Using Crocodilian Scute Tissue as an Integrative Approach to Investigating Long-term Environmental Stressors

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Introduction:

An organism's response to a stressor includes the stimulation of the hypothalamic-pituitary-adrenal (HPA) axis and secretion of glucocorticoids (Johnstone et al., 2012; Sheriff et al., 2011). A crocodilian's main glucocorticoid, corticosterone (CORT), follows a biphasic circadian rhythm (Lance and Lauren, 1984) and can increase rapidly to provide a mechanism for immediate survival by temporarily suppressing non-essential functions (e.g., reproduction; Moore and Jessop, 2003). However, long-term activation of the HPA axis can suppress the immune system, decrease growth, and reduce reproductive output of an individual (Sapolosky et al., 2000). While plasma CORT concentrations in crocodilians have been characterized in multiple studies investigating environmental stressors (Guillette et al., 1997; Lance et al., 2010), collecting baseline plasma samples in this taxa can be challenging since capturing and safely securing them prior to sample collection can be a prolonged process. Glucocorticoids in blood can change rapidly (3-5 minutes) in response to a short-term stressors such as capture and handling (Romero and Reed, 2005). Samples such as feces and keratinized tissue (e.g., nails and feathers) can be used to measure glucocorticoids deposited over extended periods of time, potentially providing a more accurate assessment of long-term stress while being less influenced by capture-induced stress (Sheriff et al., 2011).

Crocodilians are exposed to a wide array of stressors (e.g., habitat loss, contaminant exposure) that have contributed to over half of all crocodilian species being listed as a species of conservation concern. In this study, we validated the use of crocodilian scute tissue to quantify CORT concentrations as a potential measure of long-term stress. The common marking technique of removing crocodilian tail scutes provides a valuable sample for not only quantifying CORT, but to also characterize contaminant burdens (Rainwater et al., 2007) and conduct stable isotope analyses (Marques et al., 2014). As such, we designed a validation study to evaluate whether or not CORT increased in scute samples over a 2-hour capture and handling protocol (i.e., does short-term handling stress influence scute CORT concentrations). We then followed up our validation study with an applied study to determine if scute CORT was strongly associated with selenium (Se) tissue concentrations during a long-term contaminant

exposure study. Our validation (Hamilton et al., 2018) and applied (Finger et al., 2019) studies have been published and provide additional details not included in this report.

Methods:

Validation Study – We collected plasma and scute samples from 40 juvenile American alligators ranging in size from 62.2 to 111.8 cm in total length (mean 89.6 ± 8.7 cm) at the Rockefeller Wildlife Refuge in Louisiana, USA. Briefly, we collected alligators from four climate-controlled chambers (n=10 each) across two sampling days starting at 10 a.m. each day to reduce the influence of stress associated with sampling practices and circadian fluctuations on hormone concentrations. We haphazardly hand-grabbed and manually restrained alligators before collecting a blood sample from the occipital sinus and two dorsal tail scutes within three minutes of capture (i.e., baseline samples). We placed alligators in a burlap sack for 2- hours (Guillette et al., 1997) before collecting a second blood and scute sample (i.e., "post-stressor" samples). Following sample collection, samples were flash frozen in liquid nitrogen before being transported to the lab on dry ice.

Applied Study – We transported 24 juvenile alligators from the Rockefeller Wildlife Refuge to the Savannah River Ecology Lab in South Carolina, USA where animals were housed in three flow-through pens in an aquatic animal holding facility. We then divided the animals into three Se dietary treatments (i.e., mice spiked with 1000, 2000 ppm of selenomethionine or Control spiked with deionized water) for seven weeks and measured head length (HL ± SE) of the 1000 ppm group (10.99 ± 0.20 cm), 2000 ppm group (11.45 ± 0.17 cm) control group (11.78 ± 0.12 cm). At the end of the seven-week exposure, we haphazardly hand-grabbed alligators prior to collecting blood and scute tissue samples as described in the validation study.

Plasma Corticosterone – We extracted plasma CORT samples by mixing 100 µL of plasma with 900 µL of 3:2 ethyl acetate:hexane for 20 seconds on a vortexer. We then left samples undisturbed for 10 minutes before snap-freezing samples in a dry ice-acetone bath for 8-12 seconds. We then decanted the organic layer (i.e., extracted CORT) into a 1.5 mL tube and dried extracts in a laminar flow hood at 55 °C for approximately 1-hour. We calculated extraction efficiencies by adding exogenous CORT to a series of pooled alligator plasma samples prior to extraction. We determined the extraction efficiency by using the following calculation: Amount Observed/Amount Expected * 100. We also tested for parallelism by running a series of extracted samples using a 2-fold serial dilution from 1:5 to 1:80 to determine if there was a significant relationship between percent of antibodies bound and CORT concentrations. Finally, we used a 1:20 dilution with 0% steroid displacement buffer when running plasma samples on our EIA kits (ADI-900-097 Enzo, Farmingdale, NY).

Scute Corticosterone – First, we washed each scute by vortexing the sample for 10 seconds with ultra-pure water and twice with 1 mL of 100% methanol. We then dried samples in a laminar flow hood for 72 hours before cutting samples into < 3 mm pieces. After flash freezing samples in liquid nitrogen for 10 minutes, we used a steel mortar and pestle to crush each sample. We then extracted scute tissue samples using a ratio of 0.001 – 0.006 g/mL of 100% methanol for 24 – hours on an orbital shaker set at 200 rpm. Next, we centrifuged the vials for 10 minutes at 1800 g before pipetting off the organic layer (i.e., extracted CORT) and drying the supernatant in a laminar hood at 60 °C. We calculated extraction efficiencies as described in our plasma CORT methods. We conducted a parallelism test by conducting a serial dilution from 1:2 to 1:64 to run alongside the standard curve with a 15-fold pooled concentrated sample. Finally, we conducted an accuracy test by spiking eight pooled samples with one of the eight standards in the standard curve. We then calculated the recovery of exogenous CORT using the following calculation: Amount Observed/Amount Expected * 100. We followed the manufacturers recommendations when rehydrating samples and conducting the EIA (ADI-900-097 Enzo, Farmingdale, NY).

Statistics – We conducted statistical analyses using R statistical software (Validation Study) and JMP Pro 13 (Applied Study). We used non-parametric exact Wilcoxon signed-rank and parametric t-test to evaluate pre- and post-stressor CORT concentrations. We tested for individual differences in post-stressor scute CORT concentration using a linear mixed-effects model (Validation Study; please see Hamilton et al. 2018). Finally, to evaluate which stress parameter best indicated Se accumulation in the liver and kidney, we used a principal component and discriminant analyses. In addition to plasma and scute CORT, we also included heterophil to lymphocyte ratios and a body condition index in analyses but these data will not be summarized here (Applied Study; please see Finger et al., 2019). All values reported as mean ± 1 SE.

Results:

Validation Study – The mean extraction efficiency of CORT from plasma samples was 75.1 ± 7.3% and 108 ± 4.3% from alligator scute samples. A serial dilution of pooled alligator plasma and scute extracts showed parallel displacement with the standard curve ($r^2 = 0.996$, $p < 0.001$ and $r^2 = 0.987$, $p < 0.001$, respectively). Scute accuracy test yielded a significant relationship between the measured hormone in spiked samples with the expected concentrations ($r^2 = 0.999$, $p < 0.001$). Pre-stressor plasma samples ranged from 0.38 ng/mL to 2.53 ng/mL and post-stressor plasma samples ranged from 2.65 to 23.65 ng/mL. Pre-stressor and post-stressor scute samples ranged from 4.45 to 32.66 ng/g and 4.35 to 66.76 ng/g, respectively. Both pre-stressor plasma ($v = 0$, $p < 0.001$) and scute ($t = -2.49$, $df = 39$ $p = 0.017$) samples were significantly lower than post-stressor samples. 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). The calculated body condition index did contribute positively to the variation in an alligator's post-stressor scute CORT concentration ($t_{(34)}$ = 2.30, p = 0.02).

Applied Study – The liver Se concentrations (ppm) were 2.22 ± 0.14 (Control), 35.20 ± 6.31 (1000 ppm), and 49.97 ± 4.00 (2000 ppm). The kidney Se concentrations were 6.51 ± 0.22 (Control), 101.60 ± 8.64 (1000ppm), and 96.38 ± 5.81 (2000 ppm). The plasma concentrations (ng/mL) were 0.62 ±0.12 (Control), 3.00 ± 1.27 (1000 ppm), and 9.66 ± 4.55 (2000 ppm). The scute concentrations (ng/mL) were 6.20 ± 1.16 (Control), 11.19 ± 2.23 (1000 ppm), and 19.67 ± 3.87 (2000 ppm). Scute CORT was significantly correlated with liver and kidney SE concentrations (F ratio = 3.769, p = 0.049).

Discussion:

Validation Study – We were able to reliably extract CORT from all alligator scute tissues and validate the use of these extracts on our EIA. While we expected to see a significant increase in plasma CORT in response to our short-term capture and handling stress (see, Guillette et al., 1997), we also saw a significant increase in our keratinized scute tissues. Crocodilian scute tissues have a vascular bed of blood vessels that spread throughout the outer dermis layer towards the keratinized epidermis (Richardson et al., 2002). There was an 11-fold increase in plasma CORT samples, whereas scute CORT concentrations exhibited a less than 2-fold increase. It is plausible that the increased circulating CORT levels in blood influenced hormone levels in our scute samples. It will be important to evaluate how concentrations of CORT change over time in crocodilian tail scutes. Validation studies that incorporate more narrow time series (e.g., every ten minutes vs. 2- hours in this study) might provide additional information on the perfusion of blood and CORT in scute tissues.

In addition, sixteen alligators in this study also showed a decrease in scute CORT concentrations following our short-term handling stress (ranging from -12.80 to -0.05 ng/g). Even though we standardized sample collection by having the same investigator perform the same task (e.g., collecting blood or scute tissue samples) and standardizing location of scutes collected, there may have been small variations in sample collection technique (e.g., deep tissue cut) or variation in scute morphology contributing to changes in CORT concentrations. Variations in glucocorticoid concentrations based on tissue depth have been recorded in other sample types and species (e.g., blubber; Trana et al., 2015). Future studies should continue to control for tissue depth during sample collection and aim to characterize changes in CORT concentrations depending on tissue depth.

Because we report an increase in scute CORT following a short-term stress event, we suggest limiting the use of this technique to situations where baseline samples can be collected within minutes of capture (e.g., juvenile or captive animals). It might also be necessary to pool and analyze multiple scutes collected during the marking process to reduce potential influences of variation in CORT deposition across scute tissues and accurately interpret the physiological condition of an animal. Nevertheless, scute samples may be a more reliable alternative for evaluating long-term stress in crocodilians when compared to more conventional samples (e.g., blood/plasma) since hormone concentrations in scutes are not as affected by short-term stress.

Applied Study – When evaluating which parameter (H:L ratios, BCI, Plasma CORT, Scute CORT) best indicated Se accumulation in the liver and kidney of our contaminant stressor experiment, the only parameter significantly correlated with liver and kidney Se concentrations was scute CORT. While both plasma and scute had increased concentrations of CORT in our 1000 and 2000 ppm Se treatment when compared to controls, keratinized and other tissues may reflect elevated glucocorticoid concentrations over a longer period of time (e.g., exposure to contaminant stressors in the environment). Crocodilian scutes may act as a versatile sample when quantifying trace element accumulation (Nilsen et al. 2017) and evaluating stress response. However, caution should be used when evaluating scute CORT concentration until additional validation studies can characterize CORT deposition and standardize sampling techniques for larger animals (i.e., larger scute samples).

Products:

Finger, J.W., Hamilton, M.T., Kelley, M.D., Stacy, N.I., Glenn, T. C., Tuberville, T. D., 2019. Examining the effects of chronic selenium exposure on traditionally used stress parameters in juvenile American alligators (*Alligator mississippiensis*). Arch. Environ. Contam. Toxicol. 77, 14-21.

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