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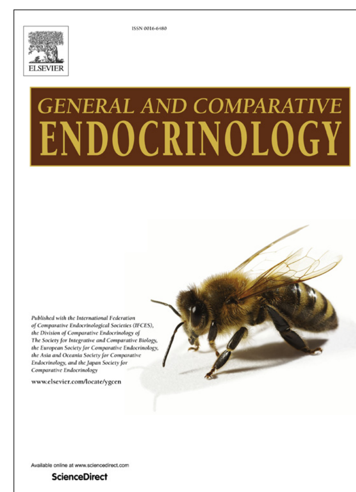

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Corticosterone in American Alligator (*Alligator mississippiensis*) Tail Scutes: Evaluating the Feasibility of Using Unconventional Samples For Investigating Environmental Stressors

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Abstract:

Baseline plasma corticosterone (CORT) concentrations have been widely used to investigate the effects of stressors in wild and captive crocodilians. However, collecting baseline plasma CORT samples from wild crocodilians may be particularly difficult due to the capture and handling protocols used for large individuals. Thus, it may prove beneficial to use recently modified techniques for extracting CORT deposited in keratinized and non-keratinized tissues to better quantify the effects of long-term stress in crocodilians. In this study, we investigated the feasibility of using American alligator (*Alligator mississippiensis*) tail scute tissues to quantify CORT by collecting blood and tail scutes from 40 alligators before and after a short-term handling stressor. The objective of the current study was to better understand CORT deposition in crocodilian scutes and whether short-term increases in CORT could be detected. We found that CORT can be reliably extracted from alligator scute tissue and quantified using a commercially available enzyme immunoassay. However, there was a significant increase in scute CORT concentrations following an alligator being exposed to a short-term stressor ($p = 0.017$), although the magnitude of change was less than observed in plasma samples from the same individuals ($p = 0.002$). Furthermore, our results indicate that there was a significant effect of body condition on an alligator's post-stressor CORT concentration ($p = 0.02$). While our study is among the first to experimentally examine the usefulness of tissue CORT in crocodilians, a combination of field and laboratory experiments are needed to better understand deposition rates of CORT in scute tissues and to further validate the usefulness of tissue glucocorticoids for evaluating the effects of stress.

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Key Words: Body Condition Index, Chronic Stress, Crocodilians, Enzyme Immunoassay, Glucocorticoids, Tissue

1. Introduction:

One of the most important responses of an organism to a stressor is the activation of the hypothalamic-pituitary-adrenal (HPA) axis and subsequent secretion of glucocorticoids (Johnstone et al., 2012; Sheriff et al., 2011). A crocodilian's primary glucocorticoid, corticosterone (CORT), naturally follows a prominent biphasic circadian rhythm where CORT concentrations peak at 0800 hr. and then again at 2000 hr. (Lance and Lauren, 1984). However, CORT levels can deviate from this circadian rhythm and increase rapidly when an individual is exposed to a short-term stressor such as capture and handling stress (Franklin et al., 2003; Pfitzer et al., 2014) and cold shock (Lance and Elsey, 1999). Increases in CORT concentrations are a critical component of an organism's daily life, and provide a mechanism for increasing survivorship by temporarily suppressing non-essential functions (e.g., growth and reproduction) to maximize resources for immediate survival (Moore and Jessop, 2003; Sheriff et al., 2011). However, elevated CORT concentrations and HPA activity over long periods of time can inhibit reproduction, suppress the immune system, and impede the growth of an animal (Sapolsky et al., 2000; Wingfield et al., 1998).

Physiological biomarkers are an important tool when evaluating the reaction of a species to an environmental stressor. In crocodilians, plasma CORT concentrations have been used to characterize an animal's response to chronic contaminant exposure (Guillette et al., 1997), extreme weather events (Lance et al., 2010), disease (Nevarez et al., 2011), and stocking densities (Elsey et al., 1990; Finger et al., 2015; Isberg and Shilton, 2013). However,

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glucocorticoid concentrations in blood or plasma can change rapidly in response to a short-term stressor such as capture and handling (3-5 min.), complicating the interpretation of an animal's response to more chronic stressors (Johnstone et al., 2012; Romero and Reed, 2005). Additionally, work published by Goessling et al. (2015) suggests that baseline plasma CORT samples may not adequately evaluate the effects of long-term stressors, thus requiring alternative or supplemental long-term physiological measurements.

Biological samples other than plasma, such as saliva, urine, and feces, have been used to quantify glucocorticoids in a wide variety of taxa. Samples such as feces, for example, provide an opportunity to measure glucocorticoids deposited over an extended period of time (hours-days) without invasive procedures (e.g., capture and blood collection), and may provide a more accurate assessment of long-term stress (Harper et al., 2016; Sheriff et al., 2011; Washburn and Millspaugh, 2002). However, fecal samples can be difficult to collect from some species (Washburn and Millspaugh, 2002). In addition, the effects of individual metabolic rate and diet, as well as sample quality and storage conditions, may limit the utility of fecal samples (Dantzer et al., 2014; Ganswindt et al., 2014). More recently, however, studies have explored the use of glucocorticoid concentrations in keratinized and non-keratinized tissues as a long-term biomarker for chronic stress. Glucocorticoids have been successfully extracted from feathers (Bortolotti et al., 2009, 2008; Lattin et al., 2011), hair (Macbeth et al., 2010; Mastromonaco et al., 2014), nails (Baxter-Gilbert et al., 2014), snakeskin sheds (Berkvens et al., 2013), blubber (Kellar et al., 2015; Trana et al., 2015), and baleen (Hunt et al., 2014).

A common method for marking crocodilians for identification purposes includes the removal of multiple keratinized dorsal tail scutes (Chabreck, 1963; Jennings et al., 1991; Finger et al., 2015, 2016). By cutting a combination of dorsal tail scutes, research and ranching

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operations can assign individual alligators with a permanent and unique identification code (Richardson 2002). Dorsal tail scutes collected during marking have been used for contaminant burden quantification (Jagoe et al., 1998; Rainwater et al., 2007) and stable isotope analyses (Marques et al., 2014), making scute samples a potentially valuable and readily available tissue for also studying long-term stress. In this study, we investigated the suitability of using crocodilian dorsal tail scute tissue samples to quantify CORT concentrations by sampling American alligator (*Alligator mississippiensis*) tail scutes before and after a short-term stressor (i.e., handling stress). The American alligator is one of the most commonly studied crocodilian species (Ryberg et al., 2002), making it an ideal model organism for crocodilian research. Thus, we used American alligators to determine whether: (1) CORT can be reliably extracted from keratinized tail scute tissue, (2) CORT concentrations increase in scutes over a two-hour period in response to a short-term stressor (i.e., capture and restraint); (3) acute stress-induced changes in scute CORT are comparable to plasma CORT; and (4) factors such as body condition influence CORT concentrations in an individual. We selected a 2-hour time frame to replicate previous experiments investigating the effects of a short-term stressor on plasma concentrations in alligators (Guillette et al., 1997; Lance et al., 2004) and because, under most circumstances, the capture, restraint, and biological sampling of wild adult alligators should be possible well within 2 hours. Finally, a two-hour time frame would allow for the opportunity to detect any increases in scute tissues caused by short-term increases in CORT that could complicate interpretation of an animal's physiological response to a long-term stressor.

2. METHODS

2.1. *Alligator Husbandry*

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On March 10th and 13th, 2014, we sampled 40 alligators housed in four climate-controlled chambers (Joanen and McNease, 1976) at the Rockefeller Wildlife Refuge (RWR) in Grand Chenier, LA. All morphometric and temperature values are reported as mean (± 1 SD). Each outdoor concrete chamber had an overall water holding capacity of 1,136 L and approximately 14.9 m^2 of surface area, which was equally divided into open water and dry basking areas. Chambers were climate controlled and had a mean air and water temperature of 16.9 ± 0.48°C and 26.6 ± 0.26°C, respectively, across the two sampling days. Additionally, atmospheric temperature ranged from 16.5°C to 19.5°C each sampling day, with a mean of 18 ± 2.12°C. Each chamber contained approximately 15 juvenile alligators. Alligators used in this study ranged in size from 62.2 – 111.8 cm, with a mean of 89.6 ± 8.7 cm. Animals were fed two-four times per week, but food was withheld for 48 hours prior to sampling. All experimental protocols were approved by the University of Georgia's Institutional Animal Care and Use Committee (approval number A2014 01-030-Y1-A3).

2.2. *Sample Collection*

We sampled 20 alligators each day, with 48 hours between sampling days (n=40 total). To reduce effects of circadian fluctuations and researcher activities on CORT concentrations (Lance and Lauren, 1984), we left alligators undisturbed for approximately 24 hours before collecting baseline (pre-stressor) samples at 10:00 hr. each sampling day (Finger et al., 2015; 2016). Additionally, we only sampled two non-adjacent chambers (n = 10 alligators per chamber) per sampling day to further minimize the effects of noise and activity associated with our sampling protocol.

We hand-captured and manually restrained each alligator before collecting a 4 mL blood sample from the occipital sinus using an 18 gauge, 3.81-cm heparinized needle (Hamilton et al.,

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2016) within 180 seconds of capture (Romero and Reed, 2005). We then used an established crocodilian identification marking technique to collect two dorsal tail scutes within 105 ± 51.9 seconds of capture (Chabreck, 1963; Jennings et al., 1991; Richardson 2002). To standardize scute tissue collection, we had the same researchers batch mark all animals using the same identification code by collecting the first two dorsal tail scutes located posterior to the bifurcated row of scutes. Furthermore, we followed the current code of practice when marking alligators for identification purposes by cutting scute tissue where the tail scutes merge with the scales at the base of the tail (Richardson 2002; Manolis and Webb 2018). The blood and scute samples collected immediately after capture represent pre-stressor samples (i.e., baseline samples). After sample collection, we placed each alligator in its own burlap sack and positioned each animal on the ground for a duration of two hours to induce a stress response (Guillette et al., 1997; Lance et al., 2004). We then removed each alligator from its burlap sack and collected a second blood and scute sample (representing "post-stressor" samples) as previously described to evaluate the influence of a short-term stressor on CORT in this keratinized tissue.

After collecting tissue and blood samples from each individual, we immediately stored samples on ice. Within 20 minutes of sample collection, we centrifuged blood samples for three minutes at 1318 g and aliquoted duplicate 1 mL plasma samples into 1.5 mL tubes. Subsequently, we flash froze all tissue and plasma samples in liquid nitrogen. We then transported all samples on dry ice to the Savannah River Ecology Laboratory near Aiken, South Carolina, and stored them at -60°C.

2.3. *Plasma Corticosterone*

We quantified plasma CORT concentrations using an enzyme immunoassay (EIA; Cat: No. ADI-900-097, Enzo Life Science, Inc., Farmingdale, NY) according to the procedures and

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guidelines outlined by the manufacturer. First, we extracted CORT from each individual at both sampling periods (pre- and post-stressor) by mixing 100 µL of plasma with 900 µL of a 3:2 ethyl acetate:hexane mixture for 20 seconds on a vortexer (Lance and Elsey, 1999; Lance et al., 2004). We left samples undisturbed for 10 min. at room temperature to allow complete separation of the aqueous and organic phases before snap-freezing samples in a dry ice-acetone bath for 8-12 seconds. We decanted the top organic layer (i.e., extracted CORT) into a new 1.5 mL tube, and dried the extracts under a laminar flow hood on a 55°C block heater for approximately 1 hour.

To determine the efficiency of our extraction protocol, we added exogenous CORT to a series of pooled alligator plasma samples prior to extraction. Briefly, we pooled plasma samples from four individuals not being analyzed as part of our experiment and aliquoted the pooled sample across 10-1.5 mL tubes. We then spiked five tubes with 20 µL of a 60 ng/mL CORT standard to obtain a known concentration of ~10 ng/mL. We left the remaining five tubes unspiked. We extracted CORT from each of the spiked and unspiked tubes as previously outlined above. Finally, we determined the extraction efficiency by using the following calculation: (Amount Observed/Amount Expected) * 100. The amount observed is the value obtained from the spiked sample, whereas the amount expected is the calculated amount of standard hormone added plus the amount of CORT in the unspiked pooled sample. We then calculated the mean (± 1 SE) extraction efficiency and used this mean extraction efficiency to correct all plasma CORT concentrations in our experiment before statistical analyses.

For each 96-well plate, we included six standards ranging from 7.82 to 10,000 pg/mL in triplicate. Assay detection limits were determined by taking two standard deviations away from the mean of the total-binding wells (BO; Wada et al., 2007) and ranged from 15.22 pg/mL to 53.69 pg/mL for our study. When CORT concentrations fell below the detection limit of the

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assay (33 out of 80 plasma samples), we used the average detection limit as the CORT concentration for that individual (Hopkins and DuRant, 2011). We tracked inter-assay and intra-assay variation by including an additional 500 pg/mL CORT standard on each plate and by running each alligator sample in triplicate, respectfully, before calculating the average coefficient of variation (CV).

2.4. Plasma Corticosterone Validation

Because sample constituents can interfere with binding antibodies, preventing accurate quantification of CORT concentrations, we employed established validation techniques for our particular assay (Hopkins and DuRant, 2011; Wada et al., 2007) to ensure accurate and reliable results with American alligator plasma samples. We conducted a parallelism test by pooling extracts from four alligators and conducting a 2-fold serial dilution from 1:5 to 1:80 run alongside the standard curve. We plotted the resulting curves and performed a linear regression to determine if there was a significant relationship between the percentage of antibodies bound and CORT concentration in our samples (Hopkins and DuRant, 2011). Using techniques established by Wada et al. (2007), we determined that a sample dilution of 1:20 with 0% steroid displacement buffer was optimal for removing any measurable effects of plasma extract components on CORT concentrations.

2.5. ***Scute Tissue Corticosterone***

We extracted CORT from alligator tail scutes based on hormone extraction techniques established for other keratinized tissues, including nails (Baxter-Gilbert et al., 2014), hair (Mastromonaco et al., 2014), and snake sheds (Berkvens et al., 2013). To prepare tissues for extraction, we measured each scute sample using calipers and recorded length, width, and height. We then washed each scute by vortexing each sample in a 2.0 mL tube for 10 seconds, once with

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1 mL of ultra pure water, and twice with 1 mL of 100% methanol. We dried each sample under a laminar flow hood for 72 hours before cutting scute samples into < 3mm pieces. Finally, we flash froze samples in liquid nitrogen for a minimum of 10 minutes before using a stainless steel mortar and pestle to crush each sample with 3-4 blows with the pestle.

After preparing tissues for extraction, we weighed and transferred each sample to a 7-mL glass scintillation vial. We then extracted samples in 100% methanol using a ratio of 0.001-0.006 g/mL and mixing for 24 hours on an orbital shaker at 200 rpm. Following CORT extraction, we centrifuged the vials for 10 minutes at 1800 g before pipetting off the supernatant into a clean 7-mL glass vial. Finally, we dried samples down using a 60°C sand bath under a laminar hood, and stored samples in a -20°C freezer until analysis. We calculated extraction efficiencies as previously described in our plasma extraction protocol by spiking samples with a 25 ng/mL spike.

To be able to compare scute and plasma samples from the same individuals, we determined scute sample CORT concentrations using the same EIA. First, we brought previously extracted scute samples to room temperature. We then reconstituted our dried-down extracts by adding 400 µL of supplied EIA buffer before vortexing twice for 10 seconds and sonicating each sample for an additional 60 seconds in a CPXH Series Ultrasonic Bath (Fisher Scientific, Suwanee, GA, USA). Reconstituted extracts resulted in a 4.5- to 13.5-fold concentration. Each 96-well plate contained six standards ranging from 7.82-10,000 pg/mL, and an additional 500 pg/mL standard run in triplicate. We assessed inter- and intra-assay variation as previously described for our plasma samples.

2.6. Scute Tissue Validation

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We validated the assay for scute tissue using established protocols for quantifying tissue hormone concentrations on an EIA (Baxter-Gilbert et al., 2014; Berkvens et al., 2013; Mastromonaco et al., 2014). First, we conducted a parallelism test by pooling scute extracts from five alligators to create a 15-fold concentrated sample. Using our pooled, concentrated scute extract, we then conducted a serial dilution from 1:2 to 1:64 to run alongside the standard curve. Finally, we plotted the resulting curves and performed a linear regression to determine if there was a significant relationship between the percentage of antibody bound and CORT concentration.

Additionally, we conducted an accuracy test by creating a pooled sample by combining extracts from five individuals. After concentrating the pooled sample 5-fold in EIA buffer, we aliquoted 200 µL of the pooled sample into eight-1.5 mL tubes. We then spiked each tube with 200 µL of one of the eight standards in the standard curve. Finally, we ran the concentrated pooled sample in triplicate to determine the percent recovery of exogenous CORT using the following calculation: (Amount Observed/Amount Expected) * 100, as described above for plasma. Finally, we conducted a linear regression to determine if there was a significant relationship between the amount of CORT added and amount recovered.

2.7. Statistical Analysis

We performed statistical analyses using R statistical software version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria). We used non-parametric analyses if data violated parametric test assumptions and could not be rectified by log transformation. For each sample type, we compared pre- and post-stressor CORT concentrations. Because normality could not be met through transformation, plasma values were analyzed using an exact Wilcoxon signed-rank test. Log-transformed scute data were analyzed using a paired t-test. Because plasma

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and tissue CORT concentrations may respond differently to a short-term stressor, we also wanted to calculate and compare the change in hormone levels between pre- and post-stressor samples. To do this, we subtracted the pre-stressor concentration from the post-stressor concentration for each individual and sample type to calculate the overall change in CORT concentration before using a paired t-test to compare this change between sample types. Finally, we tested for individual differences in post-stressor scute CORT concentrations using a linear mixed-effects model (package: nlme) that included time (min.) and body condition indexes (BCI) as fixed effects, in which time was the number of minutes for each individual between the pre- and post-stressor sample collection. We generated BCIs by using Fulton's condition factor ($K = W/L^3 * 10^n$, where W = mass in kg, L = SVL in cm, and n = 5; Rice et al., 2007; Hamilton et al., 2016). We included tank as a random effect in our model to account for alligators being housed in four communal tanks. Untransformed CORT concentrations are reported as median, interquartile range (IQR), minimum and maximum values unless otherwise specified. A significance level of 0.05 was used for all statistical tests.

3. RESULTS

3.1. Plasma Corticosterone

The mean (± 1 SE) extraction efficiency of CORT from alligator plasma samples was 75.1 ± 7.3%. Intra- and inter-assay CVs were 5.2% and 3.4%, respectively. A serial dilution of pooled alligator plasma extracts showed parallel displacement with the standard curve ($r^2 = 0.996$, $p < 0.001$), indicating that the immunological properties of our extract were similar to those of the CORT standard. Pre-stressor plasma sample CORT concentrations for this study ranged from 0.38 ng/mL to 2.53 ng/mL (Table 1) and were significantly lower ($V = 0$, $p < 0.001$;

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Fig. 1) than post-stressor CORT concentrations (range: 2.65 ng/mL to 23.65 ng/mL; Table 1), indicating that our experimental protocol did induce a stress response in juvenile alligators.

3.2. ***Scute Corticosterone***

Mean (± 1 SE) scute length was 11.56 ± 0.26 mm, with an average width of 2.70 ± 0.13 mm, and mean height of 1.68 ± 0.07 mm (n = 80). The mean (± 1 SE) extraction efficiency of alligator scute CORT samples was 108 ± 4.3%. Intra- and inter-assay CVs were 6.7% and 3.3%, respectively. Pooled alligator scute CORT serially diluted alongside the standard curve showed parallel displacement ($r^2 = 0.987$, $p < 0.001$; Fig. 2), again indicating immunological similarities between our extracted scute CORT and the assay's CORT standard. EIA accuracy tests yielded a mean recovery of known CORT concentrations at 106 ± 2.1%. In addition, our accuracy test revealed there was a significant relationship between the measured hormone concentrations in the spiked samples with the expected concentrations ($r^2 = 0.999$, $p < 0.001$; Fig. 3).

Pre-stressor scute CORT samples ranged from 4.45 ng/g to 32.66 ng/g. Post-stressor scute CORT samples range from 4.35 ng/g to 66.76 ng/g and were significantly higher than pre-stressor scute samples ($t = -2.49$, $df = 39$ $p = 0.017$; Table 1; Fig. 1). Nonetheless, after controlling for the within-tank correlations of individual alligators, variation among individuals in time between collection of pre- and post-stressor samples did not influence post-stressor CORT concentrations ($t_{(34)} = 0.02$, $p = 0.99$). However, an alligator's BCI (Table 1) contributed positively to the variation in an alligator's post-stressor scute CORT concentration ($t_{(34)} = 2.30$, $p = 0.02$).

3.3. ***Comparison of Sample Types***

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Alligator plasma samples exhibited significantly greater change in CORT concentrations (mean = 9.18 ± 0.82, range = 1.31 to 23.27 ng/mL; Table 1) than did the alligator scute samples (mean = 4.53 ± 1.57, range = -12.81 to 34.10 ng/g; $t = -3.35$, $df = 39$ $p = 0.002$; Table 1; Fig. 4).

4. DISCUSSION

Our study is the first to confirm that reliable levels of CORT can be extracted from crocodilian tail scutes and be quantified on a commercially available EIA. The standard practice of collecting and often archiving crocodilian tail scute samples when marking individuals for ecological studies or ranching purposes provides a readily available tissue for quantifying CORT. The relatively large size of scutes combined with the availability of multiple scutes creates the opportunity to quantify both CORT and other endpoints of interest from the same individual, including contaminant concentrations (Rainwater et al., 2007) or stable isotopes (Marques et al., 2014). Our study adds to the growing body of literature highlighting the potential value of non-keratinized and keratinized tissues, such as nails of freshwater turtles (Baxter-Gilbert et al., 2014) and shed skins of snakes (Berkvens et al., 2013), for quantifying glucocorticoids in reptiles.

As expected, we observed a significant increase in plasma CORT in response to our short-term capture and handling stress protocol, indicating that our experimental methodology induced a stress response in juvenile alligators. Our study yielded baseline and post-stressor plasma CORT concentrations that were similar to previous studies that used comparable techniques (Guillette et al., 1997; Lance et al., 2004). Importantly, baseline and post-stressor CORT concentrations reported here are ecologically relevant and comparable to values reported for alligators held at different stocking densities (Elsey et al., 1990), subjected to extreme

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weather conditions (Lance et al., 2010), and exposed to cold temperatures (Lance and Elsey, 1999). It is important to note, however, that baseline plasma CORT concentrations in multiple samples in our study fell below the detection limit for this assay, complicating efforts to quantify those low CORT concentrations. Alternate extraction methods may increase extraction efficiency and help reduce the influence of plasma components on antibody binding, thereby eliminating the need for diluting samples, and increasing the likelihood of samples falling above the detection limit of the assay.

Contrary to our expectations, we also saw a significant increase in alligator scute CORT concentrations following exposure to a short-term stressor. Nonetheless, the magnitude in change between pre- and post-stressor CORT concentrations was markedly different between the two sample types, with scute CORT increasing less than 2-fold compared to an 11-fold increase in plasma CORT. Generally, elevated glucocorticoid concentrations in keratinized and non-keratinized tissues are thought to reflect prolonged or repeated increases in HPA activity. For example, in a study by Ashley et al. (2011), captive Alaskan caribou (*Rangifer tarandus granti*) hair cortisol concentrations were not significantly affected by a single adrenocorticotrophic hormone (ACTH) challenge. However, the lack of effect of a short-term stressor on hair cortisol is not consistent among studies, as hair cortisol concentration in free-ranging brown bears (*Ursus arctos*) were found to be significantly influenced by multiple factors associated with capture and handling (e.g., presence or absence of capture) within minutes or hours of sample collection (Cattet et al., 2014). Our 2-hour sampling time frame may have been too long, allowing for an increase in circulating CORT in response to our short-term stressor to influence the CORT concentrations of alligator scutes. Studies incorporating a greater series of time points would help characterize when and how CORT levels rise in scutes during a short-term stress response,

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thereby informing the ideal sampling window for using glucocorticoid concentrations in scutes to characterize individual responses to chronic stressors.

Several individuals (*n* = 16) actually exhibited a decrease in scute CORT concentration following exposure to our short-term stressor protocol (Fig. 4). Crocodilian tail scutes have a thick outer layer of keratin and a dense collagen inner core that contains a network of vessels supplying blood to the outer dermis (Richardson et al., 2002). Small variations in sample collection technique (i.e., making a deep tissue cut) may also incorporate a more vascularized portion of the scute, therefore producing higher concentrations of CORT in the sample, and vice versa. For example, variation in blubber cortisol with depth of the sample has been characterized in beluga whales, with higher glucocorticoid concentrations being located in interior tissue samples collected closer to the muscle (Trana et al., 2015). In addition, changes in blood flow to the base of the scute may influence CORT concentrations. Saltwater crocodiles (*Crocodulys porosus*) were found to redirect blood flow to dorsal skin and muscle, and away from the tail during a heating treatment (Seebacher and Franklin 2007). To minimize the influence of thermal-induced changes in blood distribution, all alligators were subjected to the same ambient conditions. Additionally, we attempted to control for sample quality and measurement consistency in this study by having the same investigator perform each task in a controlled setting (e.g., climate control enclosures) and by standardizing sample collection locations and techniques following established protocols for crocodilians (Manolis and Webb 2018). However, the development of more selective sample collection techniques (e.g., analyzing only the distal end of scutes) may be necessary to alleviate the influence of circulating CORT on scute samples.

Variation in CORT deposition among scutes may have also contributed to the differences between pre- and post-stressor CORT concentrations in this study. Harris et al.

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(2016) found that there was no relationship between CORT concentrations among feathers sampled from the left and right side of tree swallows (*Tachycineta bicolor*). That same study also demonstrated a potential for greater variation in feather CORT levels among samples from the same individual than between birds (Harris et al., 2016). African house snake (*Lamprophis fuliginosus*) shed skins also showed significantly elevated CORT concentrations in the tail when compared to other regions of the animal's body (Berkvens et al., 2013). A similar issue may be contributing to the negative change in CORT concentrations following the stress event in our study, with some scutes having higher concentrations of CORT than other scutes on the same individual. Thus, it may be prudent to quantify CORT from multiple scutes collected during the marking process to create an average CORT hormone concentration to accurately interpret an individual's physiological condition.

Additionally, our study focused on analyzing scute samples from juvenile alligators. Scute samples collected from adult alligators may need to be further homogenized for a representative sub-sample to be used for quantifying hormone concentrations due to the large size of adult alligator scutes. Standardizing techniques for collecting and processing tail scute tissue samples will be important for preventing elevated scute CORT concentrations caused by circulating CORT in the blood, and to tackle the challenges of processing larger tissue samples. Methods such as those used in Nilsen et al. (2017) to standardize scute processing for quantifying trace elements may be easily adapted to processing and selecting representative samples for glucocorticoid analysis.

Glucocorticoid concentrations from several other types of keratinized and non-keratinized tissue samples have been used to evaluate the effects of long-term stressors. Baxter-Gilbert et al. (2014) found that CORT concentrations in nails did not significantly differ between

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painted turtles (*Chrysemys picta*) located near a road-impacted and a control site. Another study found that blubber cortisol concentrations in short-beaked common dolphins (*Delphinus delphis*) were correlated with the type of fatality when investigating beach-stranded and by-caught individuals (Kellar et al., 2015). Captive rhinoceros auklet (*Cerorhinca moncerata*) chicks raised on restricted diets had higher CORT in their primary feathers when compared to chicks reared on a controlled diet (Will et al., 2014). In this study, body condition was a significant factor influencing CORT concentration and could have contributed to the rise of CORT in certain individuals. Some reptiles exhibit an increased adrenocortical response in relation to factors such as body condition (Moore and Jessop, 2003). However, Jessop et al. (2003) found that a calculated body condition index was not a significant predictor of plasma corticosterone in Australian freshwater crocodiles (*Crocodylus johnstoni*). While studies incorporating alternative tissue types as biomarkers for evaluating chronic stress have been widely used across several taxa, few have investigated the rate at which glucocorticoids are deposited and to what extent these hormone levels are influenced by short-term stressors and other factors. Manipulative studies using ACTH challenges (Mastromonaco et al., 2014), CORT implants (Morici et al., 1997), or varying levels of environmentally relevant stressors could be used to investigate the rates of CORT deposition in tissues such as scutes, and to elucidate the potential utility of such samples from wild-caught individuals.

4.1. ***Summary and Recommendations***

In this study, we successfully extracted CORT from alligator scutes and validated the use of a commercially available EIA to quantify scute CORT. We also demonstrated that scute CORT experienced a lower magnitude of change in response to a short-term stressor than did plasma CORT. Reducing the effect of short-term capture stress by narrowing the sampling

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window could improve the utility of scute CORT in evaluating chronic stress. However, because some individuals exhibited decreased scute CORT levels in post-stressor samples, we suspect that deposition of CORT may vary among scutes or that scute CORT levels may be sensitive to sampling and processing techniques. These issues may be mitigated by averaging CORT levels across multiple scutes and by ensuring that scute samples are collected immediately following capture. However, further investigation is necessary to determine the extent of variation in CORT deposition between scutes in an individual, and the extent to which scute CORT levels may be prone to increases due to circulating CORT. Until the utility of crocodilian scutes as a biomarker of stress can be further validated, we recommend this technique be restricted to those scenarios where baseline samples can be quickly collected, such as wild juvenile alligators that are easy to capture and do not require prolonged restraint, or alligators in a captive or experimental setting. Future studies investigating the time frame over which capture stress induces an increase in scute CORT concentration or documenting the long-term deposition of CORT in response to exogenous CORT would increase the application and utility of tail scutes as a biomarker of long-term stress in crocodilians.

Declarations of interest: None

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Table 1. Raw corticosterone (CORT) values in plasma and scute samples of American alligators (*Alligator mississippiensis*). Body condition index (BCI) was calculated using Fulton's condition factor with SVL and mass.

CORT Sample Type	Mean (± 1 SE)	Median	Interquartile Range	Range
Baseline Plasma (ng/mL)	-	0.86	0.38 - 1.34	0.38 - 2.53
Post-Stressor Plasma (ng/mL)	-	9.85	6.52 - 12.42	2.65 - 23.65
Baseline Scute (ng/g)	-	12.80	9.84 - 18.47	4.45 - 32.66
Post-Stressor Scute (ng/g)	-	15.13	12.01 - 25.62	4.35 - 66.76
Plasma Change (ng/mL)	9.18 (± 0.82)	-	-	1.31 - 23.27
Scute Change (ng/g)	4.53 (± 1.57)	-	-	-12.81 - 34.10
Body Condition Index (BCI)	2.16 (± 0.06)			1.46 – 2.74

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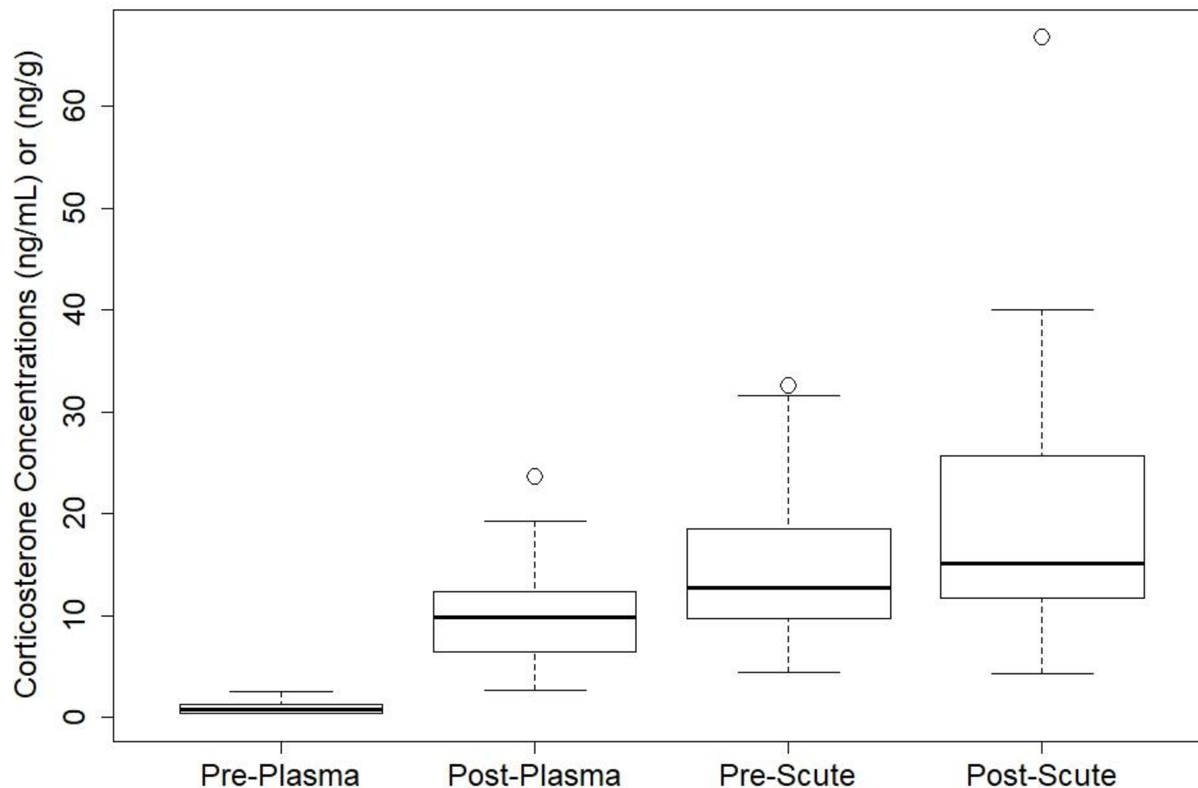

Figure 1. Boxplots of American alligator (*Alligator mississippiensis*) corticosterone (CORT) concentrations in plasma and scute samples (n = 40) before (pre-stressor) and after (post-stressor) exposure to short-term capture stress. Outliers are represented by circles above each boxplot. Post-stressor plasma and scute CORT concentrations were significantly higher than pre-stressor concentrations using exact Wilcoxon signed-rank tests and a paired t-test, respectively ($V = 0$, $df = 39$, $p < 0.001$ and $t = -2.49$, $df = 39$ $p = 0.017$).

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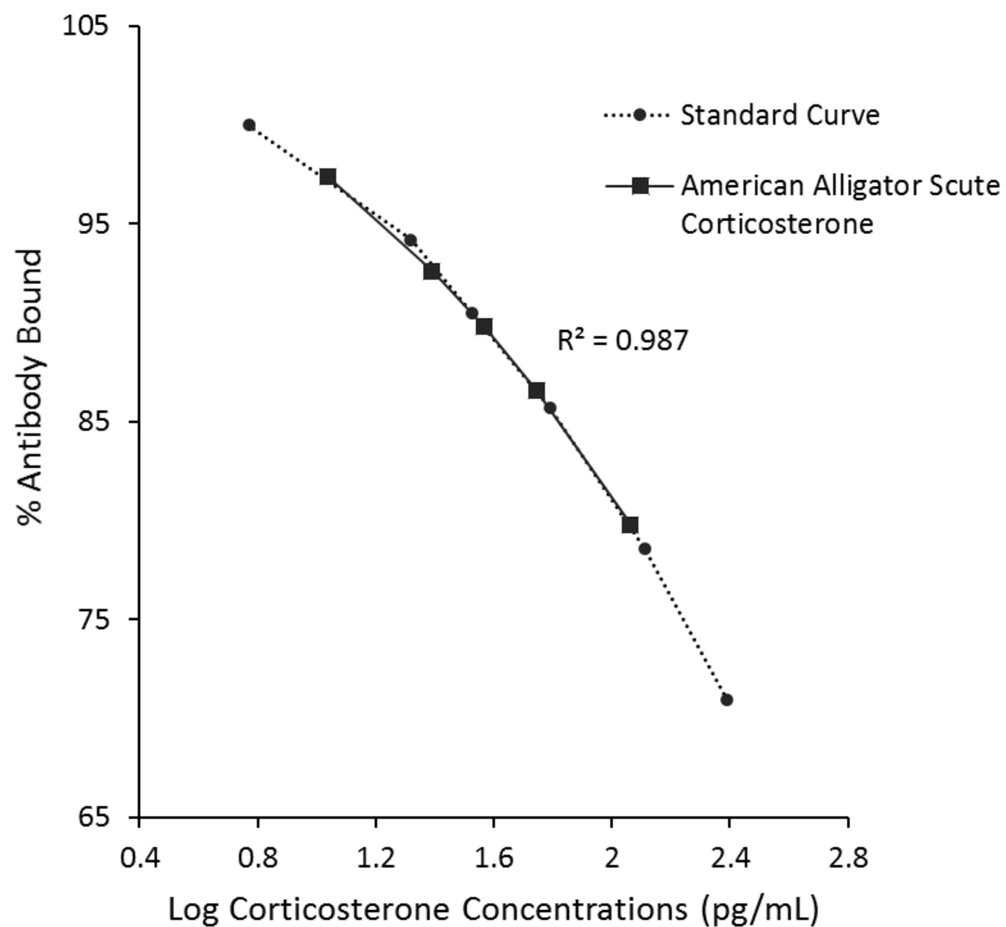

Figure 2. Parallelism between serially diluted American alligator (*Alligator mississippiensis*) scute corticosterone extract and the standard curve.

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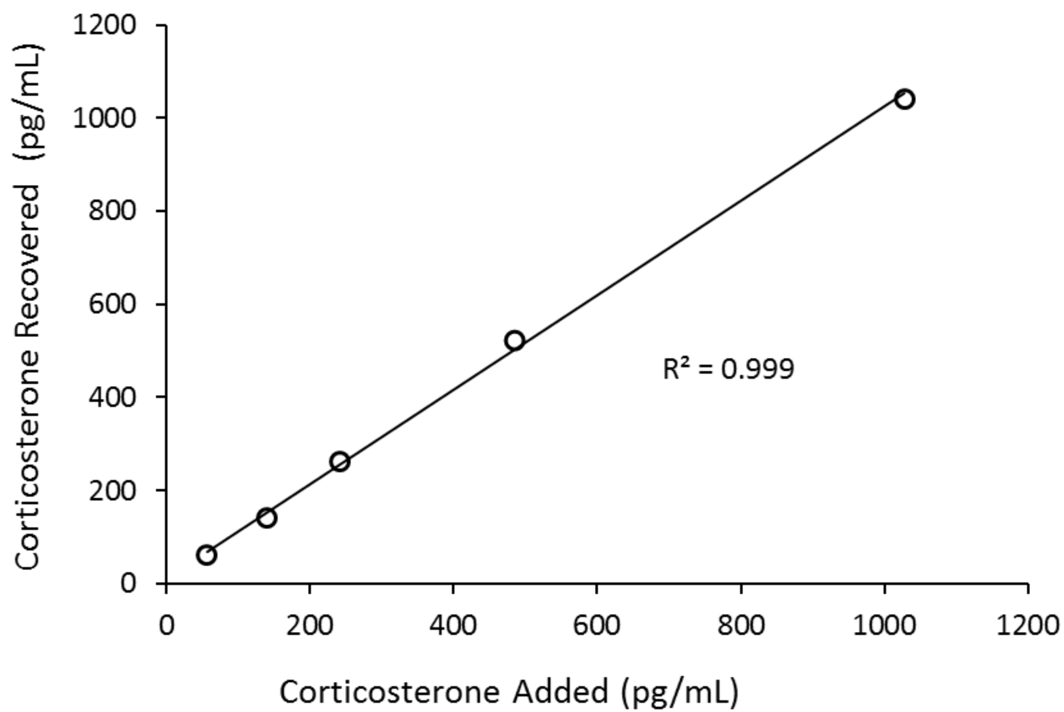

Figure 3. Exogenous corticosterone recovered from alligator scute extracts was strongly and positively correlated with amount of corticosterone added ($r^2 = 0.999$, $p < 0.001$).

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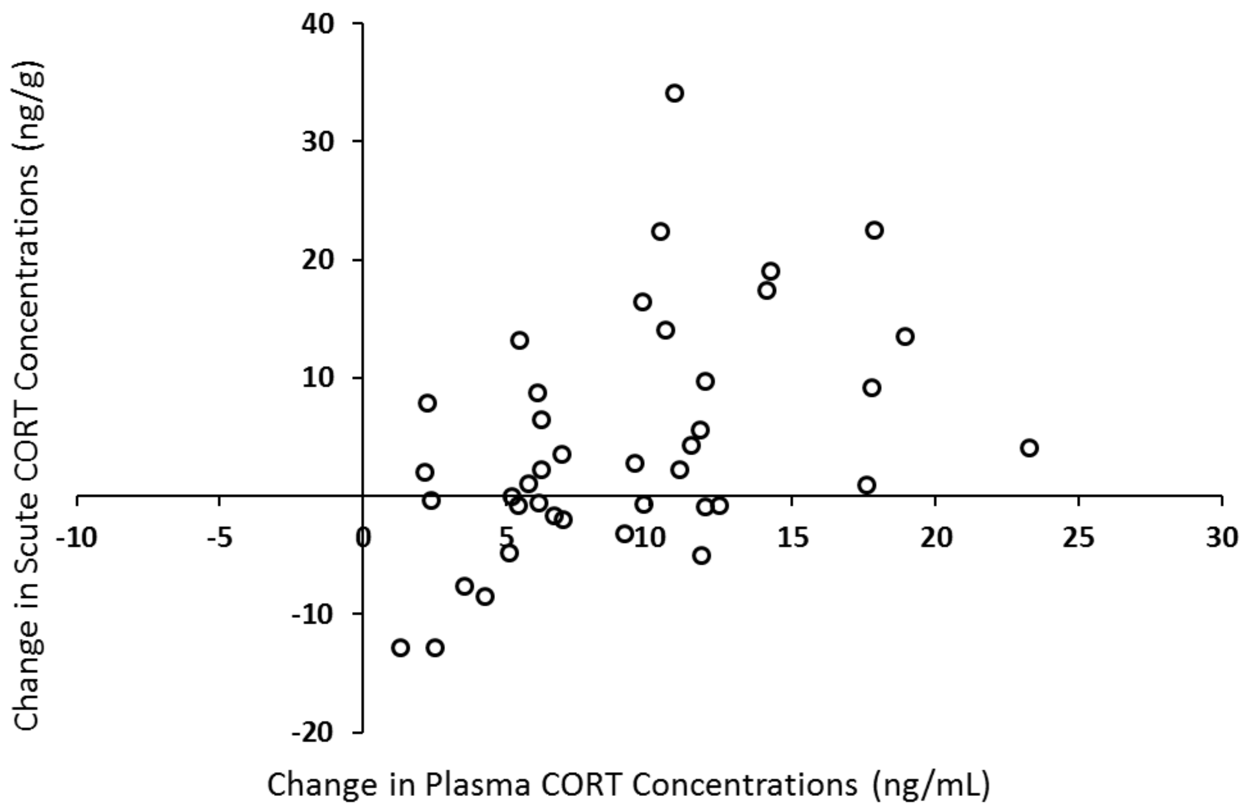

Figure 4. Change between pre- and post-stressor corticosterone (CORT) concentrations in both plasma and scutes plotted for individual juvenile alligators (n = 40) exposed to short-term capture and restraint stress.

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Highlights:

- Modified extraction techniques have allowed for tissue glucocorticoid quantification.
- We reliably extracted corticosterone (CORT) from American alligator scute tissue.
- Scute CORT levels increased less than plasma CORT after a two-hour stressor.
- Body condition and individual scutes may influence CORT concentrations.
- Crocodilian scute CORT may provide insight into stress under controlled conditions.

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