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Corticosterone measurement in Komodo dragon shed skin

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The analysis of corticosterone (CORT), the main glucocorticoid in reptiles, via blood or faeces provides an index of hormone concentrations over a relatively short time period. Unlike these conventional matrices, snake shed skin is supposed to incorporate circulating CORT over the period of skin growth, thus reflecting long-term retrospective levels of the hormone. The present study aimed to assess the feasibility to extract CORT from shed skin of Komodo dragon and biochemically validate the quantification of the hormone by enzyme immunoassay (EIA). Additionally, possible sources of variation in shed skin CORT that could reflect biological variation were examined (sex, age, body region and season of ecdysis). Results of the biochemical validation showed that CORT can be reliably measured in shed skin of Komodo dragon by EIA through the presented methodology. Males presented statistically higher levels of CORT than females, and when accounting for males' seasonal differences, concentrations decreased significantly from spring to summer. Juveniles showed higher CORT values than adults, however, results should be interpreted with caution since the model revealed that date of ecdysis was significantly influencing CORT levels. Besides that, concentrations of CORT were not influenced by body region. Overall, the present study demonstrates a potential biological source of variation in shed skin CORT concentrations due to sex, age and season of skin ecdysis. Combined with other indicators, detection of CORT concentrations in shed skin could allow a systematic control of Komodo dragon's physiology, offering a useful tool for zoo management and providing key data for the species conservation.

Key words: Chronic stress; Ecdysis; Glucocorticoid; Saurian

INTRODUCTION

The secretion and regulation of glucocorticoids (GCs) through the hypothalamic-pituitary-adrenal (HPA) axis is a fundamental endocrine response to stress (Moberg & Mench, 2000; Sapolsky, 1992). These hormones have glucoregulatory functions and mobilise energy reserves to re-establish homeostasis (Turner et al., 2012). Acute increases in GCs, such as after a severe storm or pursuit by a predator, can trigger a suit of physiological and behavioural changes that facilitate survival (Johnstone et al., 2012; Dantzer et al., 2014). Nevertheless, long-term repeated or prolonged activation of the HPA axis and high levels of these hormones, known as chronic stress, can have detrimental effects on reproduction, the immune system, growth and the general health condition (Moberg & Mench, 2000; Reeder & Kramer, 2005). While there are many environmental factors that can cause either acute or chronic stress responses, several intrinsic factors related to the animal's biology can also influence GC levels (Cockrem, 2013; Busch & Hayward, 2009). Documenting how these biological attributes (e.g. species, body condition, age, sex) influence the stress responses is imperative for properly interpreting how environmental challenges or anthropogenic disturbances impact on animals' physiology (Dantzer et al., 2014; Busch & Hayward, 2009).

In reptiles, most stress-related studies use blood samples to measure corticosterone (CORT) (Tokarz & Summers, 2011), the main GC in reptiles (Cockrem, 2013). However, this method has important limitations. Circulating CORT levels can vary significantly over short periods of time, such as in response to capture and handling, or due to the circadian cycle (Romero & Reed, 2005). To solve these issues, some studies have tested the use of faeces as a non-invasive matrix to evaluate CORT concentrations (Ganswindt et al., 2014; Halliday et al., 2015; Kalliokoski et al., 2012; Rittenhouse et al., 2003). Levels of CORT in faeces have been successfully correlated to circulating hormone levels (Halliday et al., 2015) and also to ethological indicators of reptile stress (Kalliokoski et al., 2012). Corticosterone levels assessed through both blood and faeces are, however, indicative of relatively short-term activity of the HPA axis. Recently, the assessment of CORT concentrations in snake shed skin has been suggested to inform on the long-term HPA axis activity in a variety of species, including African house snake, Eastern Massasauga rattlesnake, Dumeril's boa and European asp (Carbajal et al., 2014a; Berkvens et al., 2013). The advantages of this sample type over traditional ones are that collection is non-invasive, samples are comparatively simple to obtain and easy to store at ambient temperatures. The

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Figure 1. Collection of shed skin samples from a Komodo dragon. Samples were collected under protected contact using tweezers.

most significant improvement by analysing shed skin CORT concentrations is, indeed, that the window of hormone detection is expected to be extended to weeks or even months compared to the other conventional matrices (Berkvens et al., 2013). As hypothesised, CORT is deposited in the new epidermal layer during its differentiation and keratinisation, and possibly also while it is in place (Berkvens et al., 2013). Accordingly, shed skin CORT concentrations should reflect circulating CORT levels secreted at least since the previous moulting (ecdysis). Therefore, the measurement of shed skin CORT concentrations can be an excellent non-invasive method to assess long-term secretion of steroid hormones for both wild and captive animals, providing useful data for any conservation programme or for captive species management. However, as CORT can vary due to life-history differences or seasonal factors between individuals, validation of this matrix deserves more detailed evaluation.

The Komodo dragon (*Varanus komodoensis*), the world's largest living lizard (Auffenberg, 1981), endemic to five tiny islands in Eastern Indonesia (Purwandana et al., 2014). The species faces a variety of ecological and anthropogenic stressors, such as the loss of their natural habitat or the increasing human population and distribution, being classified as vulnerable by the IUCN (2014). Studies on free living Komodo dragons are mainly focused on the assessment of demographic parameters, such as population size and distribution (Jessop et al., 2007; Purwandana et al., 2014, 2016). When using captive individuals, most of the studies look at management issues, such as the breeding and husbandry of this species (Ley et al., 2006; Sunter, 2008; Trooper et al., 2004). Research on the endocrine activity can improve our understanding of animal biology since hormones affect many body tissues (Kersey & Dehnhard, 2014). Nevertheless, until the present, only one study has assessed the CORT stress response in a species of monitor lizards (Jessop et al., 2015), while the Komodo dragons' endocrinology remains largely unknown. The analysis of shed skin CORT concentrations in Komodo dragons could be of particular interest for non-invasively assessing stress-related hormones and thus gain further insights into the physiology of this species. Accordingly, the present study aimed to: (i) assess the feasibility to extract CORT from shed skin of this endangered species and biochemically validate the quantification of shed skin CORT concentrations by enzyme immunoassay (EIA); and (ii) to examine sources of variation in shed skin CORT that may reflect biological variation (sex, age, body region and season of ecdysis).

METHODS

Animals were managed following the principles and guidelines of the Ethics Committee of Parc Zoològic de Barcelona.

Animals and shed skin sampling

Shed skin samples were collected between March and October 2014 from five Komodo dragons; two adult males, one adult female and two juvenile females, housed at Barcelona Zoo (Spain). Over the period of the study, 39 shed skins were collected with an interval time of 10.56 ± 16.29 days (mean ± SD) between sheds and stored at room temperature. Shed skin was collected with tweezers during training sessions under protected contact (Fig. 1). This technique allowed the collection and identification of shed skin samples originated from three different body regions (head, body and limbs) of each individual (Fig. 1).

Hormone extraction and analysis

Extraction procedures were performed during the following two weeks after all the samples had been collected. The extraction methodology was performed using a methanol-based technique modified from Berkvens et al. (2013). Each sample was placed into a 15-mL conical tube and first washed with distilled water to remove sand or small stones adhered at the shed skin. Distilled water was added to the conical tube until samples were completely covered by the liquid and vortexed vigorously for 2 min. Samples were allowed to dry for 48 h on a paper tissue at room temperature (20 - 25 ˚C).

Afterwards, samples were washed with 70% methanol to remove possible external sources of CORT coming from blood or faeces. Once samples were completely dried, they were minced with a ball mill (MM200 type, Retsch, Germany) for 2 minutes at 25Hz. For steroid extraction, between 50 and 100 mg of each powdered sample were incubated with 6 mL of 80% methanol (Scharlab, Spain) for 18 h at 30˚C with gentle shaking (G24 Environmental Incubator Shaker; New Brunswick Scientific Co. Inc., Edison, USA). Following extraction, samples were centrifuged at 1750 x g for 15 min and 1.5 mL of the supernatant was transferred to a new aliquot. Samples were then placed in an oven at 38˚C. Dried extracts were reconstituted with 0.2 mL of EIA buffer provided by the EIA kit. This reconstitution volume was selected following previous hormone extraction methodologies in other keratinous samples performed by our laboratory (feathers: Carbajal et al., 2014b, Monclús et al., 2017; hair: Tallo-Parra et al., 2015) and immediately stored at -20˚C until analysis.

Hormone assay & biochemical validation of the EIA

Corticosterone concentrations from shed skin extracts and all the validation tests were determined by using competitive EIA kits (#402510 Neogen® Corporation Europe, Ayr, UK). Manufacturer reported cross-reactivity as follows: Deoxycorticosterone = 38%, 6-hydroxycorticosterone = 19%, progesterone = 5.1%, tetrahydrocorticosterone = 2.7%, prednisolone = 1.5%, cortisol = 1.1%, pregnenolone = 0.85%, 11-epicorticosterone = 0.78%, cortisone = 27%, 21-desoxycortisol = 0.24%, d-aldosterone = 0.13%, testosterone = 0.12%, 17 α-hydroxyprogesterone = 0.12%, prednisone = 0.10%, dexamethasone = 0.03%, cholesterol < 0.01%.

The biochemical validation of the EIA was carried out by following the criteria for an immunological validation: specificity, accuracy, precision and sensitivity (Reimers & Lamb, 1991). Extracts from 10 random samples were pooled for the assay validation. Intra-assay coefficient of variation (CV), and thus precision, was calculated by running samples by duplicate. The specificity was evaluated with the linearity of dilution, determined by using 1:1, 1:2, 1:5 and 1:10 dilutions of the pool with EIA buffer. Specificity was also evaluated with a parallelism test by comparing two different calibration curves (the standard curve and a pool curve created with serial dilutions following the standard curve pattern). To test for accuracy, different volumes of the pool were spiked with different volumes of standard steroids of known concentrations (0.22, 2.13 and 7.81 ng CORT/ml). The spike recovery was assessed measuring the final recovery of the known amounts of CORT added to the sample pools. The sensitivity of the test was given by the smallest amount of hormone concentration detected.

Statistical analysis

Data were analysed using R software (R-project, Version 3.0.1, R Development Core Team, University of Auckland, New Zealand) with a *P*-value below 0.05 as a criterion for significance.

For the biochemical validation, Pearson's Product Moment correlation was used to evaluate the correlation between obtained and expected values from serial dilutions and from spiked pool extracts with hormone standards. The test was also applied to calculate the relationship of the parallelism between the standards and the serially diluted pool extract.

The assumption of normality was checked using a Shapiro–Wilk test and concentrations were log-transformed to achieve normality. A linear mixed model with all data could not be performed since samples were not uniformly distributed among the independent variables of study. Accordingly, three separate linear mixed models, with Komodo subjects as a random factor to control for pseudo-replication, were used. Model 1 accounted for sex differences in shed skin CORT concentrations employing samples from adult Komodo dragons collected during spring. Model 2 tested for age effects on CORT levels from adult and juvenile females. Finally, model 3 tested for seasonal patterns in shed skin CORT concentrations from adult male samples. Shed skins from three different body regions were obtained throughout the study, therefore, this variable was included as a fixed factor in each of the three separate models. Additionally, date of ecdysis was included as covariate in each model. Selection of the best fit model was performed based upon Aikake's Information Criteria corrected for small sample size (AICc). We calculated AICc, ΔAICc (difference between each model's AICc and that of the lowest model) and Akaike weights. Candidate set models were chosen for which ΔAIC ≤ 2.

RESULTS

Biochemical validation of the EIA

The intra-assay coefficient of variation was 4.08 ± 3.35 % (mean ± S.D.). The obtained and expected corticosterone concentrations were significantly correlated (r = 0.99, P < 0.01; Fig. 2). In the spike-and-recovery test, hormone standards spiked with the pool presented a mean recovery percentage of 103.77 ± 11.82 % (mean ± S.D.) and obtained and expected values were significantly correlated (r = 0.90, P < 0.01). Corticosterone concentrations from the standard curve and the pool curve obtained in the parallelism test showed correlation (r = 0.99, P < 0.01; Fig. 3). The sensitivity of the assay was 0.22 pg CORT/mg shed skin.

Corticosterone concentration in shed skin

Corticosterone was detectable in all shed skin samples (Table 1). Shed skin CORT concentrations varied significantly between males and females (Table 2). When testing for age variances on shed skin CORT concentrations, juveniles presented significantly higher CORT levels than adult Komodo dragons although date of ecdysis showed an influence on shed skin CORT concentrations (Table 3). Season had a significant effect on CORT concentrations, with higher levels observed on skins shed during spring (Table 4). No influence of body region on shed skin CORT concentrations was detected in any of the models.

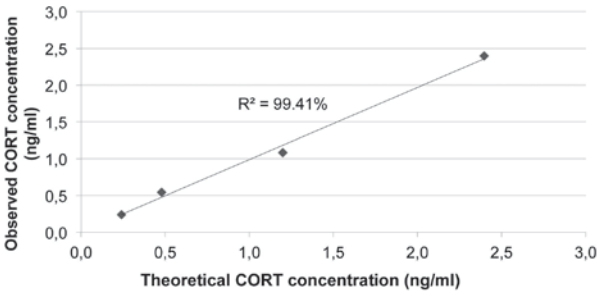

Figure 2. Correlation between observed and theoretical cortisol concentrations obtained in the dilution test (Pearson's correlation; $r = 0.99$, $P < 0.01$).

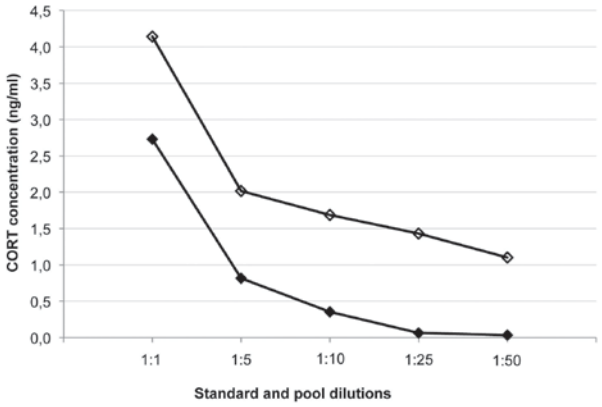

Figure 3. Parallelism relation between lines from the standard (black diamonds) and sample pool (white diamonds) curves obtained in the parallelism test (Pearson's correlation; $r = 0.99$, $P < 0.01$).

Table 1. Distribution of CORT concentrations (pg/mg) in Komodo dragon shed skin obtained for the four potential sources of variation analysed.

Variable	Level	# Samples	Mean ± S.D.	Median	Range
Sex	Female	11	11.10 ± 7.50	11.80	0.94 - 23.74
	Male	10	31.23 ± 19.70	25.78	16.9 - 76.37
Age	Adult	11	11.10 ± 7.50	11.80	0.94 - 23.74
	Juvenile	5	58.80 ± 18.50	53.66	33.73 - 78.37
Season	Spring	10	31.23 ± 19.70	25.78	16.9 - 76.37
	Summer	13	12.45 ± 7.02	11.87	4.22 - 25.58
Body	Head	10	21.87 ± 20.83	17.10	4.32 - 76.37
region	Body	14	17.87 ± 11.93	16.00	4.63 - 53.66
	Limbs	15	28.09 ± 26.16	25.58	0.94 - 78.37

DISCUSSION

Although the importance of studying threatened species endocrinology is evident, such studies are difficult to perform compared to studies on lab or domestic animals. One of the biggest challenges is that the access to samples is limited, even when using captive individuals. In addition, the invasiveness of most sampling methodologies hinders even more the study of these species physiology. In this paper we present a non-invasive and easy to obtain tool to study an emblematic threatened species which can give insight, for the first time, into their endocrine activity. Results of the current study show that CORT concentrations can be reliably measured in shed skin of Komodo dragons. To our knowledge, this is the first study reporting successful measurements of CORT levels in shed skin of any saurian. The sample processing and hormone extraction method presented here enabled the detection of CORT levels in all Komodo dragon samples. In addition, the detection of this hormone was biochemically validated, demonstrating the suitability of a commercial EIA kit in the quantification of CORT in samples processed through the stated methodology. As affirmed by Buchanan & Goldsmith (2004), this step is of primary importance when new techniques to measure steroid hormones are being developed.

In reptiles, as observed in other vertebrates, sex, age, body condition, health, season and the reproductive state can be sources of variation of the stress response (Moore & Jessop, 2003). We aimed to examine the effect of some of these factors on shed skin CORT concentrations with an attempt to explore the complex interaction between long-term levels of CORT and the physiological state of Komodo dragons. Sex, age and season of skin ecdysis had an effect on shed skin CORT concentrations. In contrast, no effect of the body region was observed.

Although sexual variation in the adrenocortical response has already been documented in different reptile species (Moore & Jessop, 2003), such information on Komodo dragons had never been addressed until the present. Here, we determined for the first time significant differences in shed skin CORT concentrations between adult males and females of Komodo dragon. Grassman & Hess (1992) also described sex differences in plasma CORT of six-lined racerunner (*Cnemidophorus sexlineatus*), detecting seasonal trends in those differences. These authors observed a decrease in circulating CORT levels from spring to summer only in males, concurring with the period of reproductive activity. To test for sex differences in the present study, only samples shed in spring could be used, hindering the detection of a potential seasonal connection with the sexual variation observed. Interestingly, we also observed seasonal differences on male shed skin CORT concentrations; levels declined significantly from spring to summer, a pattern similar to the one described in racerunners (Grasmann & Hess 1992) and in Texas horned lizards (*Phrynosoma cornutum*) (Wack et al., 2008). Reptiles can display fluctuations in circulating levels of CORT during the reproductive season (Moore & Jessop 2003), thus the decrease in male shed skin CORT concentrations could be partly influenced by the breeding period of the species. Accounting for the same seasonal fluctuations in females, in parallel with assessment of other reproductive indices, would be of interest to clarify the potential link of shed skin CORT concentrations with the reproductive activity of the species. The present study provides the first evidence that CORT levels detected on Komodo dragons shed skin could be influenced by the

Table 2. Model 1 selection and final model output for linear mixed models studying the effect of sex in SSCC of Komodo dragons.

Model	*k*	AICc	Δ AICc	wi
SSCC ~ Sex	**4**	**62.9**	**0.00**	**0.65**
SSCC ~ null	3	65.3	2.42	0.19
SSCC ~ Sex + Date	5	66.0	3.11	0.14
SSCC ~ Sex + Body region + Date	7	71.8	8.90	0.01
SSCC ~ Sex + Body region + Date + Sex*Body region	9	72.5	9.65	0.00
Parameter	**Estimate**	**S.E.**	**t (df)**	***P*-value**
Intercept	2.02	0.25	7.96 (21)	<0.01
Sex (male)	1.28	0.37	3.48 (21)	<0.01

Abbreviations: k is the number of parameters in the model; AICc is the bias-corrected Akaike's information criterion value; ΔAICc is the difference between each model and the top model; wi are the model weights.

Table 3. Model 2 selection and final model output for linear mixed models studying the effect of age in SSCC of juvenile and adult Komodo dragons.

Model	*k*	AICc	Δ AICc	wi
SSCC ~ Age + Date	**5**	**47.5**	**0.00**	**0.95**
SSCC ~ Age	4	53.7	6.19	0.043
SSCC ~ null	3	57.5	10.03	0.01
SSCC ~ Age + Body region + Date	7	59.7	11.58	0.00
SSCC ~ Age + Body region + Date + Age*Body region	9	76.9	29.35	0.00
Parameter	**Estimate**	**S.E.**	**t (df)**	***P*-value**
Intercept	2.02	0.27	7.49 (16)	<0.01
Age (juvenile)	2.01	0.49	4.13 (16)	<0.01
Date	0.06	0.02	3.86 (16)	<0.01

Abbreviations: k is the number of parameters in the model; AICc is the bias-corrected Akaike's information criterion value; ΔAICc is the difference between each model and the top model; wi are the model weights.

Table 4. Model 3 selection and final model output for linear mixed models studying the effect of the season in SSCC of Komodo dragons.

Model	*k*	AICc	Δ AICc	wi
SSCC ~ Season	**4**	**49.0**	**0.00**	**0.77**
SSCC ~ Season + Date	5	52.0	3.09	0.16
SSCC ~ null	3	54.0	5.05	0.06
SSCC ~ Season + Date + Body region	7	59.5	10.55	0.00
SSCC ~ Season + Date + Body region + Season*Body region	9	66.0	17.08	0.00
Parameter	**Estimate**	**S.E.**	**t (df)**	***P*-value**
Intercept	3.31	0.18	18.61 (23)	<0.01
Season (summer)	- 0.95	0.24	- 4.39 (23)	<0.01

Abbreviations: k is the number of parameters in the model; AICc is the bias-corrected Akaike's information criterion value; ΔAICc is the difference between each model and the top model; wi are the model weights.

season when ecdysis occurred. Results obtained here provide a further intriguing prospect for future research on the influence that reproduction can have on Komodo dragon's CORT levels.

In addition, we found that shed skin CORT concentrations differed significantly between juveniles and adult females. As reported by using short-term GC measures, baseline and stressor-induced GC levels can vary among ages (Dantzer et al., 2014; Sopinka et al., 2015). A higher metabolic rate in juvenile Komodo dragons could result in higher shed skin CORT concentrations, as previously described in other reptile species by using conventional matrices (Gregory et al., 1996; Jessop et al., 2000). Importantly, the model also detected that date of ecdysis was influencing shed skin CORT concentrations, possibly since all juvenile samples has been shed on the same day. In order to reliably confirm that juveniles differ from adults in shed skin CORT concentrations, further research should include a larger sample size shed at different periods.

Despite differences expected among body regions, we found no evidence of an effect on shed skin CORT concentrations. Previous research on snake's shed skin described higher CORT concentrations in tail sections than in the head and middle sections (Berkvens et al., 2013). Komodo dragons shed skin differs in thickness and shape of scales among body regions, but presumptively, it did not have an effect on CORT levels.

In conclusion, this paper demonstrates, for the first time, that CORT can be successfully measured in shed skin of Komodo dragon with the methodology presented above. Importantly, results demonstrate a potential biological source of variation in shed skin CORT concentrations due to the sex, age and season of skin ecdysis. The analysis of GC in shed skin may be the only method available to obtain a long-term and retrospective measurement of hormonal levels in reptiles. Combined with other indicators, detection of CORT concentrations in shed skin could allow a systematic control of Komodo dragon's physiology, offering a useful tool for zoo management and providing key data for the species conservation.

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