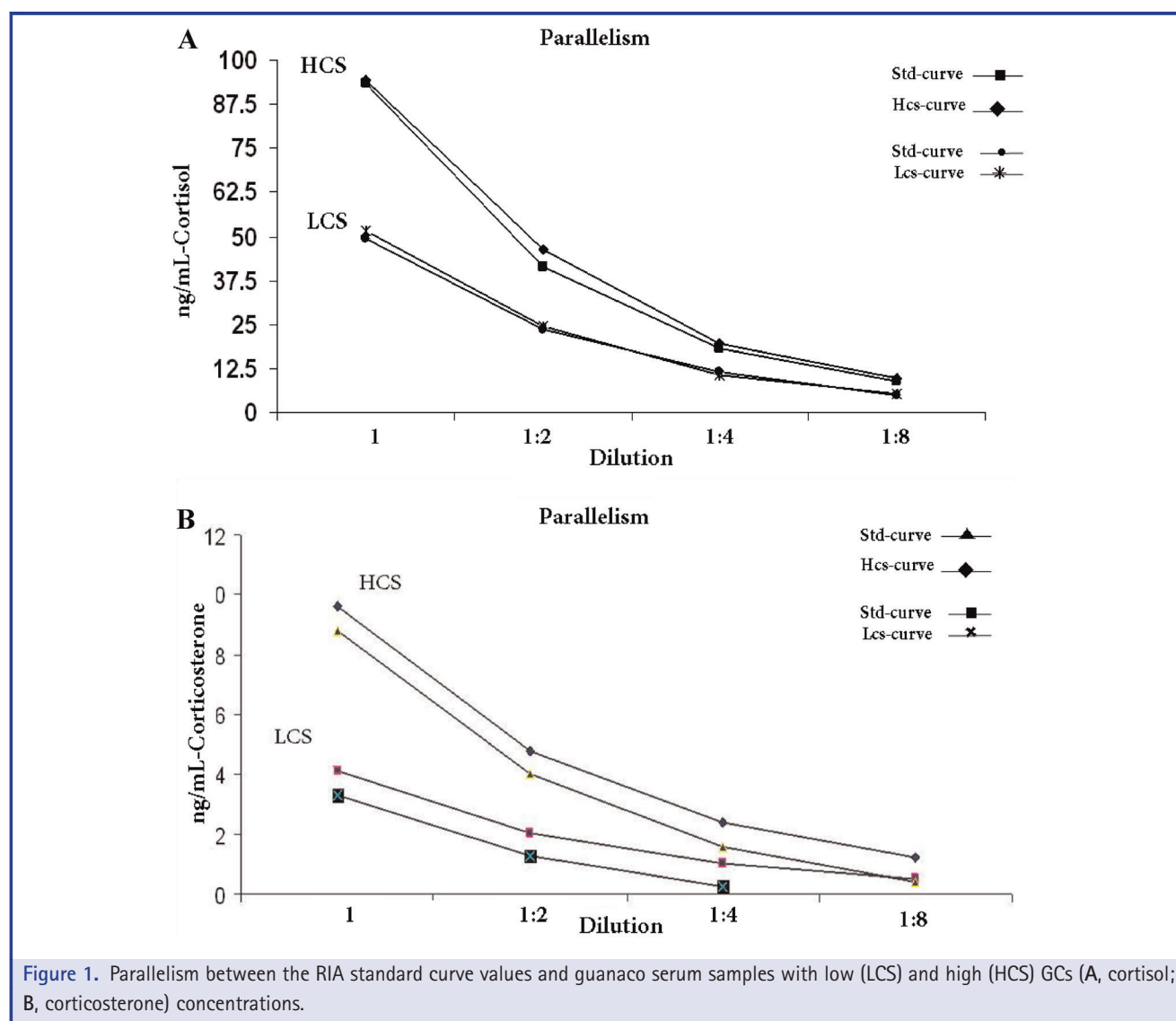
The goals of this study were to: (a) validate a radioimmunoassay (RIA) for measuring GCs levels in *L. guanicoe* serum; (b) assess cortisol and corticosterone (if present) functional levels in the

serum of free-ranging guanacos; and (c) compare *L. guanicoe*'s response to acute stressors (handling, shearing, and release).

MATERIALS AND METHODS

Study Area, Animals, and Sample Collection

The Payunia Provincial Reserve is located in the south of Mendoza province in Argentina (between 36.000°S and 36.360°S; 68.340°W and 69.230°W; Fig. 1) and covers approximately 4,500 km², of which 2,000 km² are state-owned. The climate is typically continental desert with average temperatures of 6°C in winter and 20°C in summer, and an average annual precipitation of 255 mm. The reserve is included in La Payunia Phytogeographic Province within the Andean-Patagonian domain (Martinez Carretero, 2004) and presents a semiarid landscape dominated



by grasslands and scrublands. La Payunia reserve has suffered severe land degradation due to cattle overgrazing (Paruelo, 2005). In this region, human populations are scarce and goat breeding is the basis of a subsistence economy (Lichtenstein and Carmanchahi, 2012). Nevertheless, this area supports the most numerous migratory guanaco population in the central region of Argentina, with more than 10,000 individuals (Candia et al., '93). In view of this situation, technicians of natural resource agencies with the collaboration of biologists of the Wildlife Conservation Society and Physiological Ecology Research Group bring the rural settlers of La Payunia support in implementing a sustainable program for managing, shearing and improving the conservation status of guanacos in the Patagonia (Carmanchahi et al., 2011).

We collected 115 blood (in heparinized tubes) samples from every individual that was sheared in order to assess the effect of management (during the spring season) on stress response in guanacos. In addition, we measured rectal temperature, cardiac and respiratory frequencies, and identified individuals by sex and age. Approximately 10 mL of blood was extracted (in less than 3 min) from the femoral vein of each guanaco using a 10 mL syringe. Then, the blood was allowed to clot at room temperature and centrifuged at 3,300g for 20 min using a 12 V portable centrifuge, and serum samples were immediately frozen for posterior analysis in the laboratory.

Cortisol and Corticosterone Responses to Acute Stress

Wild guanacos were captured by horseback riders driving them towards a corral trap to evaluate the role of cortisol and corticosterone in the response to acute stress (Carmanchahi et al., 2011). The animals were removed from the holding pen, immobilized by binding the legs, then placed on a gurney and taken to the shearing machines. Shearing was carried out using an electric machine fed by a generator. The shorn individuals were sampled as described below, identified with numbered and colored (according to sex) collars, and then released.

Hormone Profile Analysis

GC concentration was measured in unextracted serum using a commercial 125I-Cortisol radioimmunoassay kit (IM-1841-Immunotech; Beckman Coulter Company, Prague, Czech Republic) and RIA for corticosterone (07-120102-MP; Diagnostic, Tigre, Buenos Aires, Argentina). To validate the method for guanaco serum, total steroids were extracted (Ovejero, 2013) twice from 1 mL of selected serum samples with 5 mL diethyl ether. The organic phases were pooled, evaporated and re-suspended in 1 mL of phosphate-buffered saline (PBS, pH 7.4). Initially, serial dilutions of extracted samples were run in parallel with the unextracted samples from the same animal in the same assay. Because no significant differences were observed between extracted and unextracted samples, no further extractions were performed ($F=4.30094946$, degree of freedom (DF)=22, $P=0.1549$). The assay is capable of measuring cortisol concen-

trations up to 633 ng/mL and corticosterone concentrations up to 1,000 ng/mL. Detection limit is 3.075 and 0.625 ng/mL, respectively, as provided by the manufacture. All samples were assayed in duplicate. Cross-reactions of the antibodies used in both assays with other structurally similar molecules are very low, as reported by the manufacturers.

Validation of Assays

Both assay kits have been validated for use in *L. guanicoe* (Ovejero, 2013). We examined the parallelism between the standard curves (and the high and low concentration calibrator) of the RIA and serially diluted (1:2; 1:4; 1:8) low and high GC concentrations in serum samples collected in the field (see Results Section). We used ten serum samples of approximately 94.43/93.23 ng/mL (higher GCs values) and 51.48/49.53 ng/mL (lower GCs values); all the samples were pooled (higher and lower group samples) and diluted using PBS buffer (pH 7) before the assay.

STATISTICAL ANALYSES

We used maximum likelihood-analysis to estimate parameters for different models to find the best goodness of fit and the best function that explained GC stress response. All models were built using R-program (version 2.9.0, www.r-project.org) to compare the effects of all variables, choosing the best model according to Akaike's information criterion.

ETHICAL NOTE

The animals were cared for in accordance with the Guidelines for the use of animals in research and teaching (ASAB/ABS, 2003) and the "Good Practices of Wild Guanacos Handling Protocol" (Carmanchahi and Marull, 2012).

RESULTS

Validation of Assays

The serial dilution of pooled guanaco serum samples indicated no deviations from parallelism between regression slopes of samples and GCs standards (Cortisol- $F_{1,6}=3.4$, $P=0.24$; standard slope = -0.825; sample slope = -0.902; Corticosterone- $F_{1,6}=2.4$, $P=0.15$; standard slope = -0.735; sample slope = -0.806). Furthermore, higher and lower GCs values in both set of samples were in parallel to the standard curves of the GCs RIA (Fig. 1A,B). The inter- and intra-cortisol assay coefficients of variation were 8.8% and 3.5%, respectively; corticosterone assay coefficients were 7.5% and 2.4%, respectively. Taken together, these results show that cortisol and corticosterone can be measured directly in serum samples of *L. guanicoe* without an extra extraction step.

Cortisol and Corticosterone Responses to Acute Stress

As we expected, GCs have an acute stress response due to human activities like handling, shearing, and release. However, a nonexpected result was the different hormonal response elicited

by cortisol versus corticosterone (Table 1) under the same intensity of perturbation, with a higher response by cortisol than corticosterone (Fig. 2A,B). This differential response between both hormones under the same perturbation would be a very interesting topic for further research, in order to try and understand the adaptive significance of these responses.

Relationship between GCs and biological processes. We found that the best function that explained GCs kinetic triggered by the total retention time (handling, shearing, and release) is of polynomial (rational) type and not linear (Table 2). The function is defined by set of parameters (a , b , c , sig) and the equation is $=ax - c/b + (x - c)$, where a , individual GCs levels and their threshold response; b , total retention time; c , scaling parameter, indicated as the difference in time of the initial hormonal response; and sig = data variability.

DISCUSSION

Studies in wild species have emphasized the large interspecific variation that exists in plasma GC levels, the relative proportions of cortisol and corticosterone in plasma, the seasonal patterns of GC variation and the responses of plasma-binding globulins to stressors, among other aspects (Boonstra and McColl, 2000; Boonstra et al., 2001; Romero, 2002; Romero et al., 2008). However, the intraspecific (or intrapopulation) temporal variation in these characteristics is generally considered as very low or not considered at all. Particularly, the identity of the dominant GC in plasma, as well as the seasonal patterns of GC secretion, is assumed as characteristic for a given species. These “patterns” may be valid for some species, but our present data indicates that this view may be, at least in part, the result of the lack of long-term research program assessing variations and functions in both cortisol and corticosterone concentrations in natural populations. Therefore, this is the first study that measures and validates GC RIA for free-ranging *L. guanicoe* in South America.

It is widely assumed that cortisol and corticosterone share the same physiological roles in free-range mammals and that their relative importance depends merely on their concentrations (Bartolomucci et al., 2001; Creel, 2001; Romero, 2004; Raouf et al., 2006). This study shows that cortisol responds to the acute stress (management of guanacos is a valid process that triggers a stress response, as an alternative method to biological validation) response signal in *L. guanicoe* but corticosterone does not. This is a remarkable result considering (in face of the similar physiological role assumption) that serum cortisol levels were higher than

corticosterone concentrations (especially under field conditions). Considering that the determinations of cortisol and corticosterone levels were performed using the same serum samples, our data shows that these animals increased their plasma cortisol concentrations while keeping their corticosterone levels “stable.” The fact that cortisol and corticosterone differed in their patterns of variation in free-range individuals suggests that, under natural conditions, both hormones are affected differently by environmental stimuli or that they are subject to different endogenous regulations of their seasonal secretion. Thus, our results indicate that both hormones are differentially regulated in the species, strongly suggesting differentiated physiological roles for both hormones in *L. guanicoe*.

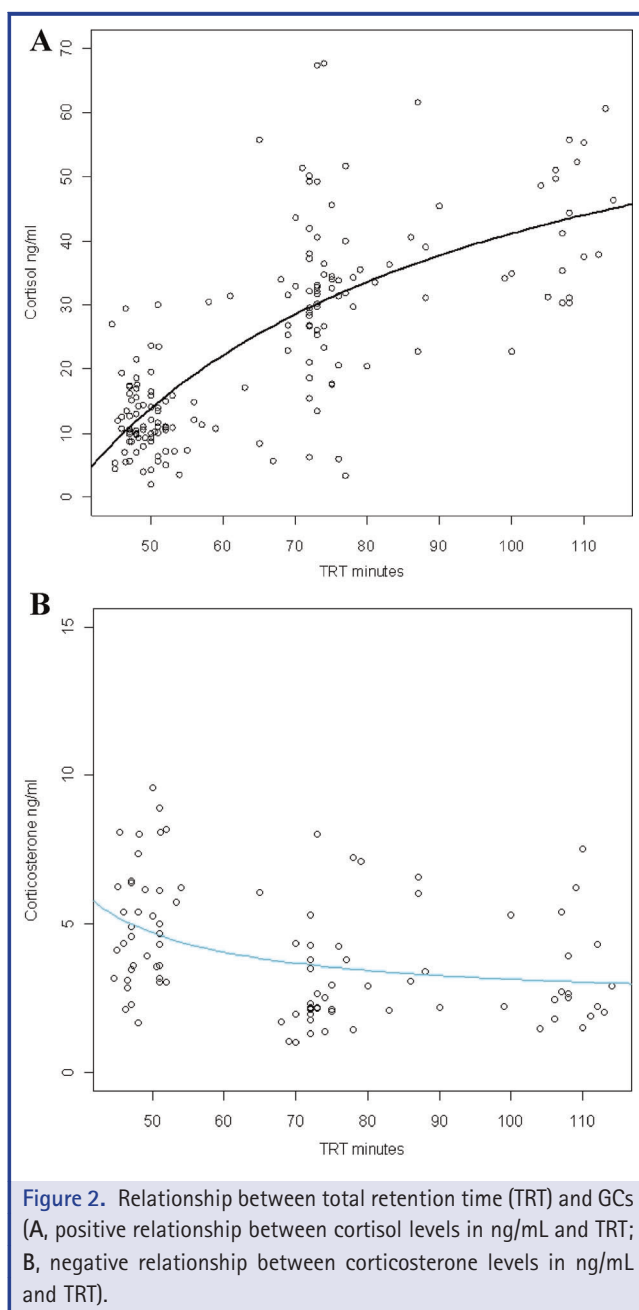
Comparisons of the responses of cortisol and corticosterone to ACTH and/or acute stress are relatively scarce in the literature. In deers (*Cervus elaphus*; Smith and Bubenik, '90) and ferrets (*Mustela putorius*; Rosenthal et al., '93), both cortisol and corticosterone increased after ACTH administration. Vera et al. (2012) observed a positive response in *Ctenomys talarum* of cortisol and a lack of response of corticosterone to ACTH, in concordance with the same pattern of responses exhibited by these hormones to acute stressors (Vera et al., 2011). The responses of both GCs to acute stressors and patterns of variations in the wild also show considerable variation among species. In golden-mantled ground squirrels (*Spermophilus saturatus*), cortisol levels showed a more robust response to restraint and handling than corticosterone (Romero et al., 2008) and both hormones showed contrasting seasonal variation patterns (Boswell et al., '94). However, in chipmunks (*Tamias amoenus*) and brown bats (*Myotis lucifugus*), both GCs showed a similar patterns of variation and response to handling and/or restraint (Kenagy and Place, 2000; Reeder et al., 2004).

Another widely known assumption is that GC are critical mediators of homeostatic balance and allostatic load (Goymann and Wingfield, 2004; McEwen and Winfield, 2003), meaning that the typical well-known roles of GCs in the mobilization of energy reserves largely prevail over other physiological functions. Therefore, increased plasma GCs levels are almost always interpreted as an indicator that the animal is in an energetically demanding situation or facing acute or chronic environmental stressors.

Nonetheless, there exists recent evidence indicating that both hormones may have different physiological functions (Schmidt and Soma, 2008; Schmidt et al., 2010; Vera et al., 2011, 2012). Moreover, there is evidence showing that GCs have also

Table 1. GCs response due to stress agent, but with different intensity.

GCs	Stress agent	<i>n</i>	Media	DE	EE	$1 - \alpha = 0.95$
Cortisol	Total retention time	94	27.01	15.19	1.56	$x \pm 3.07$
Corticosterona	TRT	94	3.98	2.08	0.21	$x \pm 0.422$



mineralotropic actions by increasing the absorption of Na⁺ in different portions of renal tubules and intestine (see Agarwal and Mirshahi, '99). Indeed, Angiotensin II stimulates the secretion of cortisol and corticosterone from the adrenal glands in bovines (Bird et al., '89; Rainey et al., '91; Rábano et al., 2004).

Accordingly, one main question emerged from our results: What are the possible role/roles of corticosterone in this free-range ungulate? Vera et al. (2012) suggested that the possible role of

Table 2. Differences between the tested models used to determine the effect of guanaco handling time on serum GCs levels.

Model/functions	GCs	AIC	df	dAIC	Weight
Rational					
Michaelis–Menten	Cortisol	408.8	3	29.7	<0.001
Michaelis–Menten	Cortisol	379.1 ^a	4	0.0	0.5
With scaling	Cortico	399.2 ^a	4	0.0	0.289
Exponential-based					
Monomolecularcortisol	Cortisol	398.6	3	19.5	<0.001
	Cortico	399.4	3	0.3	0.261
Polynomials					
Lineal	Cortisol	394.3	3	15.1	<0.001
	Cortico	400.5	3	1.3	0.154
No lineal	Cortisol	379.1 ^a	4	0.0	0.5
	Cortico	399.1 ^a	4	0.0	0.296

^aBest goodness of fit.

corticosterone in free-range *C. talarum* is mineral–water balance. We think it is remarkable that these issues have been very rarely considered in studies regarding the physiology of the HPA axis in wild species in spite of the large amount of evidence coming from studies using laboratory and domestic animals. In regards to this, Rosenmann and Morrison ('63) tested the physiological response to heat and dehydration in the guanaco and concluded that the ability of the camel and the donkey to resist severe dehydration (Schmidt-Nielsen et al., '56; Charnot, '60) appeared to be shared by the guanaco. Therefore, we suggest that the possible role of corticosterone in free-range guanacos could be mineral–water balance. Thus, this study renders guanacos as an interesting model for studying HPA axis physiology and sets the basis for future research addressing the physiological foundations of these differences.

Plasma GCs Concentration in *L. guanicoe* in Relation to Other Species

A cortisol response can be triggered by behavioral patterns (Cavigelli, '99), social dominance (Creel, 2001) or seasonality (Huber et al., 2003), and is essential for the adaptive short-term response to stressors (Sapolsky, '92). The complex interactions between these factors make baseline GC values difficult to assess in free-range mammals (Reeder and Kramer, 2005). Both acute and chronic stressors can cause increases in plasma GC levels, as well as changes in clinical parameters (Montane et al., 2003). In this study, the increase in cortisol levels with manual restraint time is consistent with data reported for other mammals, such as the black-back jackal (Van Heerden and Bertchinger, '82), bighorn sheep (Kock et al., '87), wild impala (Hattingh et al., '90), roe deer (Montane et al., 2003), and vicuñas (Bonacic et al., 2006).

CONCLUSIONS

The present study highlights some issues that may be considered in future studies that involve large free-range mammals (wild camelids, in this case).

1. We consider that management activities (time of manipulation as a stressor) are a valid method for testing the biological validation of hormonal stress response, as an alternative method that measures the same response as an ACTH treatment.
2. We consider that the validation of a relevant step (direct measure of GCs in samples) in laboratory methods are beneficial in time and money.
3. This is the first study that measures and validates GC RIA for this wild South-American camelid.
4. The fact that cortisol and corticosterone differed in their patterns of variation in free-range individuals suggests that, under natural conditions, both hormones are differently affected by environmental stimuli or that they are subject to different endogenous regulation of their seasonal secretion.
5. We consider it interesting to study the possible role/roles of corticosterone in this species and in other wild free-range mammals.
6. It should not be assumed that both cortisol and corticosterone are stress responsive and regulated mainly by the HHA axis.

Thus, a lot of work is still needed in order to have a better understanding of the roles of cortisol and corticosterone in free-range animals. We hope these models will be useful in generating predictions for future studies and resolving the inconsistencies that currently complicate interpretation for conservation endocrinologists.

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