CHARACTERIZING STRESS AND IMMUNE PARAMETERS IN THE AMERICAN ALLIGATOR (*ALLIGATOR MISSISSIPPIENSIS*)

by

MATTHEW T. HAMILTON

(Under the Direction of Tracey D. Tuberville and Robert B. Bringolf)

ABSTRACT

Crocodilians are exposed to a suite of abiotic and biotic stressors that have the potential to influence individual and population health. I collected pre- and post-stressor blood and tissue samples from 40 juvenile captive alligators using a short-term capture and handling stress protocol to evaluate the short-term effects of stress on six commonly used wildlife stress and immune metrics, and to validate the use of tail scute tissue samples for quantifying corticosterone concentrations. I found that the short-term stressor of capture and restraint caused significant increases in plasma corticosterone and lactate concentrations, percent heterophils, and H:L ratios, while a significant decrease in percent lymphocytes. I reliably extracted corticosterone from scute tissues, however the significant increase in scute corticosterone concentrations following the short-term stressor necessitates further investigation before applying this technique broadly across crocodilian research. These studies highlight the importance of evaluating the effects of capture methods when investigating environmental stressors.

INDEX WORDS: American alligator, *Alligator mississippiensis*, Stressors, Hypothalamus-Pituitary-Adrenal Axis (HPA), Glucocorticoids
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TABLE OF CONTENTS

ACkNOWLEDGEMENTS ............................................................................................................................. iv
LIST OF TABLES ....................................................................................................................................... viii
LIST OF FIGURES ..................................................................................................................................... ix

CHAPTER

1 INTRODUCTION AND LITERATURE REVIEW ................................................................. 1
   THESIS OBJECTIVES ..................................................................................................................... 6
   LITERATURE CITED ..................................................................................................................... 8

2 EVALUATION OF SHORT-TERM STRESS AND IMMUNE PARAMETERS IN THE AMERICAN ALLIGATOR (ALLIGATOR MISSISSIPPIENSIS) .............................................................................................................. 13
   INTRODUCTION .......................................................................................................................... 13
   METHODS ..................................................................................................................................... 15
   RESULTS ...................................................................................................................................... 21
   DISCUSSION ............................................................................................................................... 23
   LITERATURE CITED ..................................................................................................................... 28

3 EVALUATING THE POTENTIAL USE OF CROCODILIAN SCUTE TISSUE WHEN INVESTIGATING LONG-TERM ENVIRONMENTAL STRESSORS ........................................................................................................... 36
   INTRODUCTION .......................................................................................................................... 36
METHODS ...........................................................................................................39
RESULTS .............................................................................................................47
DISCUSSION .........................................................................................................49
LITERATURE CITED .............................................................................................55

4 CONCLUSIONS .................................................................................................64
LITERATURE CITED .............................................................................................67
Table 2.1: Summary of leukocyte differentials, heterophil:lymphocyte ratios (H:L), packed cell volume (PCV), total solids (TS), and bacteria killing ability (BKA) of American alligators (Alligator mississippiensis; n = 40) exposed to short-term handling stress and restraint for 2 hours. Reported values are medians (range) of each parameter, except for total solids, which is reported as mean (range). Significant comparisons between pre- and post-stressor samples collected from the same individuals are indicated in bold (all p ≤ 0.002) .............................................32
LIST OF FIGURES

Page

Figure 2.1: Boxplots of juvenile American alligator (*Alligator mississippiensis*; *n* = 40) corticosterone concentrations in plasma samples collected from the same individuals before (pre-stressor) and after (post-stressor) exposure to a short-term capture and restraint stress. Post-stressor corticosterone concentrations were significantly higher than pre-stressor concentrations using an exact Wilcoxon signed-rank test (*V* = 0, *p* < 0.001) .................................................33

Figure 2.2: Mean plasma pre-stressor and post-stressor lactate concentrations with 95% confidence intervals in juvenile American alligators (*Alligator mississippiensis*; *n* = 40) exposed to a short-term capture and restraint stress. Post-stressor plasma lactate concentrations were significantly higher than pre-stressor lactate concentrations using a paired *t*-test (*t*_{39} = -3.8431, *p* < 0.001) ..................34

Figure 2.3: Boxplots of juvenile American alligator (*Alligator mississippiensis*; *n* = 40) heterophil:lymphocyte (H:L) ratios before (pre-stressor) and after (post-stressor) exposure to a short-term capture and restraint stress. Using an exact Wilcoxon signed-rank test, post-stressor H:L ratios were significantly higher than pre-stressor samples (*V* = 82.5, *p* < 0.001). To reduce scale of the y-axis, we log transformed H:L ratios. .................................................................35

Figure 3.1: Boxplots of American alligator (*Alligator mississippiensis*) corticosterone concentrations in plasma and scute samples (*n* = 40) before (pre-stressor) and
after (post-stressor) exposure to short-term capture stress. Post-stressor plasma and scute corticosterone concentrations were significantly higher than pre-stressor concentrations using an exact Wilcoxon signed-rank tests ($V = 0, p < 0.001$ and $V = 217, p = 0.008$ respectively).

Figure 3.2: Parallelism between serially diluted American alligator (*Alligator mississippiensis*) scute corticosterone extract and the standard curve.

Figure 3.3: Exogenous corticosterone recovered from alligator scute extracts was strongly and positively correlated with amount of corticosterone added ($r^2 = 0.998, p < 0.001$).

Figure 3.4: Change in individual raw plasma and scute corticosterone concentrations in juvenile alligators ($n = 40$) exposed to short-term capture and restraint stress.
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Wildlife confront a suite of social, environmental, and physiological stressors over the course of their lifetime that can contribute to a cascade of behavioral and physiological changes (Moore and Jessop, 2003; Sapolsky et al., 2000). Stressors can be defined as any stimulus that either directly threatens, or is perceived to threaten, an organism’s survival or homeostasis and subsequently induces a stress response (Boonstra, 2013). Reptiles in particular are confronted with an assortment of stressors that have contributed to drastic declines in populations globally, including habitat loss and degradation, introduction of invasive species, environmental pollution, disease, unsustainable use, and climate change (Gibbons et al., 2000). Reptiles and other organisms have a set of physiological and behavioral strategies to manage abiotic and biotic stressors through the stimulation of the sympathetic nervous system and activation of the hypothalamus-pituitary-adrenal (HPA) axis (Sheriff et al., 2011). However, long-term activation of physiological and behavior mechanisms due to long-term or persistent stressors may cause reduced fitness and survival.

Physiological strategies include the stimulation of the sympathetic nervous system, which results in the release of catecholamines (e.g., epinephrine, norepinephrine; Reeder and Kramer, 2005). The HPA axis stress response begins with the release of corticotrophin releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus, which then stimulates the anterior pituitary to secrete adrenocorticotropic
hormone (ACTH) into the blood stream (Sheriff et al., 2011; Webster Marketon and Glaser, 2008). ACTH then travels to the adrenal gland to cause the synthesis and release of glucocorticoids. Under short-term stress, glucocorticoid feedback mechanisms operate normally to return systems back to homeostasis, however long-term stress can cause weak feedback signals and subsequently longer periods of HPA activation (Sheriff et al., 2011).

Glucocorticoids released during a short-term stressor help to mitigate the potential deleterious effects of stressors by reducing non-essential functions such as reproduction and growth to increase the mobilization of energy stores and promote escape behaviors (Moore and Jessop, 2003; Wingfield et al., 1998). However, long-term elevation in glucocorticoid concentrations and HPA activity can suppress the immune system, inhibit reproduction and growth, and impede territorial behaviors of an animal (Sapolsky et al., 2000; Sopinka et al., 2015; Wingfield et al., 1998). For example, short-term stress can have immunoenhancing effects by increasing the number and proportion of circulating leukocytes (Davis et al., 2008; Dhabhar, 2009), whereas increases in long-term stress can reduce the number of lymphocytes in the spleen or thymus and suppress antibody responses (Pruett, 2001; Webster Marketon and Glaser, 2008). The potential deleterious individual- and population-level effects of stress require an increased focus on characterizing the interaction between an organism and its environment.

To characterize the relationships between vertebrates and their environments, researchers have often employed tools and techniques to quantify stress through measurements collected from blood samples (Maceda-Veiga et al., 2015). Research has quantified stress through the use of several different metrics including stress hormones
(i.e., glucocorticoids), heterophil:lymphocyte ratios (H:L ratios), leukocyte counts, immune function, and stress proteins (Johnstone et al., 2012). Unfortunately, handling and capture stress associated with sample collection may cause interpretational challenges due to rapidly changing stress indices. Trapping and immobilization techniques often employed in research generate a stress response that may influence glucocorticoid concentrations in as little as 3 minutes (Breuner et al., 2008; Romero and Reed, 2005). Elevated glucocorticoid concentrations caused by capture stress can increase the number of circulating heterophils/neutrophils (Davis et al., 2008; Dhabhar, 2002), in addition to causing fluctuations in glucose concentrations (Maceda-Veiga et al., 2015). Furthermore, some species exert increased muscular activity during capture and restraint, subsequently contributing to an acid-base disturbance and release of lactic acid (Coulson and Hernandez, 1983; Franklin et al., 2003; Olsson and Phalen, 2013). Thus, it is important to account for capture and sample collection techniques when assessing wildlife stress and immune function so that baseline samples can be accurately collected and interpreted.

Alternatively, biological samples such as feces, saliva, and keratinized and non-keratinized tissue may be useful biomarkers for measuring long-term stress. Glucocorticoid concentrations measured in feces provide an opportunity to assess stress over the course of hours or days (Johnstone et al., 2012; Sheriff et al., 2011), and have been used to quantify stress associated with the effects of anthropogenic disturbance (Creel et al., 2002) and capture (Harper et al., 2016). However, sample collection for analyzing fecal glucocorticoid concentrations may be particularly difficult for some species like Alaskan brown bears (*Ursus arctos horribilis*) and white-tailed deer.
Glucocorticoids have been successfully extracted from several types of keratinized tissues including 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), snake sheds (Berkvens et al., 2013), baleen (Hunt et al., 2014), and non-keratinized tissues such as blubber (Kellar et al., 2015; Trana et al., 2015). Glucocorticoid concentrations from feathers (Bortolotti et al., 2009, 2008; Lattin et al., 2011) and hair (Macbeth et al., 2010; Mastromonaco et al., 2014) may provide insight into stress exposure over weeks or months, and have been used in studies investigating mortality risk (Koren et al., 2012), offspring investment (Fairhurst et al., 2011), and body condition (Macbeth et al., 2012). While these tissues provide a promising avenue for assessing long-term stress over extended periods of time (i.e., days to months depending on the sample), further validation is necessary to determine the rate of glucocorticoid deposition and potential sources of confounding variables. For example, Cattet et al., (2014) suggested that brown bear (Ursus arctos) hair cortisol concentrations may be influenced by the presence or absence of capture, restraint, and handling, thus implying that hair cortisol concentrations may be affected by factors other than hormones being deposited by passive diffusion over time. Additionally, levels of cortisol in beluga whale (Delphinapterus leucas) blubber seem to increase with tissue depth, and are negatively influenced by the condition of the sample (Trana et al., 2015). Thus, it is important to
further validate the use of these tissue samples by incorporating manipulative studies using ACTH challenges (Mastromonaco et al., 2014), hormone implants (Morici et al., 1997), analyses of sample collection and storage techniques (Cattet et al., 2014; Trana et al., 2015), and/or levels of environmentally relevant stressors to investigate the deposition and use of glucocorticoid hormone concentrations in tissues when investigating the effects of environmental stressors.

Crocodilian populations are exposed to a suite of environmental stressors including habitat loss and degradation (Ross, 1998; Thorbjarnarson et al., 2006), exposure to contaminants (Guillette et al., 2000; Rainwater et al., 2007), and extreme weather (Lance et al., 2010). The aforementioned stressors in combination with illegal harvest have led to declines in several crocodilian species (Ross, 1998). The American alligator (Alligator mississippiensis) was exploited heavily throughout the late 19th century until the mid-late 20th century (Thorbjarnarson, 1992) when the alligator was listed under the Endangered Species Preservation Act of 1966. Populations of American alligators have since recovered as a result of management and conservation efforts, and are now a hunted species in several states throughout its range.

American alligators inhabit a wide range of wetland ecosystems, including freshwater sloughs, marshes, swamps, lakes, and rivers. The alligator’s large distribution across the southeastern United States spans across ten states, including Alabama, Arkansas, North and South Carolina, Florida, Georgia, Louisiana, Mississippi, Oklahoma, and Texas (Richardson et al., 2002). These long-lived predators are not only of economic importance because of sustainable management programs, but alligators are also an indicator of environmental quality and an important component of wetland
ecosystems (Milnes and Guillette, 2008). The American alligator is one of the most studied crocodilian species in the world (Brisbin et al., 1986; Ryberg et al., 2002), with research focused on a wide array of topics including behavior (Lange, 1976; Vliet, 1989), reproduction (Guillette et al., 1997; Lance et al., 2009; Lance, 1989), genetics (Green et al., 2014), and stress physiology (Coulson and Hernandez, 1983; Guillette et al., 1997; Lance et al., 2004). As a model species for crocodilians, it is important to identify the effects of environmental stressors and validate the use of tools and techniques to quantify the effects of long-term stressors in crocodilians.

THESIS OBJECTIVES

To properly manage and conserve crocodilians, an array of tools and techniques must be used to assess the health status of individuals exposed to environmental stressors. My research focused on investigating the effects of capture and restraint on a suite of stress and immune parameters to evaluate their effectiveness when investigating stress in the American alligator. To do this, I exposed forty juvenile captive alligators to a 2-hour short-term stressor (capture and restraint) and collected blood and tissue samples pre- and post-stressor to evaluate any changes in stress and immune parameters. In Chapter 2, I used pre- and post-stressor blood and plasma samples to investigate short-term changes in six commonly used stress and immune metrics. My objectives were to: (1) examine how a short-term stressor affects plasma corticosterone and lactate concentrations, bacteria killing ability, blood differentials (including H:L ratios), total solids, and packed cell volume values, and (2) to determine their potential utility when characterizing stress. In Chapter 3, I used modified tissue glucocorticoid extraction techniques to extract...
corticosterone from alligator tail scute tissues to evaluate scute tissue corticosterone concentrations as a measure of long-term stress. My objectives were to determine: (1) whether corticosterone can be reliably extracted from alligator tail scute tissue, (2) whether corticosterone concentrations increase in scutes over a 2 hr period in response to a short-term stressor (i.e., capture and restraint); and (3) whether increases in plasma and scute corticosterone concentrations within individuals are correlated.


CHAPTER 2

EVALUATION OF SHORT-TERM STRESS AND IMMUNE PARAMETERS IN THE AMERICAN ALLIGATOR (ALLIGATOR MISSISSIPPIENSIS)

INTRODUCTION

Over the course of their lifetime, vertebrates confront a suite of short- and long-term stressors that can contribute to a cascade of behavioral and physiological changes (Sapolsky et al., 2000). Glucocorticoids, regulated by the hypothalamic-pituitary-adrenal (HPA) axis, help to mitigate the effects of stressors by increasing gluconeogenesis, suppressing reproductive behavior, and by aiding in the regulation of the immune system to increase short-term survival of an individual (Moore and Jessop, 2003; Wingfield et al., 1998). However, recurring or chronically elevated glucocorticoid concentrations associated with long-term stressors may negatively influence the physiology, behavior, and overall fitness of an animal (Breuner et al., 2013; Moore and Jessop, 2003; Romero et al., 2009; Sopinka et al., 2015). The aforementioned effects on individual health have generated an increase in research focused on characterizing the relationship between an organism and its environment by investigating the use of stress and immune metrics (Johnstone et al., 2012).

However, capture and handling stress associated with these investigations can influence a suite of stress and immune metrics within minutes of capture (Johnstone et al., 2012; Romero and Reed, 2005), complicating efforts to evaluate the effects of
environmental and physiological stressors. Glucocorticoid concentrations are one of the most commonly used stress metrics. However, hormone levels can increase rapidly following capture and handling, requiring a baseline sample to be collected within three minutes of capture for some species (Romero and Reed, 2005). Additionally, stress and elevated glucocorticoid concentrations can increase the number of circulating heterophils and cause a decline of circulating lymphocytes, thus potentially making leukocyte profiles (including leukocyte differentials and heterophil:lymphocyte ratios) an attractive alternative to sampling glucocorticoid concentrations, which can change rapidly following capture (Davis et al., 2008; Dhabhar, 2002). Moreover, the capture and restraint associated with sampling wildlife can also cause an animal to exert intense muscular activity, and contribute to acid-base disturbance and a release of lactic acid (Coulson and Hernandez, 1983; Franklin et al., 2003; Olsson and Phalen, 2013).

Certain taxa—due to their size, strength, speed or the habitat in which they occur—can be particularly challenging to capture and restrain quickly enough to obtain baseline samples. American alligators (Alligator mississippiensis), for example, are commonly captured using baited snare traps and may be confined to a trap for several hours after capture (Webb et al., 1987). Even active noosing of alligators by researchers from land or boat can involve a lengthy pursuit and capture process, consequently making it difficult or impossible to collect baseline samples. Plasma concentrations of corticosterone, an alligator’s main glucocorticoid, have shown to increase substantially and rapidly when individuals are exposed to capture and restraint stress (Guillette et al., 1997; Lance et al., 2004). Several studies have also characterized the effects of short-term stress on alligator plasma catecholamine levels, glucose concentrations, leukocyte
counts, and sex hormones (Guillette et al., 1997; Gunderson et al., 2003; Lance and Elsey, 1999b; Lance et al., 2004). However, there is a need to characterize the effects of stress associated with capture and restraint in other commonly employed stress and immune metrics to better interpret results when evaluating stress in alligators.

In this study, we examined plasma corticosterone levels, lactate concentrations, packed cell volume, total solids, bacteria killing ability (bacteria killing assays), and leukocyte differentials (including heterophil:lymphocyte ratios) in juvenile American alligators using blood and plasma samples collected before and after capture and restraint. We chose these metrics due to their common use and recommended use in wildlife studies evaluating stress and individual health (Breuner et al., 2013; Demas et al., 2011; Johnstone et al., 2012), as well as their use in studies evaluating potentially long-term stressors specifically in alligators (Guillette et al., 1997; Lance et al., 2010; Murray et al., 2015). Using capture and restraint protocols already established for alligators (Guillette et al., 1997; Lance et al., 2004), we wanted to characterize the change in a suite of stress and immune parameters after alligators were exposed to the short-term stressor, and determine their potential utility when measuring stress.

METHODS

Alligator Husbandry

We sampled 40 juvenile alligators in March 2014 at the Rockefeller Wildlife Refuge (RWR) in Grand Chenier, LA. Alligators were housed in four outdoor environmentally controlled chambers (Joanen and McNease, 1976). Each 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 across both sampling days, with a mean air and water temperature (±1 SD) of 16.9 ± 0.48°C and 26.6 ± 0.26°C, respectively. Additionally, the mean atmospheric temperature (±1 SD) was 18 ± 2.12°C, and ranged from 16.5°C to 19.5°C each sampling day. Each chamber had approximately 20 juvenile alligators. The alligators in this experiment ranged in size from 62.2 – 111.8 cm, with a mean (±1 SD) of 89.6 ± 8.7 cm. Prior to the experiment, alligators were fed 2 – 4 times per week, but food was withheld for 48 hrs 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).

**Pre- and Post-stressor Sample Collection**

We sampled 20 alligators each day, with 48 hours between the two sampling days. To reduce the effects of circadian fluctuations and researcher activities on corticosterone concentrations (Lance and Lauren, 1984), we left alligators undisturbed for approximately 12 hours before collecting baseline (pre-stressor) samples at 1000 hr each sampling day (Finger et al., 2015). We also 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 individual alligator before collecting a 4 mL blood sample from the occipital sinus using an 18 gauge, 3.81-cm heparinized needle (Hamilton et al., 2016) within three minutes of capture (Romero and Reed, 2005). Blood samples collected immediately after capture represent pre-stressor samples (i.e., baseline samples). We then immediately placed alligators in a burlap sack
and positioned each individual on the ground (Guillette et al., 1997; Lance et al., 2004). Two hours after capture, we removed alligators from their burlap sack and a second blood sample was collected using the same protocol, including blood collection site, as described above (representing the post-stressor samples). Following sample collection, we measured alligator total length (TL) to the nearest 0.1 cm. Finally, we used blunt-nosed tweezers to determine the sex of all alligators in this study using methods previously described in Allsteadt and Lang (1995).

After each blood collection, samples were immediately placed in a cooler wrapped in a towel on ice packs for approximately 20 minutes before being centrifuged for three minutes at 1318 g. We then aliquoted duplicate 1 mL plasma samples into a pair of 1.5 mL tubes before flash freezing each sample in liquid nitrogen. We transported plasma samples on dry ice until placing samples in a −60°C freezer for later analysis.

**Blood Differentials, Packed Cell Volume, & Total Solids**

Prior to centrifugation, we used whole blood samples to make two blood smears per individual at each sampling period. After air-drying each smear, we fixed slides with 100% methanol while in the field. Blood smears were then submitted to the University of Georgia’s Tifton Veterinary Diagnostic Laboratory for white blood cell quantification. Briefly, slides were stained with Wright-Giemsa and examined using oil immersion at 1000X magnification until 100 cells were counted. Cells were identified as heterophils, lymphocytes, monocytes, eosinophils, and basophils. The percent of each cell type was then determined. We calculated the ratio of heterophils to lymphocytes (H:L) for each alligator based on the percentages of these cells (Davis and Maerz, 2010). Additionally, we determined the packed cell volume (PCV) of each alligator by centrifuging a blood
sample in a heparinized micro-hematocrit tube (#15401-628; VWR Scientific Inc. Drummond Scientific Co., Broomall, PA, USA) at 10,000 rpm using a LW Scientific ZIPocrit microcentrifuge (Lawrenceville, GA, USA) for 180 seconds. PCV (in percent) was determined using the provided ZIPocrit reading chart. Finally, we used a Reichert VET 360 refractometer to measure total solids (TS).

*Plasma Corticosterone*

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

We determined extraction efficiencies by adding exogenous corticosterone to a serious of pooled alligator plasma samples prior to extraction. Briefly, alligator plasma samples from four individuals not being analyzed as part of this experiment were pooled and aliquoted into 10-1.5 mL tubes. Five tubes were spiked with 20 μL of a 60 ng/mL corticosterone spike to obtain a known concentration of ~10 ng/mL. The remaining five tubes were left unspiked. We extracted corticosterone from each spiked and unspiked tube as previously outline above. The extraction efficiencies were calculated as a percent using the following formula: (Amount Observed/Amount Expected) * 100, where the amount observed is the value obtained from the spiked sample, and the amount expected
is the calculated amount of standard hormone added plus the amount of corticosterone in the un-spiked pooled sample. Finally, we calculated the mean (± 1 SE) extraction efficiency and used this mean extraction efficiency to correct all plasma corticosterone concentrations from our experiment before statistical analyses.

We quantified plasma corticosterone concentrations using an enzyme immunoassay (EIA; Cat: No. ADI-900-097, Enzo Life Science, Inc., Farmingdale, NY) following the procedures and guidelines outlined in the manufactures instructions. Following validation techniques established in Chapter 3 we ran plasma samples using a 1:20 dilution to remove any measureable effects of plasma extract components on corticosterone concentrations. Each 96-well plate included six standards ranging from 7.82 to 10,000 pg/mL in triplicate. Assay detection limits ranged from 15.22 to 53.69 pg/mL. When corticosterone concentrations fell below the detection limit of the assay (33 out of 80 plasma samples), we used the plate’s detection limit as the corticosterone concentration for that individual (Hopkins and DuRant, 2011). Briefly, assay detection limits were determined by taking two standard deviations away from the mean of the total-binding wells (BO; Wada et al., 2007). Finally, to estimate inter-assay variation, we included an additional 500 pg/mL corticosterone standard that we ran in triplicate on each plate. We assessed intra-assay variation by calculating the average coefficient of variation (CV) across all samples, and inter-assay variation across plates by calculating the CV of the 500 pg/mL standards.

**Plasma Lactate Concentrations**

To quantify lactate concentrations, we used an i-STAT portable analyzer and CG4+ cartridge according to protocols established by Hamilton *et al.* (2016), which
previously validated the use of frozen plasma as an appropriate sample type for quantifying lactate with the CG4+ cartridge. We thawed plasma samples for approximately 15 minutes prior to transferring a 95 μL sample to a CG4+ cartridge for analysis. Alligator body temperatures were not collected during this study, thus samples were run on the CG4+ cartridge at a constant 37°C using internal thermal controls incorporated by the analyzer and were not corrected to incorporate fluctuations in animal body temperature when calculating lactate concentrations.

**Bacteria Killing Assay**

We performed bacteria killing assays (BKAs) to assess bacteria killing ability according to Finger et al. (2015) using frozen plasma and *Esherichia coli* (ATCC 8739; Microbiologics, USA). Briefly, we aliquoted 7 μL of thawed plasma into a 1.5 mL tube with 133 μL of CO2-independent media before mixing with 10 μL of *E. coli* and plating onto tryptic-soy agar plates. We plated this mixture on a control plate at time zero (i.e., immediately following the addition of *E. coli*), and again on a second plate after a 60-minute incubation period at room temperature. We then inverted and incubated each plate at 30°C for 24 hours. Finally, we counted colonies and used the following calculation for quantifying the percent bacteria killed: (1 - [Colonies at 60 min./Colonies at 0 min.]) X 100 (Finger *et al.*, 2015).

**Statistical Analysis**

All statistical analyses were performed using R Version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria). We assessed the validity of the assumptions of parametric tests using Shapiro-Wilk test for normality for each parameter. To compare pre- and post-stressor samples collected from the same individual, we proceeded with
either a paired t-test, or if violations were observed, an exact Wilcoxon signed-rank test (the non-parametric equivalent of a paired t-test; package: exactRankTests) without attempting to transform data to meet the assumptions of parametric testing. We first compared pre- and post-stressor corticosterone concentrations between males and females using an exact Wilcoxon rank sum test. Because no differences between sexes were detected, we then proceeded to compare pre- and post-stressor corticosterone, blood differential, H:L ratio, bacteria killing assay, and TS data, using an exact Wilcoxon signed-rank test for all individuals. We compared pre- and post-stressor lactate and PCV values using paired t-tests. Finally we applied a Bonferroni correction to account for multiple comparisons. A significance level of 0.004 was used for all statistical tests. Test statistics were reported as $W$ for the Wilcoxon rank-sum test, $V$ for the Wilcoxon signed-rank test, and $t_{df}$ for the paired t-test. Non-parametric test results are reported as medians and interquartile range, whereas parametric results are reported as mean ($\pm$ 1 SE).

RESULTS

The mean ($\pm$ 1 SE) extraction efficiency of corticosterone from plasma samples was 75.1 $\pm$ 7.3%. Additionally we observed an intra-assay CV of 5.2% and inter-assay CV of 3.4%. Neither pre- ($W = 142, p = 0.888$) nor post-stressor ($W = 108, p = 0.321$) plasma corticosterone values were significantly different between males ($n = 31$) and females ($n = 9$). Pre-stressor plasma corticosterone concentrations ranged from 0.38 ng/mL to 2.53 ng/mL with a median concentration of 0.85 ng/mL (IQR = 0.38 – 1.34 ng/mL). Post-stressor plasma corticosterone sample concentrations ranged from 2.65 ng/mL to 23.65 ng/mL with a median concentration of 9.85 ng/mL (IQR = 6.52 – 12.42
ng/mL). Post-stressor plasma corticosterone concentrations were significantly higher than pre-stressor corticosterone concentrations ($V = 0, p < 0.001$; Fig. 2.1).

Lactate concentrations were also significantly higher in post-stressor samples, with a mean of 8.01 mmol/L ($\pm 0.49$) compared to pre-stressor samples with a mean of 6.06 mmol/L ($\pm 0.45$; $t_{39} = -3.8431, p < 0.001$; Fig. 2.2). There was no significant difference in bactericidal killing ability between pre- and post-stressor samples, with a median of 95.7% (IQR = 90.3 – 98.4%) for pre-stressor samples and a median of 96.9% (IQR = 91.5 – 98.4%) for post-stressor samples ($V = 378, p = 0.673$; Table 1). Additionally, pre-stressor packed cell volume samples had a mean value of 39% ($\pm 1.94$) and were not significantly different than post-stressor values, which had a mean value of 35% ($\pm 2.51$; $t_{39} = 1.09, p = 0.282$; Table 1). Pre-stressor total solids were not significantly different than post-stressor samples with a median of 4.4 (IQR = 4.18 – 4.60) and 4.10 (IQR = 4.00 – 4.60) respectively ($V = 379.5, p = 0.472$; Table 1). There was a significant difference between pre- and post-stressor heterophils ($V = 172.5, p = 0.002$) and lymphocytes ($V = 761.5, p < 0.001$), but not monocytes ($V = 303.5, p = 0.336$), eosinophils ($V = 242.5, p = 0.239$), or basophils ($V = 326, p = 0.525$; Table 2.1). Additionally, we saw a significant difference between pre- and post-stressor H:L ratio values ($V = 82.5, p < 0.001$; Table 2.1), with a lower H:L ratio in pre-stressor samples (median = 2.85, IQR = 1.58 – 3.70) when compared to post-stressor samples (median = 5.00, IQR = 3.60 – 7.1).
DISCUSSION

Our results demonstrate that a short-term stressor, such as that associated with capture and handling stress, has the potential to influence several physiological measurements in alligators. Plasma corticosterone concentrations increased significantly in response to our experimental capture and handling stress protocol. Juvenile alligator studies incorporating the same methodology as used in our study, observed similar plasma corticosterone concentrations when investigating the effects of short-term stress on plasma sex hormones (Guillette et al., 1997; Gunderson et al., 2003; Lance et al., 2004). Baseline plasma glucocorticoid concentrations, such as corticosterone, are one of the most commonly used stress metrics. However, it is recommended that baseline samples be collected within three minutes of capture (Johnstone et al., 2012; Romero and Reed, 2005). Baseline corticosterone samples may be difficult to collect and accurately interpreted from wild alligators, depending on the capture technique and size of the animal. Quantifying corticosterone concentrations in tissue or fecal samples may provide a longer-term measure of stress and may also be less affected by short-term changes in stress hormones (Bortolotti et al., 2008; Johnstone et al., 2012; Sheriff et al., 2011).

Leukocyte profiles can be a useful tool when exploring the effects of stress, as profiles have shown to be directly related to stress hormone levels in some taxa (Davis et al., 2008; Johnstone et al., 2012; Morici et al., 1997). Not surprisingly, we observed a significant increase in juvenile alligator H:L ratios following exposure to capture and restraint stress. Changes in glucocorticoid concentrations can cause an increase in heterophil numbers in addition to decreases in lymphocyte counts (Davis et al., 2008; Johnstone et al., 2012). As such, H:L ratios have been commonly used to measure the
magnitude of a stressor. Increased H:L ratios have been previously reported for alligators affected by extreme weather such as the effects of hurricanes (Lance et al., 2010). Additionally white blood cell differential values have been reported to fluctuate in alligators exposed to cold-water temperatures (Lance and Elsey, 1999), 48-hours of restraint (Lance and Elsey, 1999b), and in *Aeromonas hydrophila* infected individuals (Glassman et al., 1981). While the magnitude of change we observed in H:L ratios over a 2 hr period in this study (median increase from 2.85 to 5.00 between pre- and post-stressor samples) was not as drastic as the changes in corticosterone concentrations over the same time period (median increase from 0.86 to 9.85 ng/mL between pre- and post-stressor samples), caution should be employed when interpreting H:L ratios from animals that may have experienced prolonged handling and restraint stress.

Elevated lactate concentrations can indicate tissue hypoxia, hypoperfusion, and damage that is often associated with strenuous activity or capture and handling stress (Coulson and Hernandez, 1979; Franklin et al., 2003; Lewbart et al., 2015). In alligators, concentrations of lactate have been documented to exceed 30 mmol/L following short bursts of activity (Coulson and Hernandez, 1983). Although we observed relatively modest levels of lactate in juvenile alligators after capture-induced stress (2.42 – 13.43 mmol/L) with a mean increase of only 1.95 mmol/L in juvenile alligator lactate concentrations between pre- and post-stressor samples, mature alligators may exhibit a more drastic increase in lactate concentrations following capture and restraint. It is important to note that the degree of plasma lactate rise following capture and restraint has been shown to be influenced by both the size of an individual and time to exhaustion (Bennett et al., 1985; Franklin et al., 2003). Therefore, study design and sampling
technique is of utmost importance when assessing lactate concentrations, particularly when collecting baseline values from larger individuals. We attempted to minimize these sources of variation by sampling only juvenile alligators that could be easily captured and subdued within a short time period in this study.

Short-term responses to stress can have immune enhancing effects by increasing the number and proportions of circulating leukocytes (Davis et al., 2008; Dhabhar, 2009). However, long-term stress is thought to be immunosuppressive by reducing natural killer cell activity, lymphocyte populations, and antibody production (French et al., 2006; Pruett et al., 1993; Webster Marketon and Glaser, 2008). BKAs quantify the in vitro antimicrobial capacity of blood or plasma as a means to characterize immunological variation among and within species (Demas et al., 2011; Matson et al., 2006; Millet et al., 2007). In this study, we did not observe a significant difference in bacteria killing ability between alligator plasma samples collected pre- or post-stressor. Thus, a short-term stressor such as capture and restraint might not influence constitutive immune mediators in alligator plasma. However, saltwater crocodiles have demonstrated variation in bactericidal capabilities with differences between 4 and 9 months of age (E. coli, 32 – 58%), perhaps highlighting potential fluctuations in bactericidal killing efficiency due to environmental fluctuations and early development in crocodilians (Finger et al., 2015). Additionally, we used E. coli due to its standard use in BKAs. However, using alternative bacteria, such as Providencia rettgeri, may provide a more biologically-relevant measure of immune function because crocodilians have been reported to succumb to septicemia associated with P. rettgeri (Finger et al., 2015; Huchzermeyer, 2003).
Finally, we did not observe an increase in either total plasma solids or packed-cell values in response to a short-term stressor. Packed cell volume and total plasma solids can be useful when assessing anemia, hydration, and the general health status of reptiles (Mader, 2006; Thrall et al., 2012). Total solid values in this study fell within the reference intervals suggested for reptiles, however several individuals had PCV values (Range: 12-74%) outside the reference values reported for American alligators (Mader, 2006; 12-40%), 42.5% of individuals in this study had higher % PCV than the established reference values in either pre- or post-stressor samples. PCV values less than 20% might suggest anemia, whereas a PCV greater than 40% can suggest either hemoconcentration or erythrocytosis (Thrall et al., 2012). It is important to note that we used pre-heparinized syringes during this study. Although efforts were made to standardize the heparinization of syringes and sample collection volume, variation in dilution by this anti-coagulant among samples could have influenced these results (Johnson et al., 2014), in addition to lymph dilution and/or an underlying health condition.

Our study characterized the effects of a short-term stressor on American alligators by evaluating the change in six commonly used stress and immune metrics of interest. One of the most significant interpretational challenges that physiological studies must confront is the ability to account for the influences of capture and handling techniques when assessing the effects of environmental stressors in wildlife (Johnstone et al., 2012). As mentioned previously, and as observed in this study, glucocorticoids can rise rapidly (within 3-5 minutes) and subsequently cause an increase in leukocyte metrics (e.g., H:L ratios; Davis et al., 2008; Johnstone et al., 2012). Additionally, long-term shifts in glucocorticoid concentrations can influence physiological processes such as digestion,
reproduction and immune function (Romero and Butler, 2007). Thus, it is important to understand and characterize the influences of short-term stressors, like capture and restraint stress, on stress and immune indices of interest to appropriately apply the use of these metrics when assessing stressors. In this study, we collected baseline and post-stressor samples, however to better characterize the temporal response of each parameter to capture stress, studies focused on collecting samples over the course of multiple time points are needed. Likewise, using multiple metrics rather than relying on a single metric, may provide a more comprehensive assessment of the effects of stressors on individual organisms. The evaluation of other stress and immune metrics not included in this study are needed to better understand the effects of capture and handling stress, and to evaluate the effectiveness of each parameter when assessing the health of an organism.
LITERATURE CITED


Table 2.1. Summary of leukocyte differentials, heterophil:lymphocyte ratio (H:L), packed cell volume (PCV), total solids (TS), and bacteria killing ability (BKA) of American alligators (*Alligator mississippiensis*; *n* = 40) exposed to short-term handling stress and restraint for 2 hours. Reported values are medians (range) of each parameter, except for total solids, which is reported as mean (range).

Significant comparisons between pre- and post-stressor samples collected from the same individuals are indicated in bold (all *p* ≤ 0.002).

<table>
<thead>
<tr>
<th>Alligator Samples</th>
<th>Heterophils (%)</th>
<th>Lymphocytes (%)</th>
<th>Eosinophils (%)</th>
<th>Basophils (%)</th>
<th>Monocytes (%)</th>
<th>H:L</th>
<th>PCV (%)</th>
<th>TS (g/dL)</th>
<th>BKA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Stressor</td>
<td>55.5 (19-72)</td>
<td>19.5 (4-42)</td>
<td>7 (1-23)</td>
<td>6.5 (0-19)</td>
<td>9 (1-19)</td>
<td>2.9</td>
<td>39 (16-64)</td>
<td>4.3 (2.6-5.4)</td>
<td>95.7 (5.2-99.1)</td>
</tr>
<tr>
<td>Post-Stressor</td>
<td>61 (35-89)</td>
<td>12 (1-29)</td>
<td>8.5 (0-20)</td>
<td>8.5 (0-27)</td>
<td>10 (2-26)</td>
<td>5.0</td>
<td>35 (12-74)</td>
<td>4.2 (3.2-5.3)</td>
<td>96.9 (23.7-99.7)</td>
</tr>
</tbody>
</table>
Figure. 2.1. Boxplots of juvenile American alligator (*Alligator mississippiensis; n = 40*) corticosterone concentrations in plasma samples collected from the same individuals before (pre-stressor) and after (post-stressor) exposure to a short-term capture and restraint stress. Post-stressor corticosterone concentrations were significantly higher than pre-stressor concentrations using an exact Wilcoxon signed-rank test ($V = 0, p < 0.001$).
**Figure 2.2.** Mean plasma pre-stressor and post-stressor lactate concentrations with 95% confidence intervals in juvenile American alligators (*Alligator mississippiensis*; *n* = 40) exposed to short-term capture and restraint stress. Post-stressor plasma lactate concentrations were significantly higher than pre-stressor lactate concentrations using a paired t-test (*t*<sub>39</sub> = -3.8431, *p* < 0.001).
Figure 2.3. Boxplots of juvenile American alligator (*Alligator mississippiensis*; *n* = 40) heterophil:lymphocyte (H:L) ratios before (pre-stressor) and after (post-stressor) exposure to a short-term capture and restraint stress. Using an exact Wilcoxon signed-rank test, post-stressor H:L ratios were significantly higher than pre-stressor samples (*V* = 82.5, *p* < 0.001). To reduce scale of the y-axis, we log transformed H:L ratios.
CHAPTER 3
EVALUATING THE POTENTIAL USE OF CROCODILIAN SCUTE TISSUE WHEN INVESTIGATING LONG-TERM ENVIRONMENTAL STRESSORS

INTRODUCTION
The response of a species to an environmental stressor has most commonly been evaluated through the use of individual physiological assessments. One of the most important physiological 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 main glucocorticoid, corticosterone, follows a prominent biphasic circadian rhythm where corticosterone concentrations peak at 0800 hr and then again at 2000 hr (Lance and Lauren, 1984). Corticosterone can also 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 corticosterone 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 corticosterone 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).
In crocodilians, plasma corticosterone concentrations have been used to characterize the effects of several potentially long-term stressors, including chronic exposure to contaminants (Guillette et al., 1997), extreme weather events (Lance et al., 2010), disease (Nevarez et al., 2011), and ranching operations (Elsey et al., 1990; Finger et al., 2015; Isberg and Shilton, 2013). However, glucocorticoid concentrations in blood or plasma can change rapidly in response to a short-term stressor such as capture and handling stress (3-5 min.), complicating the interpretation of an animal’s response to more chronic stressors (Johnstone et al., 2012; Romero and Reed, 2005). Additionally, recent work published by Goessling et al., (2015) suggests that baseline plasma corticosterone samples may not adequately evaluate the effects of long-term stressors, thus requiring alternative or supplemental long-term physiological measurements.

In a wide variety of taxa, types of biological samples other than plasma, including saliva, urine, and feces, have been used to quantify glucocorticoids. Samples such as feces 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, numerous 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), snake skin sheds (Berkvens et al., 2013), blubber (Kellar et al., 2015; Trana et al., 2015), and baleen (Hunt et al., 2014).

Although we are unaware of any studies attempting to quantify corticosterone concentrations in crocodilians using sample types other than plasma and feces, tissue samples are regularly collected for marking and individual identification purposes. A common method for marking crocodilians for research and ranching operations includes the removal of dorsal tail scutes (Chabreck, 1963; Jennings et al., 1991). Crocodilian caudal scutes have also been used for quantifying contaminant burdens (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 caudal scute tissue samples to quantify corticosterone concentrations by sampling American alligator (*Alligator mississippiensis*) tail scutes before and after a short-term stressor (i.e., handling stress). The American alligator is the most studied crocodilian species (Ryberg et al., 2002), making it an ideal model organism for crocodilian research. As such, we used American alligators as our study organism to determine: (1) whether corticosterone can be reliably extracted from alligator tail scute tissue, (2) whether corticosterone concentrations increase in scutes over a 2 hour period in response to a short-term stressor (i.e., capture and restraint); and (3) whether increases in plasma and scute corticosterone concentrations within individuals are correlated. 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 individual wild adult alligators should be possible well within 2 hours. Based on previous literature, we expected plasma corticosterone levels to be significantly higher following the short-term stressor. However, because corticosterone is presumably deposited over longer time periods in scutes relative to plasma, we did not expect scute tissue samples to be significantly different between pre- and post- stressor samples.

METHODS

Alligator Husbandry

In March 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. Each outdoor concrete chamber had an overall water holding capacity of 1,136 L and approximately 14.9 m² 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 (± 1 SD) of 16.9 ± 0.48°C and 26.6 ± 0.26°C, respectively, across sampling days. Additionally, atmospheric temperature ranged from 16.5°C to 19.5°C each sampling day, with a mean temperature (± 1 SD) of 18 ± 2.12°C. Each chamber contained approximately 20 juvenile alligators. Alligators used in this study ranged in in size from 62.2 – 111.8 cm, with a mean (± 1 SD) 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).

**Sample Collection**

Sampling protocols have been previously described in Chapter 2. Briefly, we sampled alligators over a course of two days ($n = 20$ alligators per day), with 48 hours between sampling days. To minimize effects of circadian fluctuations and researcher activities on circulating corticosterone concentrations (Lance and Lauren, 1984), we left alligators undisturbed for approximately 12 hours before collecting baseline (pre-stressor) samples at 1000 hr (Finger *et al.*, 2015). We also limited our sampling efforts to two nonadjacent 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 alligators one at a time by manually restraining the individual, and collecting a 4 mL blood sample from the occipital sinus using a heparinized, 18 gauge, 3.81-cm needle (Hamilton *et al.*, 2016) within three minutes of capture (Romero and Reed, 2005). We then used an established crocodilian identification marking technique to collect two dorsal tail scutes (Chabreck, 1963; Jennings *et al.*, 1991). To standardize scute tissue collection, we batch marked all individuals using the same identification code by collecting the first two caudal scutes located posterior to the bifurcated row of scutes. The blood and scute samples collected immediately after capture represent pre-stressor samples (i.e., baseline samples). After sample collection, we placed alligators in a 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. Finally, we measured alligator total length (TL) to the nearest 0.1 cm, and used blunt-nosed tweezers to determine the sex of each alligator (Allsteadt and Lang, 1995).

After collecting each tissue and blood sample from each individual, we immediately stored samples in a cooler wrapped in a towel on ice packs for approximately 20 minutes. We then centrifuged blood samples for three minutes at 1318 g before aliquoting duplicate 1 mL plasma samples into a pair of 1.5 mL tubes. Finally, we flash froze all tissue and plasma samples in liquid nitrogen before transporting samples on dry ice for storage in a -60°C freezer for later analysis.

**Plasma Corticosterone**

We quantified plasma corticosterone concentrations using an enzyme immunoassay (EIA; Cat: No. ADI-900-097, Enzo Life Science, Inc., Farmingdale, NY) according to the procedures and guidelines outlined in the manufactures instructions. First, we extracted corticosterone for each individual and sampling period (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 mixture for 8-12 seconds. We decanted the top organic layer (i.e., extracted corticosterone) 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 corticosterone 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 corticosterone spike to obtain a known concentration of ~10 ng/mL. We left the remaining five tubes unspiked. We extracted corticosterone 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 corticosterone in the unspiked pooled sample. We then calculated the mean (± 1 SE) extraction efficiency and used this mean extraction efficiency to correct all plasma corticosterone 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 corticosterone concentrations fell below the detection limit of the assay (33 out of 80 plasma samples), we used the assay’s detection limit as the corticosterone concentration for that individual (Hopkins and DuRant, 2011). We tracked inter-assay variation by including an additional 500 pg/mL corticosterone standard on each plate in triplicate. We tracked intra-assay variation by running each alligator sample in triplicate. We assessed intra-assay variation by calculating the average coefficient of variation (CV) across all samples, and inter-assay variation across plates by calculating the CV of the 500 pg/mL standards.
**Plasma Corticosterone Validation**

Validation of an EIA for every species and sample type analyzed on an assay is necessary for accurate and reliable results. As such, we employed protocols outlined in Hopkins and DuRant (2011), to validate the assay for use with American alligator plasma samples. To detect immunological similarities between the corticosterone standard provided with the assay and a serially diluted pooled sample from the target species, we conducted a parallelism test by pooling extracts from four alligators and conducted 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 corticosterone concentration.

Plasma constituents can interfere with binding antibodies, preventing accurate quantification of corticosterone concentrations. The degree of interference can vary among species and between assays depending on the reagents and antibodies provided in the assay. Using techniques established by Wada et al., (2007), we determined that a sample dilution of 1:20 was optimal for removing any measurable effects of plasma extract components on corticosterone concentrations. Briefly, we stripped a pooled sample of its corticosterone with 1% Norit A Charcoal and 0.1% dextran in ultra-pure water. We then spiked the stripped pooled sample with corticosterone standard to a concentration of ~500 pg/mL before conducting a 2-fold serial dilution from 1:10 to 1:80. Finally, we selected our dilution factor by comparing the corticosterone values quantified from the different dilutions to the known concentration of the corticosterone spike.
**Scute Tissue Corticosterone**

We extracted corticosterone 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 tissue 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 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 by mixing for 24 hours on an orbital shaker at 200 rpm. Following corticosterone extraction, we centrifuged the vials for 10 minutes at 1800g 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 compare scute and plasma samples from the same individuals, we determined scute sample corticosterone concentrations using the same EIA. First, following storage in a -20°C freezer, 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.

**Scute Tissue Validation**

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 test for parallelism to detect immunological similarities between the standard curve and a serial dilution of alligator scute extracts. We pooled scute extracts from five alligators to create a 15-fold concentrated sample. We then conducted a serial dilution of our pooled, concentrated scute extract 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 corticosterone concentration.

Additionally, we conducted an accuracy test to examine the potential interference of antibody binding due to components located in the tissue extract by analyzing the recovery of exogenous corticosterone added to pooled alligator scute extracts. Briefly, we created 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 assayed the concentrated pooled sample in triplicate to determine the percent recovery of exogenous corticosterone using the following calculation: \( \frac{\text{amount observed}}{\text{amount expected}} \times 100 \), as described above for plasma. Finally, we conducted a linear regression to determine if there was a significant relationship between the amount of corticosterone added and recovered for our accuracy test.

**Statistical Analysis**

All statistical analyses were performed using R Version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria). We assessed the validity of the assumptions of parametric tests using a Shapiro-Wilk test for normality, and proceeded with non-parametric analyses due to observed violations in all parameters without attempting data transformation to meet the assumptions of parametric testing. First, we compared pre- and post-stressor concentrations between males and females using an exact Wilcoxon rank sum test (package: exactRankTests). Because no differences between males and females were observed, we pooled all individuals to compare pre- and post-stressor corticosterone concentrations and overall change in corticosterone concentrations of alligator plasma and scute samples using an exact Wilcoxon signed-rank test. In addition, we used a Spearman’s rank correlation analysis to calculate the correlation between the change in plasma and the change in scute corticosterone concentrations.

To calculate the overall change in corticosterone concentration for each sample type (i.e., plasma and scute), we subtracted the pre-stressor concentration value from the post-stressor concentration. Due to the non-overlapping ranges in values between scutes and plasma, we also calculated the percent change in plasma and scute concentrations.
using the following calculation: \(((\text{POST} – \text{PRE})/\text{PRE})\times100 = \text{Percent Change}\). The PRE value is the corticosterone concentration obtained from samples prior to an individual being exposed to the short-term stressor (i.e., placed in a burlap sack), whereas the POST value is the corticosterone concentration obtained from samples collected following exposure to the short-term stressor. Concentrations are reported as median (IQR) and the total range of values recorded. Test statistics are reported as \(W\) for the Wilcoxon rank-sum test and \(V\) for the Wilcoxon signed-rank test. A significance level of 0.05 was used for all statistical tests.

RESULTS

**Plasma Corticosterone**

The mean (± 1 SE) extraction efficiency of corticosterone from alligator plasma samples was 75.1 ± 7.3%. Additionally we observed an intra-assay CV of 5.2% and inter-assay CV of 3.4%. 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 the provided corticosterone standard. Neither pre- \((W = 142, p = 0.888)\) nor post-stressor \((W = 108, p = 0.321)\) plasma corticosterone values were significantly different between males \((n = 31)\) and females \((n = 9)\). Pre-stressor plasma sample corticosterone concentrations for this study ranged from 0.38 ng/mL to 2.53 ng/mL with a median concentration of 0.85 ng/mL (IQR = 0.38 – 1.34 ng/mL; Fig. 3.1). Post-stressor plasma corticosterone sample concentrations ranged from 2.65 ng/mL to 23.65 ng/mL with a median corticosterone concentration of 9.85 ng/mL (IQR = 6.52 – 12.42 ng/mL; Fig. 3.1). Post-stressor plasma corticosterone concentrations
were significantly higher than pre-stressor corticosterone concentrations ($V = 0, p < 0.001$), indicating that our experimental protocol did induce a stress response in juvenile alligators.

**Scute Corticosterone**

We recorded a mean ($\pm$ 1 SE) scute length of 11.56 ± 0.26 mm, an average width of 2.70 ± 0.13 mm, and mean height of 1.68 ± 0.07 mm ($n = 80$). The mean ($\pm$ SE) extraction efficiency of alligator scute corticosterone samples was 108 ± 4.3%. We observed an intra-assay CV of 6.7% and inter-assay CV of 3.3%. Pooled alligator scute corticosterone serially diluted alongside the standard curve showed parallel displacement ($r^2 = 0.987, p < 0.001$; Fig. 3.2), again indicating immunological similarities between our extracted scute corticosterone and the assay’s corticosterone standards. EIA accuracy tests yielded a mean recovery of known corticosterone concentrations at 106 ± 2.1%. In addition, we detected a significant relationship between the measured hormone concentrations in the spiked samples with the expected concentrations for our accuracy test ($r^2 = 0.998, p < 0.001$; Fig. 3.3). Neither pre- ($W = 156, p = 0.610$) nor post-stressor ($W = 127, p = 0.698$) scute corticosterone values were significantly different between males and females. Pre-stressor scute corticosterone samples ranged from 4.45 ng/g to 32.66 ng/g with a median of 12.8 (IQR = 9.84 – 18.47 ng/g; Fig. 3.1). Post-stressor scute corticosterone samples range from 4.35 ng/g to 66.76 ng/g. Post-stressor scute samples had a median of 15.13 ng/g (IQR = 12.01 – 25.62 ng/g) and were significantly higher than pre-stressor scute samples ($V = 217, p = 0.008$; Fig. 3.1).
**Comparison of Sample Types**

The median change in alligator plasma corticosterone concentrations was 9.34 ng/mL (IQR = 5.45 – 11.85 ng/mL) and ranged from 1.31 to 23.27 ng/mL, whereas the median change in alligator scute corticosterone concentrations was 2.23 ng/g (IQR = -0.82 – 9.35 ng/g) and ranged from -12.81 to 34.1 ng/g. There was a significant positive correlation between the change in plasma corticosterone concentrations and the change in scute corticosterone concentrations when using a Spearman’s rank correlation analysis ($r = 0.486$, $p = 0.002$; Fig. 3.4). However, the percent change in alligator plasma corticosterone concentrations was significantly greater than the percent change in alligator scute corticosterone concentrations ($V = 819$, $p < 0.001$). The median percent change in alligator plasma corticosterone concentrations was 1108.02 % (IQR = 430.92 – 2620.20 %) with a range of 93.69 to 6119.45 %, whereas the median percent change in alligator scute corticosterone concentrations was only 20.12 % (IQR = -8.40 – 71.47 %) with a range of -67.62 to 277.39 %.

**DISCUSSION**

Our study is the first to validate that reliable levels of corticosterone can be extracted from alligator caudal scutes and 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 corticosterone. Our study adds to the growing body of literature highlighting the value of keratinized tissues for quantifying glucocorticoids. Specifically in reptiles, corticosterone has been successfully extracted and quantified
from nails of freshwater turtles (Baxter-Gilbert et al., 2014) and shed skins of snakes (Berkvens et al., 2013). In addition, the relatively large size of scutes and the availability of multiple scutes to sample, provides the opportunity to quantify both corticosterone and other endpoints, including contaminant concentrations (Rainwater et al., 2007) or stable isotopes (Marques et al., 2014), from the same individual.

As expected, we observed a significant increase in plasma corticosterone in response to short-term capture and handling stress, indicating that our experimental methodology did induce a stress response in juvenile alligators. The experimental protocol employed in this study (i.e., exposure to a short-term stressor) yielded baseline and post-stressor plasma corticosterone concentrations similar to studies that used comparable capture and restraint techniques (Guillette et al., 1997; Lance et al., 2004). Additionally, corticosterone concentrations reported here are within the range of values described for alligators held at different stocking densities (Elsey et al., 1990), alligators exposed to extreme weather conditions (Lance et al., 2010), and animals exposure to cold temperatures (Lance and Elsey, 1999). It is important to note, however, that multiple plasma sample corticosterone concentrations fell below the detection limit for this assay, and that quantifying low corticosterone concentrations in baseline samples may be difficult using the extraction protocol and assay employed in this study. Alternate extraction methods may help reduce the influence of plasma components on antibody binding, and thereby eliminate the need for diluting samples.

Contrary to our expectations, we did see a significant increase in scute corticosterone concentrations following the exposure to a short-term stressor. Crocodilian tail scutes have a tough keratinized epidermis with a central vascular bed from which
blood vessels spread throughout the outer dermis layer (Richardson et al., 2002). Thus, increases in blood corticosterone concentrations may contaminate scute samples. Furthermore, our 2-hour sampling time frame may have been too extensive, and allowed for rises in corticosterone circulating in the bloodstream to influence the corticosterone concentrations of alligator scutes. Nevertheless, the change in corticosterone concentrations between the two sample types was markedly different. Plasma exhibited an 11-fold increase in corticosterone concentration, whereas scute corticosterone concentrations increased less than 2-fold.

Glucocorticoid concentrations from several 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 corticosterone concentrations did not significantly differ between 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 corticosterone in their primary feathers when compared to chicks reared on a controlled diet (Will et al., 2014). Although several studies have incorporated the use of alternative tissue types as a biomarker for chronic stress, few have actually experimentally determined that glucocorticoids concentrations increase over time or to what extent they are influenced by short-term stressors such as capture stress. Manipulative studies using adrenocorticotropic hormone (ACTH) challenges (Mastromonaco et al., 2014), corticosterone implants (Morici et al., 1997), or varying levels of environmentally
relevant stressors should be used to investigate the rates of corticosterone deposition in tissues such as scutes, and to elucidate the potential use of such a sample from wild-caught individuals.

Several individuals ($n = 16$) exhibited a decrease in scute corticosterone concentration (from $-12.80$ to $-0.05$ ng/g) following exposure to the short-term stressor (Fig. 3.4). Although we made efforts to control for sample quality and measurement consistency in this study by having the same investigator perform each task and by standardizing sample collection locations and techniques, variations in alligator scute morphology or sample collection may have occurred. Consequently, small variations in sample collection technique (i.e., making a deep tissue cut) may incorporate the more vascularized portion of the scute, therefore producing higher concentrations of corticosterone 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). A similar issue may occur in alligator caudal scutes, thus it is critical for studies to control for tissue depth during sample collection, and enforces the need for further research investigating the deposition of glucocorticoids in tissues. Finally, our study focused on analyzing scute samples from juvenile alligators. Scute samples collected from adult alligators may need to be homogenized due to the size of the tissue sample for a representative sub-sample to be used for quantifying hormone concentrations. Standardizing techniques for collecting and processing caudal scute tissue samples will be important for preventing elevated scute corticosterone concentrations.
caused by corticosterone in the blood, and to tackle the challenges of processing larger tissue samples.

**Summary and Recommendations**

Baseline plasma corticosterone concentrations have been widely used to investigate the effects of stressors on wild and captive crocodilians (Finger *et al.*, 2016, 2015; Guillette *et al.*, 1997; Lance *et al.*, 2004; Nevarez *et al.*, 2011). However, these studies have highlighted the importance of samples being collected within three minutes of capture to correctly reflect baseline hormone concentrations (Johnstone *et al.*, 2012; Romero and Reed, 2005). Crocodilians often exert intense muscular activity during capture and restraining protocols, which may lead to increases in plasma corticosterone concentrations prior to blood collection (Franklin *et al.*, 2003). Collecting baseline plasma corticosterone samples from wild crocodilians may be particularly difficult due to the capture and handling protocols (e.g., snares, steel traps, hook and line) often used for larger individuals. Thus, an alternative sample type for measuring stress that isn’t as prone to influence by short-term increases in hormone levels, such as caudal scutes, may prove valuable for crocodilian research.

Using modified extraction protocols established for quantifying tissue glucocorticoid concentrations, we reliably extracted and validated the use of corticosterone from American alligator scute samples. Until further investigation can determine whether there may be variation in deposition among scutes and the extent to which scutes may be prone to the influence of or contamination by circulating corticosterone in blood, it is critical to both standardize sample collection and processing techniques and minimize time between capture and sample collection. Thus, 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 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 where individuals can be quickly sampled. Future studies investigating the time frame over which capture stress induces an increase in scute corticosterone concentration or documenting the long-term deposition of corticosterone in response to exogenous corticosterone would increase the application and utility of caudal scutes as a biomarker of long-term stress in crocodilians.
**LITERATURE CITED**


Figure 3.1. Boxplots of American alligator (*Alligator mississippiensis*) corticosterone concentrations in plasma and scute samples \((n = 40)\) before (pre-stressor) and after (post-stressor) exposure to short-term capture stress. Post-stressor plasma and scute corticosterone concentrations were significantly higher than pre-stressor concentrations using exact Wilcoxon signed-rank tests \((V = 0, p < 0.001\) and \(V = 217, p = 0.008\) respectively).
Figure 3.2. Parallelism between serially diluted American alligator (*Alligator mississippiensis*) scute corticosterone extract and the standard curve.
Figure 3.3. Exogenous corticosterone recovered from alligator scute extracts was strongly and positively correlated with amount of corticosterone added ($r^2 = 0.998$, $p < 0.001$).
**Figure 3.4.** Change in individual raw plasma and scute corticosterone concentrations in juvenile alligators (n = 40) exposed to short-term capture and restraint stress.
CHAPTER 4
CONCLUSIONS

Crocodilian populations worldwide are continuously being affected by the influence of abiotic and biotic stressors. The decline of several species due to illegal harvest (Ross, 1998) and habitat loss and/or degradation (Thorbjarnarson et al., 2006) highlights the need to evaluate stressors that may impact the survival and health of a species. Researchers have commonly used blood and plasma indices to characterize the effects of stressors on an organism (Johnstone et al., 2012; Maceda-Veiga et al., 2015). However, some stress indices can increase rapidly due to the processes of capture and restraint, thus causing interpretational issues (Maceda-Veiga et al., 2015; Romero and Reed, 2005). Therefore, it is important to evaluate the effects of capture and handling on stress metrics of interest, and to develop new tools and techniques to evaluate stress in wildlife.

The American alligator (Alligator mississippiensis) is one of the most studied crocodilian species in the world (Brisbin et al., 1986; Ryberg et al., 2002). As such, I used the American alligator to explore the effects of stress associated with capture and restraint by collecting pre- and post-stressor blood and tissue samples from 40 juvenile alligators at the Rockefeller Wildlife Refuge in Grand Chenier, LA for Chapter 2 and Chapter 3. In Chapter 2, I evaluated the influence of capture and restraint stress on six commonly used stress and immune metrics, including plasma corticosterone concentrations, leucocyte differentials (including H:L ratios), lactate concentrations,
bacteria killing ability (BKAs), total solids (TS), and packed cell volume (PCV). When comparing pre- and post-stressor samples, this study detected significant increases in corticosterone concentrations, lactate concentrations, increases in percent of heterophils and H:L ratios, and a significant decrease in percent lymphocytes following our stress protocol. However, I did not see a significant difference between BKA, TS, and PCV when comparing pre- and post-stressor values. Characterizing the effects of capture and handling on stress and immune metrics is important to reduce the influence of confounding variables and interpretational errors. Future studies should account for capture and handling techniques when evaluating physiological measurements by incorporating a suite of stress and immune metrics to not only evaluate the effects of environmental stressors, but to also quantify the influence of capture and handling. Additionally, the development and evaluation of other stress and immune metrics that aren’t as influenced by capture and handling techniques in crocodilian species may be necessary. In this study I used primarily male juvenile alligators ($n = 31$ males, $n = 9$ females), however future research should evaluate the influence of gender and size on stress and immune values.

Glucocorticoid concentrations can change rapidly in blood samples (3-5 minutes), causing interpretational issues when evaluating the physiological status of an individual (Johnstone et al., 2012; Romero and Reed, 2005; Chapter 2). Recent research has assessed the use of glucocorticoid concentrations in a range of keratinized and non-keratinized tissues (Baxter-Gilbert et al., 2014; Bortolotti et al., 2008; Kellar et al., 2015; Macbeth et al., 2010), however no such tissue sample has been validated for use with crocodilians. Thus, in Chapter 3, I explored the use of American alligator tail scute
tissues as a viable tissue sample for extracting reliable concentrations of corticosterone and potentially evaluating long-term stress. Corticosterone was reliably extracted from all tissue samples. However, in contrast to our expectations, I observed a significant increase in scute corticosterone concentrations when comparing pre- and post-stressor tissue samples. Additionally, there was a significant positive correlation between the change in scute corticosterone concentrations and change in plasma corticosterone concentrations, although the change in scute corticosterone was much smaller in magnitude than the change in plasma corticosterone. Finally, I did observe a decrease in corticosterone concentration in some scute tissue samples, potentially highlighting the need to further standardize sample collection and processing techniques. Future studies evaluating the rate of corticosterone deposition in scute tissues are needed to determine their suitability as a long-term measure of stress, and to define a time frame for collecting samples. Sample collection and processing techniques may need to be modified for evaluating larger samples and to reduce any potential contamination from circulating corticosterone in the blood. My study highlights the importance of experimentally validating the appropriateness of tissue samples as biomarkers of chronic stress.
LITERATURE CITED


