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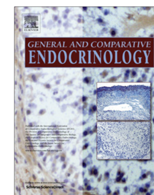

Validation of a shed skin corticosterone enzyme immunoassay in the African House Snake (*Lamprophis fuliginosus*) and its evaluation in the Eastern Massasauga Rattlesnake (*Sistrurus catenatus catenatus*)

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ABSTRACT

This study investigates the use of an enzyme immunoassay to measure keratin glucocorticoid concentrations in reptilian shed skins. Keratin glucocorticoid concentrations were compared to fecal glucocorticoid concentrations during the period of keratin growth in the African House Snake (*Lamprophis fuliginosus*) and the Eastern Massasauga Rattlesnake (*Sistrurus catenatus catenatus*). Biochemical validation was performed for the shed skin and fecal corticosterone enzyme immunoassays in the African House Snake. Biological and physiological validations were attempted in the African House Snake. A statistically significant positive association was detected between shed skin corticosterone and the mean fecal corticosterone metabolites from 3 weeks before to 1 week after the previous ecdysis in the African House Snake. A statistically significant difference was not detected between the shed skin corticosterone concentrations of the minimally handled control and the weekly handled (or experimentally stressed) African House Snakes. Adrenocorticotropic hormone stimulation did not result in the physiological validation anticipated for shed skin corticosterone concentrations in the African House Snake.

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1. Introduction

Corticosterone is the primary glucocorticoid produced in snakes (Moore et al., 2001). Methods similar to those used to quantify cortisol in mammals and corticosterone in birds, have been developed to assay this hormone or its metabolites in reptilian plasma (Sanford and Stephens, 1988; Tyrrell and Cree, 1998; Mathies et al., 2001; Romero and Wikelski, 2002; Preest et al., 2005; Sykes and Klukowski, 2009) and feces (Rittenhouse et al., 2005; Lentini, 2008). As in mammals and birds, levels of corticosterone in reptilian plasma reflect events occurring over the short term, values may increase within minutes and handling and blood sample collection have the potential to increase concentrations (Romero and Reed, 2005). Blood sampling to measure corticosterone concentrations may be detrimental or difficult to perform in some small reptilians. Thus, non-invasive ways to measure corticosterone such as fecal hormone analysis or shed skin corticosterone are potentially very useful.

Recently, keratin glucocorticoid concentrations have been investigated as a biomarker of chronic stress or persistent elevation in glucocorticoid concentrations in a variety of mammals, including Rhesus Macaques (Davenport et al., 2006; Dettmer et al., 2009), Crab eating Macaques (Herod et al., 2011), Common Marmosets (Clara et al., 2008), Vervet Monkeys (Fairbanks et al., 2011; Laudenslager et al., 2011), Domestic Dogs (Accorsi et al., 2008; Bennett and Hayssen, 2010), Domestic Cats (Accorsi et al., 2008; Finkler and Terkel, 2010), Eastern Chipmunks (Martin and Reale, 2008), Rock Hyrax (Koren et al., 2002), Caribou and Reindeer (Ashley et al., 2011), Grizzly Bears (Macbeth et al., 2010) and Polar Bears (Bechshoft et al., 2011), as well as birds (Bortolotti et al., 2008, 2009; Harms et al., 2010).

Snakes possess a distinct cycle of ecdysis, in which episodic loss (or shedding) of a keratinized old outer epidermal generation occurs following formation of a new keratinized inner epidermal generation. The complete cycle is comprised of six stages, a resting stage with three subdivisions and five discrete renewal stages. This culminates in ecdysis, or complete shedding of the old outer keratinized epithelial layer (Maderson, 1965). The renewal stage lasts approximately 6–14 days. The shed outer keratinized layer of skin is readily collected at ecdysis. Health, disease, age, frequency of feeding and amount consumed, and environmental temperature

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may all influence shedding frequency (Jacobson, 2007), though ecdysis is ultimately under thyroidal control (Chiu and Lynn, 1970).

Uptake of glucocorticoids by the outer epithelial generation of the snake's skin while it is differentiating and keratinizing, and perhaps while it is in place, may reflect and integrate the status of the hypothalamic–pituitary–adrenal gland (HPA) axis over that time frame. The corticosterone concentration in a shed skin was hypothesized to reflect circulating corticosterone levels, at least during the approximately 2 week period that the outer epithelial generation was forming prior to the previous ecdysis.

Our principal aim was to validate the relationship of shed skin corticosterone concentrations to fecal corticosterone metabolite (FCM) concentrations over time in the African House Snake (AHS). Biochemical validation and biological validation was attempted by comparing shed skin corticosterone levels in a control group of AHS to an experimentally manipulated group of AHS. Physiological validation via ACTH stimulation was also attempted in the AHS. The African House Snake, a laboratory snake, was chosen as a model for wild snake species, particularly endangered or threatened species, such as the Massasauga Rattlesnake. To our knowledge this is the first published study of corticosterone extraction and enzyme immunoassay using shed snake skins.

2. Materials and methods

All chemicals used for corticosterone extraction and analysis were obtained from Sigma–Aldrich Canada Ltd., Oakville, Ontario, Canada.

All animal procedures and experiments were approved by the University of Guelph Animal Care Committee and the Toronto Zoo Animal Care and Research Committee.

2.1. African House Snakes

Thirty sexually mature, adult female AHS were housed individually in 49.6 L Rubbermaid Cleverlatch™ containers measuring 59 cm by 45 cm by 29 cm (Rubbermaid Canada, Mississauga, ON) in the Toronto Zoo's Animal Health Center. The room temperature was set at 25 °C. Heating pads set at 33 °C were placed below each container, along one margin, with temperatures of hot spots in the containers ranging from 31–34 °C. Lighting was provided by fluorescent room lights. Photophase commenced at 07:30 h and scotophase at 16:30 h. All snakes were provided plastic hide boxes measuring 16.5 cm by 11.4 cm by 6.4 cm, and a shallow water dish. Snakes were offered two whole 20 g mice weekly and consumption was sporadic.

Snakes were assigned to a control group ($n = 10$) and an experimentally stressed group ($n = 20$) using a random number generator. Fecal and shed skin samples were collected from the snakes for approximately 1 year. The control snakes were handled as little as possible, except when needed for husbandry purposes. The experimentally stressed snakes were deliberately handled weekly as follows: snakes were caught up and placed in individually marked bags, transported to another room at the zoo and placed into a snake tube as per the venomous or dangerous snake handling protocol; the snakes then were replaced in their bags and returned to their individual container once the entire group had been handled. The elapsed time was approximately 60 min for the group of experimentally stressed snakes.

An adrenocorticotropic hormone (ACTH) challenge (see below) was performed using the control snakes at the end of the trial as a means of physiological validation of fecal and shed skin corticosterone concentration analysis.

2.1.1. Shed skin and fecal sample collection

Over the period of the study all shed skins and fecal samples produced by the snakes were collected, individually identified, placed in 10.2 cm by 15.2 cm sealed "ziploc-type" polyethylene plastic bags (EJ Bags and Boxes Company, Scarborough, Ontario, Canada), and stored at −20 °C.

2.2. Massasauga Rattlesnakes

Twenty five unsexed Eastern Massasauga Rattlesnakes less than 1 year of age, temporarily held at the Toronto Zoo, were individually housed in 18 L polycarbonate boxes measuring approximately 30 cm by 45 cm by 23 cm (Cambro Manufacturing Company, Huntington Beach, CA) in the Zoo's Animal Health Center quarantine.

Ambient room temperature ranged daily from a mean low of 25.4 °C to a mean high of 28.2 °C. Containers were illuminated with 40 W double strip fluorescent lights suspended approximately 20 cm above. Photophase commenced at 07:00 h and scotophase at 19:00 h. All snakes were provided plastic hide boxes measuring 16.5 cm by 11.4 cm by 6.4 cm and a shallow water dish. These snakes were fed whole pinkie mice weekly.

2.2.1. Shed skin and fecal sample collection

All skin sheds and feces produced by these snakes over a 125 day period were collected for corticosterone extraction and assay. Attempts were made to avoid contamination of AHS fecal samples with urates during fecal sample collection. All samples were individually identified and placed in 10.2 cm by 15.2 cm sealed "ziploc-type" bags and stored at −20 °C.

2.3. Corticosterone extraction from shed skins

Shed skin samples for the AHS and Massasauga Rattlesnakes were analyzed within 1 year and 1.5 years respectively, following collection. To avoid contamination of shed skin samples by biological fluids which might artificially elevate corticosterone levels, all shed skins were hand rinsed by personnel wearing disposable nitrile gloves in a distilled water bath for 2 min, air dried for 2 h and then spray rinsed with 100% methanol for 1 min followed by air drying in a fume hood overnight. The lengths of the shed skins were measured with a metric ruler. Whole shed skins were cut transversely into quarters to facilitate hormone extraction. Hormone concentration in each quarter of the shed skin was assayed separately. The weight of the shed skins were measured to within one ten-thousandth of a gram using a Mettler Toledo balance, model AB54-S (Mettler Toledo International, Inc., Columbus, Ohio). Each quarter of a shed was cut into pieces, each less than 5 mm^2, using surgical scissors. The fragments of shed skin were collected into screw-capped Falcon polystyrene centrifuge tubes (AHS: 50 ml tubes; Massasauga Rattlesnake: 15 ml tubes; VWR, Mississauga, ON, Canada) and stored at −20 °C until used. Samples were extracted in those tubes using 80% methanol in distilled water at a ratio of 0.02 g of keratin per 1 ml of 80% methanol-distilled water. Due to the large mass of shed AHS skins a large volume of methanol was required for extraction; thus multiple large tubes were required to extract a single shed. The samples were agitated for 24 h on a shaker. Extracts were poured off through a 40 μm cell strainer (Fisher Scientific, Ottawa, ON, Canada) and stored in polystyrene centrifuge tubes at −20 °C until analysis. Due to the large number of shed skins analyzed and the amount of time required to process each individual shed, sheds were not all washed and extracted on the same day.

A variety of extraction methods were tested (manuscript in preparation). The technique used was chosen based on the efficacy of extraction along with being time- and cost-effective for hair, feathers and shed skins.

2.4. Corticosterone extraction from feces

All fecal samples from AHS were extracted and analyzed individually. Fecal samples were extracted on a wet weight basis using a ratio of 0.5 g of feces per 5 ml of 80% methanol-distilled water. These samples were weighed to within one ten-thousandth of a gram with a Mettler Toledo balance, model AB54-S (Mettler Toledo International, Inc., Columbus, Ohio). A metal spatula, cleaned with 80% methanol between samples, was used to homogenize the sample and the sample was vortexed for 5 s. Samples were then extracted for 18 h (overnight) at room temperature while agitated on a platelet rotator at 3 rpm. Afterward samples were centrifuged at 2300*g* for 10 min and the extract was decanted. Extracted samples were stored at −20 °C until analysis.

Fecal samples from Massasauga Rattlesnakes were pooled monthly, based on dates of ecdysis, in an effort to provide at least 0.1–0.2 g of sample required for extraction and analysis, as the majority of individual fecal samples from some these snakes were quite small (0.01–0.10 g). The pooled sample weights were taken and an appropriate amount of 80% methanol-distilled water was added to provide a ratio of 0.1 g of feces per 1 ml of 80% methanol-distilled water. Samples were then extracted for 18 h (overnight) at room temperature while agitated on a platelet rotator at 3 rpm. Afterward samples were centrifuged at 2300g for 10 min and the extract was decanted. Extracted samples were stored in capped glass scintillation vials at −20 °C until analysis.

2.5. Enzyme immunoassay

Each extracted sample was analyzed using an enzyme immunoassay (EIA) for corticosterone. Fecal and shed skin glucocorticoid metabolites were quantified using modifications of EIA methods previously described (Munro and Lasley, 1988). Corticosterone antiserum (CJM006; C. Munro, University of California, Davis, CA, USA) was diluted 1:15,000 in coating buffer (50 mM bicarbonate buffer, pH 9.6). The cross-reactivities of the antiserum (as provided by C. Munro, University of California Davis, CA, USA) were: corticosterone, 100%; desoxycorticosterone, 14.3%; tetrahydrocorticosterone, 0.9%; cortisol, 0.2%; cortisone, <0.01%; progesterone, 2.7%; testosterone, 0.6%; others, <0.1%. Horseradish peroxidase conjugates (C. Munro, University of California, Davis, CA, USA) were diluted 1:70,000 in EIA buffer (0.1 mM sodium phosphate buffer, pH 7.0, containing 9.0 g of NaCl and 1.0 g of Bovine Serum Albumin [BSA] per liter). The standard used was corticosterone (Steraloids Inc., Newport, RI, USA; cat # Steraloids Q1550; 0.078–20 ng/ml or 78–20,000 pg/ml). Controls consisted of laboratory stocks of pooled fecal extracts obtained from female spotted necked otters and were run at 60% binding.

Fecal samples were diluted routinely in EIA buffer prior to hormone analysis. This produced a hormone concentration within the reliable reading range, and prevented methanol from interfering with the antigen–antibody bonds in the enzyme immunoassay. AHS and Massasauga Rattlesnake fecal extracts were diluted 10-fold with several exceptions where only 5-fold dilutions were required.

African House Snake and Massasauga Rattlesnake shed skin extracts were concentrated 10-fold by drying overnight in a fume hood and reconstituting in the appropriate amount of EIA buffer.

Microtitre plates (Nunc Maxisorp, VWR, Mississauga, ON, Canada) were coated with 50 μl of antiserum diluted in coating buffer and incubated overnight at 4 °C. Unbound antiserum was washed from coated plates with 0.02% Tween 20 solution using a Bio-Tek ELx 405VR microplate washer (Bio-Tek Instruments, Winooski, VT, USA) programmed to wash the wells three times with each well completely filled (450 μl total) each time. Immediately, 50 μl of shed (or fecal) extracts, standards, and controls diluted in EIA buffer were added to wells in duplicate, followed by 50 μl of horseradish peroxidase conjugate diluted in assay buffer. Plates were incubated for 2 h at room temperature. Following incubation, the plates were washed 3 times and 100 μl of substrate solution (50 mM citrate, 1.6 mM hydrogen peroxide, and 0.4 mM 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, pH 4.0; (Munro et al., 1991) was added. Absorbance was measured at 405 nm using a spectrophotometer (MRX microplate reader, Dynex Technologies, Chantilly, VA, USA) 30–45 min after the substrate was added. Fecal and snake shed skin hormone levels are presented as mass/g wet weight.

All enzyme immunoassays were run on the same day, to minimize possible variation associated with day-to-day differences in testing sensitivities and efficiencies. All samples were assayed in duplicate and the mean of the two values is presented. Complete assay validation was performed for AHS shed skin corticosterone and FCM assays.

2.6. Corticosterone biochemical validation – African House Snake shed skin and feces

2.6.1. Extraction efficiency

Efficiency of the extraction procedure was analyzed through recovery of exogenous corticosterone added to the shed (and fecal) samples before extraction. Efficiency of extraction from shed skin (and feces) was determined from five shed (and fecal) samples. The percent efficiency was calculated using the following formula: amount observed/amount expected × 100%; where amount observed is the value obtained from the spiked sample minus background and amount expected is the calculated amount added.

2.6.2. Parallelism

Parallel displacement between the standard curve and a serial dilution of a pooled shed (and fecal) extract was used to detect immunological similarities between the standard and sample hormones. The resulting curves were plotted as relative dose versus percent antibody bound and linear regression analysis was performed. Sample dilution was selected based on 50% binding of the pooled sample curve.

2.6.3. Precision

To assess the repeatability of results, calculation of intra- and inter-assay coefficient of variation (CV) was performed. Intra-assay CVs were consistently monitored on each plate in real time by examining the CV of each duplicate run on the plate. Only values from duplicates with <10% CV were recorded as data. Intra-assay CVs were further evaluated using a pooled extract at 50% binding loaded repeatedly across the plate and repeated three times. The inter-assay CV was evaluated using fecal extract controls (60% binding) loaded in duplicate on each plate. The average intra-assay CV was evaluated from the 60% control.

2.6.4. Accuracy

Possible interference of components within the extract with antibody binding was analyzed through recovery of exogenous corticosterone added to the shed (and fecal) extracts using concentrations within the range of the standard curve (Kummrow et al., 2011). A pooled sample of shed extract concentrated to the usual range for the assay for unknown samples and a pooled sample of fecal extract diluted to the usual range for the assay for unknown samples were used. The percent recovery was calculated using the following formula: amount observed/amount expected × 100%, where amount observed is the value obtained from the spiked sample and amount expected is the calculated amount of standard hormone added plus the amount of endogenous hormone in the unspiked sample. The percent recovery is presented as mean ± standard error of the mean. The graph was plotted as hor-

mone added versus hormone recovered, and regression analysis was used to determine if there was a significant relationship between them.

2.7. Corticosterone biological validation – African House Snake shed skin

Corticosterone levels in feces and in shed skins were compared to determine the association of FCM concentrations with corticosterone concentrations in the outer epithelial layer during shed skin growth and differentiation in the AHS and Massasauga Rattlesnake. Fecal and shed skin corticosterone levels were compared between a control group of AHS which were minimally handled and an experimentally stressed group which were handled once weekly.

A previous study on Massassauga Rattlesnakes (Lentini, 2008) indicated that FCM analysis was an effective method for evaluating a biological stress response in snakes. Lentini et al. 2008 (Lentini, 2008) observed an elevation in blood and fecal CORT in Massasauga Rattlesnakes following ACTH stimulation.

2.8. Corticosterone physiological validation – African House Snake shed skin

Physiological validation of AHS shed skin corticosterone was attempted. At the end of the study, five AHS from the control group were administered 5 IU of a synthetic ACTH analog (Synacthen Depot, Novartis, Canada) per kilogram of body weight intramuscularly, three times weekly. Another five AHS were administered saline intramuscularly at an identical volume to that of the ACTH dose. Injections all commenced on the same date and were continued for each individual snake until it shed its skin. If snakes shed in less than 3 weeks from the initiation of the ACTH or saline injections, then injections were continued until a second ecdysis. Based on our knowledge of skin growth and ecdysis in snakes we hypothesized that the corticosterone concentration in a shed skin would reflect circulating corticosterone levels during the approximately 2 weeks prior to the previous ecdysis.

African House Snakes included in the ACTH trial all shed in numerous small pieces throughout this portion of this study. This was probably due to injury of the fragile skin during the pre-ecdysis period caused by handling for injection three times per week (Mader, 2006).

2.9. Statistical analysis of shed skin and fecal glucocorticoid concentrations

The following statistical analyses were performed using Intercooled Stata 9.1 (StataCorp LP, College Station, Texas):

1. A multi-level linear regression model with random intercept for snake identity was created to examine associations between mean sectional/random shed skin corticosterone concentrations and mean FCM concentrations during skin shed growth over a period of approximately 1 year. The mean corticosterone concentrations in feces collected from 3 weeks before to 1 week after the previous shed were compared to the mean of the sectional shed skin and the random shed skin corticosterone concentration of the current shed. Random shed skin corticosterone values are single values obtained from snakes which shed in multiple small sections rather than one whole section, thus preventing analysis by section. The mean corticosterone concentration of the sectional shed skins was the mean of the individually assayed head, proximal middle, distal middle and tail shed skin sections. Multiple sheds were included in the analysis for each individual AHS. The control and experimentally stressed AHS were included in this model.
2. For both snake species, multi-level linear regression models with random intercept for snake identity were examined for associations between corticosterone concentrations in different sections (head, proximal middle, distal middle and tail) of the shed skin.
3. A multi-level linear regression model with random intercept for snake identity was examined for associations between corticosterone concentrations (dependent variable) and the following independent variables (shed skin and feces; and the ACTH- and saline-injected groups). FCM concentrations collected from 3 weeks before to 1 week after the previous shed were examined for associations with the corticosterone concentrations of the current shed skin. In the ACTH trial, the shed skin corticosterone concentrations of the AHS were included in the study if at least 2 weeks of ACTH injections had been administered prior to the collection of the shed skin. If only 2 weeks of ACTH injections had been given prior to collection of the previous shed (based on the timing of initiation of ACTH injection), then only 2 weeks of FCM concentrations from before to 1 week after the previous shed were included in the values for comparison to the shed corticosterone concentrations. The first shed obtained after the ACTH injections were initiated was not included in this portion of the trial, as it was not expected to have grown during the time in which synthetic ACTH would have been in the snake's blood stream.
4. Massasauga Rattlesnake FCM concentrations from 1 month before the previous shed and 1 month before the current shed were examined for associations with the mean and the median shed skin sectional corticosterone concentrations of the current shed. These Massasauga FCM values were the results of pooled fecal samples extracted and analyzed with the corticosterone EIA.

For all regression models, the standardized residuals were examined for outliers and to assess that the models met the assumptions of normality and homogeneity of variance. In addition, for multi-level models the best linear unbiased predictors (BLUPs) were examined for normality and homogeneity of variance. Where continuous predictors were considered for inclusion in the model, a lowess curve of the independent variable and the outcome were examined to make certain the assumption of linearity was not violated. If necessary, a transformation, the addition of a quadratic term, or the categorization of the independent variable were considered if this assumption was violated. The coefficients of the linear and multi-level linear models provide an estimate of the difference between categories of each variable to a referent value (e.g., treated versus non-treated) and their statistical difference. Where more than two categories exist for a variable (e.g., section of shed) model based contrasts were performed to determine the magnitude and statistical difference among all categories. For all statistical tests, the significance level was set at $\alpha \leqslant 0.05$.

All graphs and tables were prepared in Microsoft Excel 2007 (Vista). Descriptive statistics and linear regression models in the biochemical validation were also performed in Microsoft Excel 2007 (Vista). Descriptive statistics concerning the mean and range of values of glucocorticoids for the different sample types along with the mean number of samples per snake were estimated.

3. Results

3.1. Corticosterone biochemical validation – African House Snake shed skin

The efficiency of corticosterone extraction from AHS shed skin was 108.1 ± 3.9%. A plot of corticosterone concentration in serial dilutions of a pooled shed extract showed parallel displacement

with the standard curve ($r = 0.99$) (Fig. 1A). Corticosterone recovery from a pool of AHS shed skin extracts was 86.4 ± 1.9%. The hormone concentrations in the spiked samples were associated with the expected concentrations ($r = 0.99$; $p < 0.01$) (Fig. 1B). The inter-assay CV was 10.4% and the intra-assay CV was 7.6%.

3.2. Corticosterone biochemical validation – African House Snake feces

The efficiency of extraction of corticosterone from AHS feces was 49.4 ± 1.0%. A plot of corticosterone concentration in serial dilutions of a pooled fecal extract showed parallel displacement with the standard curve ($r = 0.99$) (Fig. 1C). Corticosterone recovery from a pool of AHS fecal extracts was 81.6 ± 1.1%. The African House Snake fecal extracts were diluted 10 times in buffer according to the parallelism results. Exogenous hormone standard added to buffer alone was recovered at 89.9 ± 1.5%, but when added to 80% methanol diluted 10 times with buffer, only 52.3 ± 1.0% was recovered. The low FCM extraction efficiency may be the result of a methanol effect in our assay system, as a decrease in apparent hormone recovery of 30% was observed when using only a 10-fold dilution of methanol extract in buffer compared to drying down the methanol and reconstituting in buffer alone. The hormone concentrations in the spiked samples correlated with the expected concentrations ($r = 0.99$; $p < 0.01$) (Fig. 1D). The inter-assay CV was 10.2% and the average intra-assay CV was 2.0%.

3.3. African House Snake shed skin and FCM concentrations

Corticosterone was detected in all of the 444 shed skins samples collected from the AHS. Sectional and random shed skin corticosterone concentrations ranged from 4.42–254.07 ng/g with a mean value of 22.15 ± 0.89 ng/g ($n = 444$). FCM concentrations ranged from 0.0–1150.14 ng/g ($n = 357$) with a mean value of 71.34 ± 5.37 ng/g ($n = 357$). There were on average 4.7 shed skins per snake (with an average of 14.8 shed skin sections per snake) and 11.9 fecal samples per snake.

Using a multi-level linear regression model with a random intercept for snake identity, a statistically significant positive association was found between the $\log_{10}$ transformed AHS shed skin corticosterone concentration and the $\log_{10}$ corticosterone concentration in feces collected from 3 weeks before to 1 week after the previous ecdysis (Table 1). The $\log_{10}$ transformed shed skin and FCM data were plotted with a fitted line (Fig. 2). There was no significant difference in corticosterone concentrations between the control and weekly handled snakes (Table 1). The intraclass correlation coefficient for shed skin corticosterone concentration within a snake was 45.79%. Based on the residual analysis, no model assumptions were violated and we found no justification to remove any observations from the final analysis.

A multi-level linear regression model with a random intercept for snake identity did not find significantly different shed skin corticosterone concentrations along the length of the snake's body, when comparing the distal middle section to the head and proximal middle sections. However, the tail section was shown to have a significantly higher corticosterone concentration than the distal middle section (Table 2). The tail section was also shown to have higher corticosterone concentrations than the head ($\beta = 4.42$, 95% CI: 0.98–7.87, $p = 0.012$) and the proximal middle ($\beta = 6.53$, 95% CI: 3.08–9.97, $p = 0.000$) sections based on contrasts performed among categories in the model. The intraclass correlation coefficient for corticosterone concentrations in sections of a skin shed within a snake was 35.92%.

Sectional shed corticosterone concentrations were not available for the ACTH study snakes, because all snakes (ACTH and saline control groups) shed in numerous small pieces; therefore, samples from each snake were extracted and analyzed as a single, random

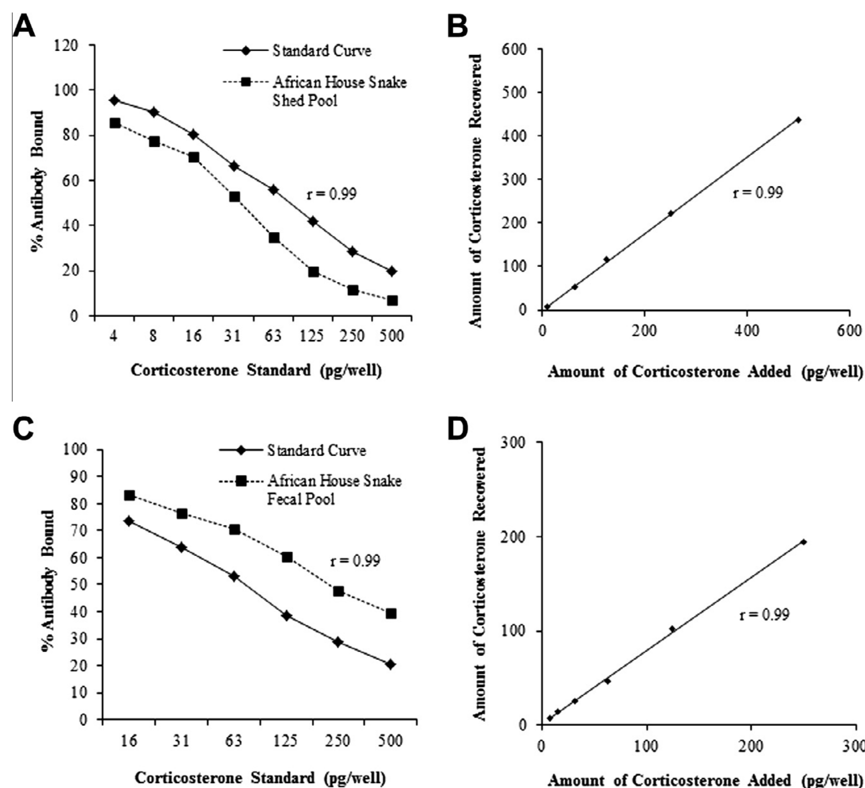

Fig. 1. Graphs of parallelism (A) and recovery (B) of corticosterone from the shed skins of African House Snakes; serial doubling dilution of pooled extract showing parallel displacement with the standard curve for corticosterone (repetitions = 2; $r = 0.99$); and fecal corticosterone parallelism (C) and recovery (D): corticosterone added versus corticosterone recovered ($r = 0.99$).

Table 1
Results of a multi-level linear regression model examining associations between $\log_{10}$ mean fecal corticosterone concentrations from samples collected 3 weeks before to 1 week after the previous shed and $\log_{10}$ re-growth shed skin corticosterone concentrations (dependent variable) in the control and experiment African House Snakes. Statistically significant results are indicated via an asterisk *; $n = 103$.

Independent variables	Coefficient (β)	95% Confidence interval	p value
$\log_{10}$ mean fecal corticosterone	0.17	0.05–0.29	0.007*
Control versus experimentally stressed	0.04	−0.10–0.18	0.551
Random effects	Variance	Standard error	95% Confidence interval
Snake identity	0.03	0.01	0.01–0.06
Shed skin	0.03	0.01	0.02–0.04

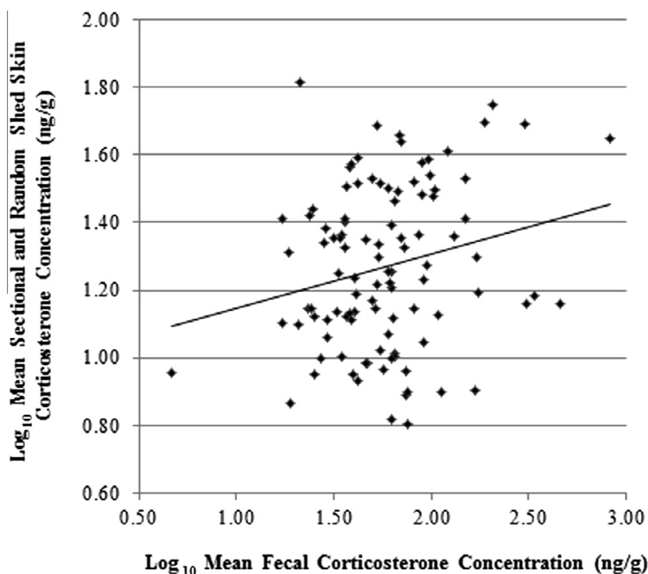

Fig. 2. $\log_{10}$ mean fecal corticosterone concentrations (from 3 weeks before to 1 week after the previous ecdysis) plotted against $\log_{10}$ mean sectional and random shed skin corticosterone concentrations for the African House Snakes ($\beta = 0.17$, 95% CI: 0.05–0.29, $p = 0.007$). Random shed skin corticosterone values are single values obtained from snakes which shed in multiple small sections rather than one whole section, thus preventing analysis by section.

shed skin sample. Shed skin corticosterone ranged from 9.24 to 82.12 ng/g with a mean value of 33.87 ± 21.59 ng/g ($n = 24$). FCM concentration ranged from 4.63 to 229.71 ng/g with a mean value of 50.16 ± 7.18 ng/g ($n = 42$). There were on average 2.7 shed skin samples and 4.7 fecal samples per snake.

A multi-level regression model with a random intercept for snake identity did not reveal a significant difference in corticosterone concentrations between fecal and shed skin samples or between the ACTH-treated and saline control groups (Table 3). The intraclass correlation coefficient for corticosterone concentrations within a snake was 1.24%.

3.4. Massasauga Rattlesnake shed and FCM concentrations

Corticosterone was detected in all of the shed skins and fecal samples collected from the Massasauga Rattlesnakes. Sectional shed skin corticosterone concentrations ranged from 3.82–22.85 ng/g with a mean value of 9.84 ± 0.48 ng/g ($n = 80$). Pooled monthly FCM concentrations ranged from 33.81–191.72 ng/g with a mean value of 98.15 ± 8.81 ng/g ($n = 30$). There were an average of 8 shed skin samples per snake and 3 monthly pooled fecal samples per snake.

A statistically significant association was not found between the mean and the median sectional shed skin corticosterone concentrations and the FCM concentrations from either 1 month before the previous ecdysis or 1 month before the current ecdysis in the Massasauga Rattlesnakes (Table 4). Nor was a significant difference identified between the different shed skin sections examined (Table 5). The intraclass correlation coefficient for corticosterone concentrations in different shed sections within the same snake was 25.02%.

4. Discussion

To our knowledge, there are no published studies identifying corticosterone in the shed skins of reptiles and this is the first to find a positive association between shed skin corticosterone and FCM concentrations in snakes. Previously published studies have identified an association between keratin (hair) glucocorticoid and fecal glucocorticoid concentrations (Accorsi et al., 2008), hair glucocorticoid and salivary glucocorticoid concentrations (Davenport et al., 2006; Bennett and Hayssen, 2010; D'Anna-Hernandez et al., 2011) and hair glucocorticoid and urinary glucocorticoid concentrations (Sauvé et al., 2007). Despite these findings, there is some inconsistency in the literature. For example, although (Sauvé et al., 2007) found a correlation between hair and urinary cortisol concentrations, they failed to find correlations between either hair or urinary cortisol concentrations and serum or salivary cortisol concentrations. Another study failed to find a correlation between baseline plasma corticosterone and feather corticosterone, but found a correlation between stress-stimulated plasma and feather corticosterone concentrations (Bortolotti et al., 2008).

Table 2
Results of a multi-level linear regression model created to examine associations between corticosterone concentrations (dependent variable) in shed skin along the length of the African House Snake's body. Statistically significant results are indicated via an asterisk *; $n = 396$.

Independent variables	Coefficient (β)	95% Confidence interval	p value
Shed location: Distal middle (Referent)			
Head	1.63	−1.81–5.08	0.353
Proximal middle	−0.47	−3.92–2.97	0.787
Tail	6.05	2.61–9.50	0.001*
Random effects	Variance	Standard error	95% Confidence interval
Snake identity	85.73	26.82	46.43–158.28
Shed skin	152.94	11.37	132.20–176.94

Table 3
Results of a multi-level linear regression model created to examine associations between corticosterone concentrations (dependent variable) in feces and shed skins in African House Snakes treated with ACTH and saline controls. *r* Refers to the referent group and *n* refers to the number of samples of each type.

Independent variables	Coefficient (β)	95% Confidence interval	*p* value
Sample type (Shed skin ($n = 24$) and fecal – *r* ($n = 42$))	−9.38	−35.22–16.46	0.477
Treatment group (Saline ($n = 30$) and ACTH – *r* ($n = 36$))	−8.24	−29.45–12.97	0.446
Random effects	Variance	Standard error	95% Confidence interval
Snake identity	51.07	146.04	0.19–13870.22
Sample	1525.06	290.80	1049.49–2216.12

Table 4
Results of univariable linear regression models examining the association between the mean (A) and the median (B) corticosterone (CORT) concentrations of sectional shed skin (dependent variable) and the pooled fecal corticosterone concentration from 1 month before the previous ecdysis and 1 month before the current ecdysis in Massasauga Rattlesnakes.

Independent variable		Coefficient (β)	95% Confidence interval	*p* value
A	Fecal CORT 1 month before previous ecdysis ($n = 8$)	<0.01	−0.03–0.03	0.972
	Fecal CORT 1 month before current ecdysis ($n = 16$)	0.01	−0.03–0.05	0.619
B	Fecal CORT 1 month before previous ecdysis ($n = 8$)	<0.01	−0.02–0.03	0.646
	Fecal CORT 1 month before current ecdysis ($n = 16$)	0.01	−0.03–0.06	0.491

Table 5
Results of multi-level linear regression model comparing corticosterone concentrations (dependent variable) in shed skin along the length of the Massasauga Rattlesnake's body; $n = 80$.

Independent variables	Coefficient (β)	95% Confidence interval	*p* value
Shed location: Distal middle (Referent)			
Head	−1.87	−4.21–0.47	0.118
Proximal middle	−0.18	−2.52–2.16	0.879
Tail	−1.95	−4.30–0.39	0.102
Random effects	Variance	Standard error	95% Confidence interval
Snake identity	4.76	3.10	1.33–17.05
Shed skin	14.26	2.46	10.17–20.01

Given our knowledge of skin growth and ecdysis in snakes (Maderson, 1965; Jacobson, 2007), the corticosterone concentration in a shed skin was hypothesized to reflect circulating corticosterone levels, at least during the 2 week period prior to the previous ecdysis. The gastrointestinal transit time in reptiles is generally slow and variable, particularly in snakes, as noted with the AHS in this study, which defecated from once every few days to once every few weeks. As a result, shed skin corticosterone concentrations were compared to the mean FCM concentrations from fecal samples collected 3 weeks before to 1 week after the previous ecdysis in the AHS. These samples were expected to most accurately reflect circulating corticosterone concentrations during the time frame of shed skin growth. The marked variation in defecation frequency among the snakes could potentially impact the hormone concentrations in the fecal samples, thus complicating comparison.

Due to the small size of the fecal samples of the Massasauga Rattlesnakes, they had to be pooled to provide a sample large enough for extraction and analysis. Therefore, fecal samples collected 1 month before the previous ecdysis and 1 month before the current ecdysis were analyzed as individual pooled samples and used for comparison to shed skin corticosterone concentrations. A significant association was not found between either of these FCM measures and the shed skin corticosterone concentration. In addition to the impact of the large variation in fecal deposition on fecal hormone concentrations, the sample size was quite small resulting in low statistical power.

Little information exists on fecal hormones in reptiles. Studies validating assays for hormone measurement in reptilian feces include quantification of fecal corticosterone metabolites in three-toed box turtles (Rittenhouse et al., 2005) and Massasauga Rattlesnakes (Lentini, 2008), and fecal reproductive hormone metabolites in veiled chameleons (Kummrow et al., 2011). No studies seeking an association between fecal and blood (serum or plasma) corticosterone concentrations in reptiles were found in the current literature, though species differences in fecal glucocorticoid metabolism and excretion are known to exist in birds and mammals (Mostl and Palme, 2002; Palme et al., 2005; Touma and Palme, 2005; Keay et al., 2006).

In the African House Snake, the tail section of the shed skins had a significantly higher corticosterone concentration than the head, proximal middle and distal middle sections. As a result we used mean shed skin corticosterone concentrations for comparison to FCM concentrations and among skin from control and experimentally stressed snakes, in an attempt to avoid the effect of variation in corticosterone concentration along the snake's body. Corticosterone concentrations were not found to differ significantly between the other shed skin sections in the AHS. Nor was a significant difference found along the length of the shed skins of the Massasauga Rattlesnakes. The cause of this finding in the AHS is unknown; however, one possible explanation is that fecal and urate hormones may have been absorbed or incorporated into the skin around the cloaca. Surface contamination is unlikely, since all shed skins were washed thoroughly prior to analysis. Hair cortisol concentrations have been noted to differ across different regions of the body in people (Sharpley et al., 2010).

No difference was found in skin corticosterone concentrations between the control and experimentally stressed AHS in this study. Due to the signs of nervousness noted in all the AHS, and the way in which they were housed and managed during this study, once weekly handling may not have been sufficiently stressful to increase skin corticosterone concentration compared to the control group.

Attempts to perform physiological validation of shed skin corticosterone assays in the AHS did not provide the anticipated results. There was no significant difference between the fecal or shed skin corticosterone concentrations of the saline control and the ACTH injected AHS. The results of the ACTH trial are difficult to interpret. If the ACTH dose and/or dose frequency were too low no difference in fecal or shed skin corticosterone would be expected between the ACTH and the saline injected AHS. If the ACTH dose and/or dose frequency were too high, an initial more dramatic increase in FCM concentration would be expected in the ACTH group and the negative feedback loop would be expected to lead to a decrease in the fecal and shed corticosterone concentrations. None of these findings were noted. Given the marked variation in gastrointestinal transit time among snakes, an association between ACTH administration and FCM levels may not be as straightforward as expected to be the case in birds or mammals. Additionally, the sample size limited statistical power.

Shed skin corticosterone concentrations have the potential to be useful as a non-invasive biomarker of chronic stress, particularly in captive reptiles held under differing management conditions or in different stages of health. Its use in wild species may be limited, given the difficulty in collecting shed skin from free ranging species, although it may prove useful in specific circumstances.

5. Conclusions

Corticosterone concentration in shed skins of African House Snakes appear to be associated with the concentration of corticosterone in feces during the time of shed skin growth. Further research, including biological and physiological validation, is necessary to determine whether corticosterone concentrations in shed skin are representative of chronic activation of the HPA axis in this species, particularly given the unexpected results of attempted physiological validation in this study. Future studies should include more drastic differences in management to provide biological validation. Validation of FCM concentration analysis in snakes would also be beneficial and might provide guidance in regards to the appropriate dose and frequency of ACTH required to yield chronic elevation of corticosterone in the snake.

The present study highlights the importance of validation prior to drawing conclusions regarding the significance of keratin glucocorticoid concentrations as a biomarker of chronic stress.

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