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Plasma and Shed-Skin Corticosterone Levels in a Population of Louisiana Pine Snakes (*Pituophis ruthveni*)

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Abstract

Measurement of corticosterone in various tissues has been used to investigate the stress response in reptile and amphibian species for decades. The tissue source from which corticosterone is measured reflects different periods of time and chronicity of stress levels in the subject, and different tissue-collection methods differ in degree of invasiveness. Studies of corticosterone in keratinized tissues of reptiles, such as shed skin, are limited in number compared to hair and feather glucocorticoid studies in avian and mammalian species, but warrant continued research, as they may reflect more different periods of time and chronicity of corticosterone levels than plasma or other tissues, and can be obtained in a minimally invasive manner. In this study, we measured corticosterone concentrations in both plasma and shed skin of Louisiana pine snakes (*Pituophis ruthveni*) that were all previously diagnosed with subclinical *Cryptosporidium serpentis* infection. We also tracked stressors experienced by different individuals to identify potential relationships between periods of increased stress and corticosterone levels in plasma and shed skin. There were no significant correlations between individual plasma and shed-skin corticosterone levels, or between corticosterone levels in either tissue type and stressors experienced. This is the first study where corticosterone levels were measured in plasma and shed skin of Louisiana pine snakes, and is the first known evaluation of plasma and shed-skin corticosterone levels in a snake population previously testing positive for *Cryptosporidium serpentis*.

Key Words: *Cryptosporidium serpentis*, glucocorticoid, hypothalamic–pituitary–adrenal axis, keratin, *Pituophis ruthveni*, stress

Introduction

Across vertebrate animals, the physiologic response to an acute stressor results in activation of the hypothalamic–pituitary–adrenal axis, resulting in increased production of glucocorticoids from the adrenal glands (Sheriff *et al.*, 2011; Cockrem, 2013; Kennedy *et al.*, 2013; Hunt, 2019). In reptiles and amphibians, corticosterone is the predominant glucocorticoid secreted, and measurement of corticosterone concentrations, usually in plasma, has been used to investigate stress in reptile and amphibian species for decades (Moore *et al.*, 1991; Romero and Wikelski, 2001; Cockrem, 2013; Tylan *et al.*, 2020). Several previous investigations in reptiles and amphibians have also focused on corticosterone as a marker of physiologic stress and how this may reflect on the health status of an individual or population by comparing hormone titers to other means of assessing health (e.g., body condition score, survival, hematology, blood biochemistry, infection rates, and clinical expression of disease; Romero and Wikelski, 2001; Hunt *et al.*, 2012; de Assis *et al.*, 2015; Lind *et al.*, 2018).

Although it might be expected that the lower metabolic rate of most reptiles would delay the speed of the acute stress response and subsequent rise in corticosterone, several studies have indicated that many reptiles exhibit a rise in corticosterone levels within a similar time frame as mammals and birds (Dallman and Bhatnagar, 2001; Romero and Reed, 2005; Sheriff *et al.*, 2011; Cockrem, 2013; Flower *et al.*, 2018; Boers *et al.*, 2020; Tylan *et al.*, 2020). Based on these findings, it is generally accepted that plasma samples obtained within 3 min of capture or handling will accurately reflect baseline circulating corticosterone levels in most reptile species (Romero and Reed 2005; Zachariah *et al.*, 2009; Cockrem, 2013; Flower *et al.*, 2018; Tylan *et al.*, 2020). Factors influencing both baseline circulating glucocorticoid concentrations as well as the duration of elevated glucocorticoid concentrations in reptiles include sex, health, body condition, reproductive stage, season, and stressor duration (Sapolsky, 1992; Romero, 2002; Reeder and Kramer, 2005; Sheriff *et al.*, 2011; Kennedy *et al.*, 2013; Cockrem, 2013). For example, female animals often exhibit higher circulating glucocorticoid concentrations than males,

and most animals experience an increase in circulating glucocorticoid concentrations during their active reproductive season (Romero, 2002; Reeder and Kramer, 2005).

The tissue source from which glucocorticoids are quantified reflects different periods of time and chronicity of stress levels in the subject (Möstl and Palme, 2002; Reeder and Kramer, 2005; Mormède *et al*., 2007; Sheriff *et al*., 2011; Ganswindt *et al*., 2012; Palme, 2019; Gormally and Romero, 2020). Additionally, tissue-sample collection methods differ in degrees of invasiveness; in wildlife, a minimally invasive approach is often desired to lessen effects of investigators on study subjects (Sheriff *et al*., 2011; Palme, 2019). Circulating glucocorticoids can accumulate in keratinized tissues such as skin and nails, although how this occurs is still not completely understood (Lattin *et al*., 2011; Romero and Fairhurst, 2016; Gormally and Romero, 2020; Dillon *et al*., 2021). Because glucocorticoid accumulation begins at the inception of tissue growth and only ends once the blood supply to that tissue is cut off or the tissue is removed, corticosterone measured in keratinized tissues represent levels reflecting longer periods of time (i.e., often weeks to months) than can be measured in plasma (Lattin *et al*., 2011; Kennedy *et al*., 2013; Romero and Fairhurst, 2016; Gormally and Romero, 2020). The strength of keratin proteins due to disulfide bonds suggests that hormones stored within these tissues can endure for years without degradation, and concentrations are not influenced by short-term factors such as handling stress, hydration status, or time of day (Kennedy *et al*., 2013; Dillon *et al*., 2021). This means that measurements of glucocorticoid concentrations in keratinized tissues represent weeks, months, or years of accumulation (depending on the subject's molt or shed cycle and growth rate), and can potentially be utilized to retrospectively evaluate stressor exposure and monitor an individual's long-term health (Ganswindt *et al*., 2012; Berkvens *et al*., 2013; Kennedy *et al*., 2013; Gormally and Romero, 2020; Zena *et al*., 2022).

Studies of corticosterone in keratinized tissues of reptiles are limited in number compared to hair and feather glucocorticoid studies in avian and mammalian species, but warrant continued research, as they can be obtained in a minimally invasive manner and may reflect more chronic glucocorticoid levels than can be measured in plasma or other tissues (Berkvens *et al*., 2013; Baxter-Gilbert *et al*., 2014; Matas *et al*., 2016; Carbajal *et al*., 2018; Hamilton *et al*., 2018; Gormally and Romero, 2020; Dillon *et al*., 2021). The chronic presence of a stressor can lead to a persistent physiologic stress response and continuous activation of the hypothalamic–pituitary–adrenal axis due to reduced efficacy of negative feedback mechanisms (Sapolsky, 1992; Sheriff *et al*., 2009). This in turn can have a negative impact on the overall health of an animal, contributing to secondary conditions such as immunosuppression, decreased growth rates, and pathologies of the cardiovascular, reproductive, and digestive systems (Sheriff *et al*., 2009; Hunt, 2019). Being able to detect chronic stress using a more integrated measure of long-term corticosterone like keratinized tissues would be a valuable tool for conservation physiology.

In reptiles, corticosterone has been quantified from turtle and chameleon claws (Baxter-Gilbert *et al*., 2014; Matas *et al*., 2016), as well as the shed skin of lizards (Carbajal *et al*., 2018; Zena *et al*., 2022) and snakes (Berkvens *et al*., 2013). No effect of a chronic stressor was reflected in shed-skin corticosterone in snakes in the study by Berkvens *et al*. (2013). Studies on lizard have demonstrated seasonal variations in shed-skin corticosterone (Carbajal *et al*., 2018; Zena *et al*., 2022). Further research is needed regarding the ability of shed-skin corticosterone to reflect chronic stress and its relationship to circulating corticosterone levels in reptiles.

The Louisiana pine snake (*Pituophis ruthveni*) is a colubrid species native to Louisiana and eastern Texas, and is one of the rarest species of snakes in North America (Conant, 1956; Thomas *et al*., 1976; Reichling, 1995; Rudolph *et al*., 2006; Hibbits *et al*., 2016). The objective of this study was to measure corticosterone concentrations in the plasma and shed skin of Louisiana pine snakes using a commercially available enzyme immunoassay (EIA). We hypothesized that extraction methods validated for other species would work similarly for the Louisiana pine snake and that measurable amounts of plasma and shed-skin corticosterone would be detected using these methods. Additionally, we hypothesized that periods of increased stress (due to disease, changes in husbandry or routine, or increased handling) in individual snakes would correlate with changes in both plasma and shed-skin corticosterone levels.

Materials and Methods

Study population: Eight adult Louisiana pine snakes (five males, three females) were included in this study. These snakes were bred in captivity at a separate zoological facility and maintained as a research population in a vivarium at Louisiana State University (Baton Rouge, LA, USA). These animals were the only captive research population besides those housed in zoo breeding colonies. Snakes were housed in racks in individual cages (170 cm high × 127 cm wide × 76 cm deep [66.9 × 50 × 29.9 in.], Freedom Breeder, Turlock, CA, USA) with heat tape. Snakes experienced the following light/heat/humidity schedules: 12 h light, 12 h darkness; a temperature range of 27–29°C (80.6–84.2°F); and 50% humidity. Snakes were fed frozen-thawed rats once weekly and provided with *ad libitum* access to tap drinking water. The snakes were housed on shredded aspen substrate (Envigo Teklad, Madison, WI, USA) and provided with a plastic hide. Each of these snakes had previously tested positive more than once for *Cryptosporidium serpentis* via PCR of gastric swabs. No snakes exhibited any clinical signs of illness on previous medical histories, physical exams, or blood testing (CBC and chemistry panel). When handling these snakes, animal care providers and investigators wore gowns and gloves. Gloves were changed between handlings of each animal. Animal care throughout this study and all procedures described here were approved by the Institutional Animal Care and Use Committee at Louisiana State University, Baton Rouge, Louisiana; the population was maintained

under an annually renewed permit from the Louisiana Department of Wildlife and Fisheries (WDP-22-017).

There were no inclusion criteria; however, in the 6 wk prior to initiation of the study each snake underwent a health evaluation including a physical examination, complete manual blood count, and blood chemistry analysis (AU680 Clinical Chemistry Analyzer; Beckman Coulter, Brea, CA, USA; blood was collected from the ventral coccygeal vein). Hematology and blood chemistry values were compared to the only published values available for this species (Giori *et al*., 2020). Because of the small sample size of the published values, any deviations from these values were characterized as "suspected abnormal" if they were higher or lower than the minimum and maximum values reported. Heterophil-to-lymphocyte (H:L) ratio was manually calculated for each sample. At the time of physical examination and blood collection, each snake was sedated with 12–18 mg/kg alfaxalone (Alfaxan; Jurox Inc., North Kansas City, MO, USA) and 0.2 mg/kg hydromorphone (HYDROmorphone; Pfizer, Lake Forest, IL, USA) administered intramuscularly for endoscopic examination of the esophagus and stomach with gastric and esophageal biopsies obtained from each snake. Different endoscopes were used for each animal and endoscopic equipment was washed using a Reliance™ EPS Endoscope Processing System (STERIS Corporation, Mentor, OH, USA) scope washer, which uses a neutral-based detergent with protease activity (Klenzyme; STERIS Corporation, Mentor, OH, USA) for 5 min at 48°C (118.4°F), and a dry chemistry disinfection solution, and an acid-based cleaner (CIP 200; STERIS Corporation, Mentor, OH, USA) for 6 min at 50°C (122°F) to sterilize the endoscopy equipment so that each snake was scoped with clean instruments. At least two biopsies were collected from each individual, one from the esophagus (to evaluate esophageal tonsils), and one from the mid-stomach; however, more samples were collected from some individuals if gross abnormalities were visualized. Biopsy samples were evaluated histologically; gastric samples were also submitted for *Cryptosporidium serpentis* qPCR testing to the University of Florida Zoological Medicine Diagnostic Laboratory (Gainesville, FL, USA).

Blood collection and processing: Eight months prior to the start of sample collection, a pilot study was conducted with the same population of snakes to ensure corticosterone extraction methodology and EIAs would be successful in obtaining measurable concentrations of corticosterone in snake plasma. Once the year-long sample collection period started, blood was collected from each snake once per month from January through December 2022. Each snake was manually restrained and blood collected from the ventral coccygeal vein within 3 min after the drawer housing the snake was opened to reduce the possibility of a stress-induced increase in corticosterone resulting from handling (Romero and Reed, 2005; Tylan *et al*., 2020). The blood collection site on the tail was cleaned with 70% ethanol prior to collection. Blood was collected with a 1-ml or 3-ml syringe using a 21-gauge or 25-gauge needle. Volume of blood collected from each snake at each collection time did not exceed 0.5 ml (less than 0.1% of the body weight of the smallest individual in the study). If a blood sample could not be collected within the 3-min time frame, the snake was returned to the drawer, and a second attempt to collect blood from that individual was made 2 wk later.

Blood was stored in a lithium heparin tube with a plasma separator (BD Microtainer PST Tubes with Lithium Heparin; Mississauga, Ontario, Canada). Tubes were stored on wet ice or freezer packs until centrifugation within 1–2 h of blood collection. Tubes were centrifuged at 5,241 × *g* (Gusto High-Speed Mini Centrifuge; Heathrow Scientific, Vernon Hills, IL, USA) for 2 min to separate plasma from red blood cells. Plasma was stored in sterile 2-ml cryogenic vials (Fisherbrand, ThermoFisher Scientific, Waltham, MA, USA) at −80°C (−112°F) for up to 12 months until corticosterone extraction and assay.

Over the year-long course of sample collection, two snakes died (one in February, and one in May), and were therefore only represented in early sample collection months. Complete postmortem examinations were performed for these snakes. Both individuals initially presented with dermal ulcerations and dysecdysis; however, these disease processes did not occur at the same time. After signs were noted, the first snake was hospitalized; diagnostics and treatments were performed. However, the snake declined and died. Postmortem examination revealed sepsis (multiple bacterial species identified) to be the cause of death. The second snake was also hospitalized as soon as the dermal lesions were noted; this snake's clinical signs did not progress as rapidly as the first and more extensive diagnostics could be performed. However, ultimately the animal's quality of life declined and the dermal lesions did not resolve; the animal was euthanized after 3 months of hospitalization. Postmortem examination revealed atherosclerosis and colloid goiter, but the exact pathogenesis and mechanism for the ulcerative dermatitis could not be determined. Neither snake's death was determined to be due to cryptosporidiosis, and both snakes were negative for *C. serpentis* on PCR performed on gastric biopsies collected postmortem.

Shed-skin collection and processing: Shed skins were collected from each snake's enclosure when the snake happened to shed from January to December 2022. If a shed-skin sample was partial, this was noted during collection. One of the two snakes that died during the study had not produced any shed, and therefore shed skins from only seven individuals were included in this study. Shed skins were stored individually in sealed plastic freezer bags (Ziploc; SC Johnson, Racine, WI, USA) at 4°C (39.2°F) at the animal care facility for up to 2 wk until they could be transferred to laboratory storage. After transfer, they were maintained at room temperature until extraction (Baxter-Gilbert *et al*., 2014; Carbajal *et al*., 2018).

Tracking of stressors: Any potential stressors that might affect the snakes outside of daily routine handling for husbandry were recorded over the year-long course of sample collection. Stressors were categorized as either direct or indirect stressors. Direct stressors included any handling of snakes for a reason other than routine husbandry, exhibition

of an abnormal behavior (e.g., increased aggression), decreased appetite, mortality, signs of clinical illness (e.g., lethargy, visible skin lesions), a period of hospitalization due to any of the above clinical concerns, or when potentially physiologically stressful events such as reproductive activity were noted (e.g., egg-laying behavior). An indirect stressor to individual snakes was considered any instance when the snake room was entered for reasons other than routine husbandry (such as sample collection), but that individual snake was not handled during that event. Total numbers of stressors per month were calculated by adding direct and indirect stressors during each month of the study.

Plasma corticosterone extraction and EIAs: Plasma corticosterone extractions were performed as previously for wild birds (Kelly *et al.*, 2022). Corticosterone was extracted from each sample using triple ethyl acetate extraction and dried down samples stored at −80°C until EIAs could be performed. Samples were assayed for corticosterone using a commercially available EIA (Arbor Assays K014; Ann Arbor, MI, USA). According to the manufacturer, cross-reactivity of this kit is 1-dehydrocorticosterone: 18.9%; desoxycorticosterone: 12.3%; 1α-hydroxycorticosterone: 3.3%; 11-dehydrocorticosterone: 2.4%; tetrahydrocorticosterone: 0.8%; aldosterone: 0.6%; cortisol: 0.4%; progesterone: 0.2%; dexamethasone: 0.1%; corticosterone-21-hemisuccinate: <0.1%; cortisone, estradiol: <0.08%; testosterone: 0.03%; allopregnanolone, dehydroepiandrosterone sulfate, estrone-3-glucuronide, estrone-3-sulfate, and 17-hydroxyprogesterone: <0.01%.

All samples and standards were run in duplicate following the manufacturer's protocol. Samples were run on four EIA plates; plate assignment and sample order were randomly determined. To estimate extraction efficiency, two samples of house sparrow (*Passer domesticus*) plasma stripped of corticosterone using dextran-coated charcoal were spiked with a known amount of corticosterone and run on each plate. Because average extraction efficiency was ∼100% for each plate, we did not correct final plasma corticosterone values. A sample of pooled plasma from all snakes was included on each plate as a measure of interplate variability; the coefficient of variation of these pooled samples averaged 5%. Intraplate variability was calculated as the coefficient of variation of duplicate samples and averaged 5%, 5%, 6%, and 6% for the four different plates. The sensitivity of this assay was 20.9 pg/ml. Graphs of corticosterone concentrations and the associated standard curve for each assay plate revealed parallel displacement for each plate (Fig. 1).

Shed-skin corticosterone extraction and EIA: A pilot study was conducted using shed snake skins that had been collected April–December 2021 to refine the corticosterone extraction protocol to provide the highest corticosterone recovery. The extraction protocol used was based on methods for corticosterone extraction from shed skin described by Baxter-Gilbert *et al.* (2014), Berkvens *et al.* (2013), Carbajal *et al.* (2018), Hamilton *et al.* (2018), and Zena *et al.* (2022), with some modifications. The final extraction protocol is described in detail in Table 1.

Shed-skin samples were run in random order on a single EIA plate. To estimate recovery, two spiked samples were created by adding a known amount of corticosterone to tubes containing 6 ml methanol that were run with the rest of the samples. Average extraction efficiency was ∼100% for each plate. Because extraction efficiency was high, we did not correct final plasma corticosterone values. Once extracted, samples were reconstituted with assay buffer, the assay protocol was followed according to the manufacturer's protocol, as for the plasma samples. All samples and standards were run in duplicate. Intraplate variability was determined using the coefficient of variation of duplicate shed-skin samples and averaged 6%. A graph of corticosterone concentrations and the associated standard curve for this assay revealed parallel displacement from the standard curve (Fig. 2).

Data analyses: All statistical analyses were performed with JMP Pro 16.2.0 (SAS Institute Inc., Cary, NC, USA). To assess the relationship between plasma and shed-skin corticosterone and snake physiology, mixed ANOVA models were used for plasma and skin corticosterone with various factors (including sex, month of collection, mortality, hematologic abnormalities, H:L ratio, endoscopic and histopathologic abnormalities, *Cryptosporidium* sp. status, direct stressors, and total stressors) as fixed effects and animal ID and EIA plate as random effects. The relationship of H:L ratio and skin corticosterone was also evaluated via a linear regression. To assess potential relationships between plasma and shed skin within individuals, the most recent plasma corticosterone level at the time of shed-skin collection and average of plasma corticosterone levels since last shed-skin collection were assessed via Pearson correlation coefficients with the corresponding shed-skin corticosterone values. The normality of the residuals from the ANOVA models was confirmed by examining quantile plots. Correlations among the noncontinuous fixed effects were evaluated via Spearman correlation coefficients. $P < 0.05$ was considered statistically significant. Intra-individual coefficient of variation (CV%) was calculated for plasma and shed skin within each snake.

Results

Health evaluations: Results of hematologic evaluation for each snake are reported in Table 2. These were compared to the only published values available for this species (Giori *et al.*, 2020), and are interpreted as follows: Snake 2 had a suspected anemia, Snakes 5 and 12 had a suspected polycythemia. Snakes 16 and 17 had a suspected leukocytosis. Snake 4 had a suspected heterophilia. Snakes 2 and 5 had a suspected lymphocytopenia. Snakes 16 and 17 had a suspected lymphocytosis. Snakes 2, 4, 5, 6, 12, and 16 had suspected monocytosis. Snakes 5 and 16 had suspected basophilia. All snakes except Snake 5 had elevated total solids. Results of endoscopic and histopathologic evaluation for each snake are reported in Table 3. No biochemistry abnormalities were found in any snake. Snake 17 was the only snake that tested positive for *C. serpentis* based on

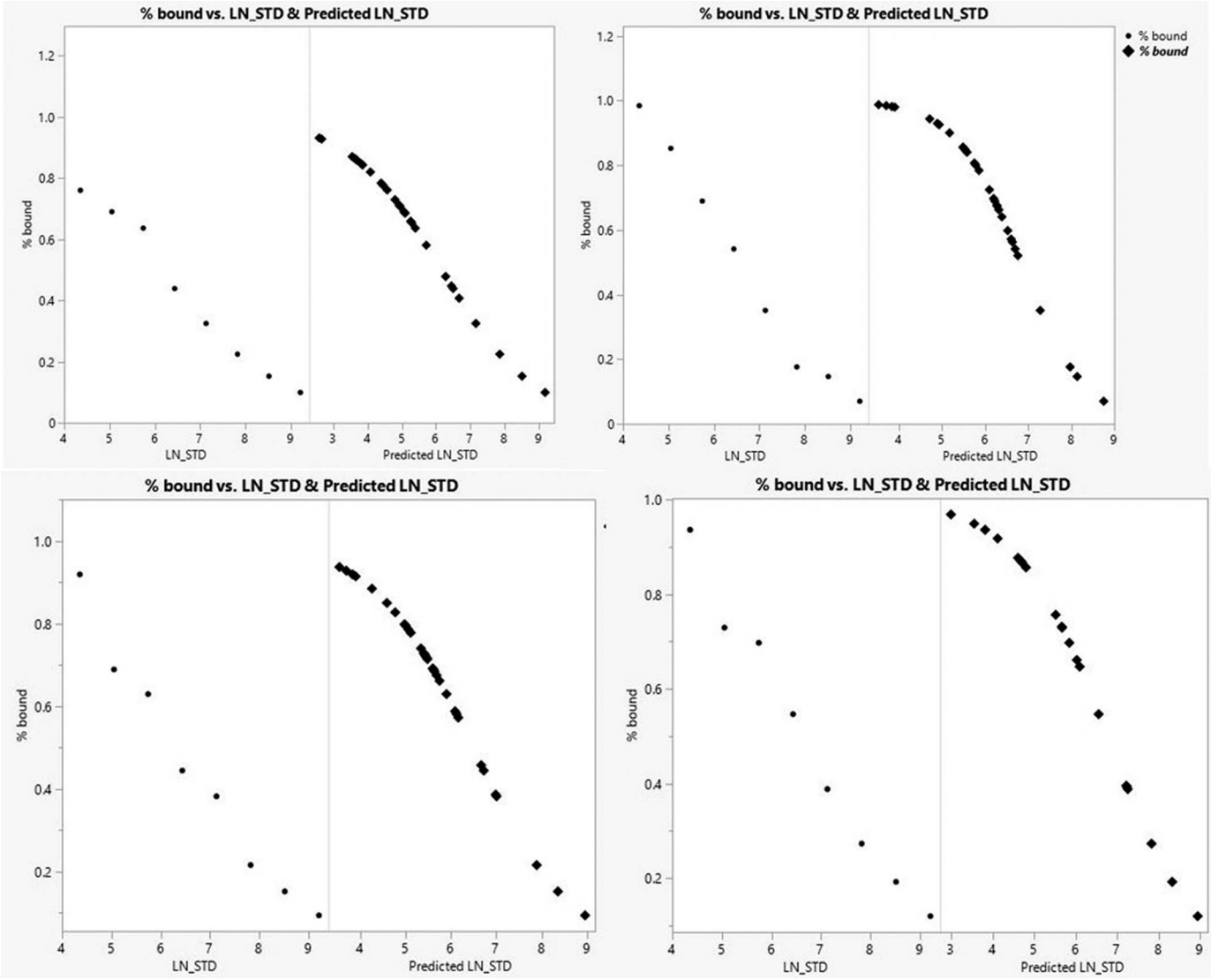

Figure 1. Parallelism of natural logarithm transformed corticosterone standards (LN_STD, points) and measured plasma corticosterone (predicted LN_STD, diamonds) in Louisiana pine snakes (*Pituophis ruthveni*), divided across four plates (each graph represents one plate).

PCR performed on the biopsy samples. For all health criteria evaluated prior to the initiation of the study, only suspected monocytosis was correlated with sex. Males were more likely to exhibit suspected monocytosis than females (male: 5/5, female: 1/3; $P = 0.034$). There were no other statistically significant correlations between these variables and sex ($P > 0.05$).

Plasma corticosterone: Snakes showed high individual variation in plasma corticosterone levels from month to month (Fig. 3); the coefficient of variation in corticosterone from individual snakes ranged from 60% to 92%. There were few statistically significant relationships between plasma corticosterone and different measured variables; the only statistically significant correlation was with heterophilia, which was inversely correlated with plasma corticosterone concentrations ($P = 0.024$, $F_{1,3.2} = 16.76$; see Fig. 4); however, it is important to note that only one individual exhibited suspected heterophilia. No correlations were found between plasma corticosterone and snake sex, month of collection, or H:L ratio ($P > 0.05$).

Shed-skin corticosterone: Shed skins could be collected from seven individuals; snakes produced between one and six skins total over the course of the year. Sheds were produced at variable intervals by each snake. Snakes showed high individual variation in shed-skin corticosterone levels (Fig. 5); the coefficient of variation in corticosterone from individual snakes ranged from 34% to 78%. There were few statistically significant correlations between shed-skin corticosterone and other variables measured. H:L ratio was positively correlated with shed-skin corticosterone (a slope of 1.73 ng/g per H:L unit, $P = 0.007$; see Fig. 6). Survivorship to the end of the study was also inversely correlated with shed-skin corticosterone ($P < 0.001$, $F_{1,18} = 36.50$), and anemia was positively correlated with shed-skin corticosterone ($P < 0.001$, $F_{1,18} = 36.50$); however, this reflects data from

Table 1. Final methods used for corticosterone extraction from shed snake skin. Specific materials and products used in this study are included under descriptions in parentheses.

Extraction steps	Description	References
Step 1: Wash shed skins	Submerge shed skins in 50-ml conical tubes filled with distilled water, vortex 2 min	Baxter-Gilbert *et al*. (2014); Berkvens *et al.* (2013); Carbajal *et al*. (2018); Hamilton *et al*. (2018); Zena *et al*. (2022)
Step 2: Dry shed skins	Lay shed skins flat overnight in a fume hood to dry	Baxter-Gilbert *et al*. (2014); Berkvens *et al*. (2013)
Step 3: Flash freeze and mince skins	Submerge individual samples briefly in liquid nitrogen, then mince with an electric coffee grinder (12 cup fine grind setting; Mr. Coffee 12 Cup Electric Coffee Grinder with Multi Settings; Sunbeam Products, Boca Raton, FL, USA)	Baxter-Gilbert *et al*. (2014); Carbajal *et al*. (2018); Hamilton *et al*. (2018)
Step 4: Vortex and store minced skin	Vortex each minced sample for homogenous distribution; store samples individually in plastic sealed freezer bags at room temperature	Baxter-Gilbert *et al*. (2014); Carbajal *et al*. (2018)
Step 5: Methanol immersion	Place 0.1 g of each minced shed-skin sample into a 15-ml conical tube containing 6 ml methanol (Avantor Performance Materials, LLC; Radnor, PA, USA)	Carbajal *et al*. (2018); Dillon *et al*. (2021)
Step 6: Incubation	Incubate all tubes for 18 h in a 30°C (86°F) shaking water bath (45 rpm, Precision Shaking Water Bath 15; ThermoFisher Scientific, Waltham, MA, USA)	Carbajal *et al*. (2018)
Step 7: Separate skin from extracted corticosterone in methanol	Decant each sample through a 40-μm cell strainer (Greiner Bio-One EASYstrainer Cell Strainer, ThermoFisher Scientific) into a clean 15-ml conical tube and flush an additional 1 ml of methanol through the strainer into the conical tube	Berkvens *et al*. (2013)
Step 8: Divide samples into microcentrifuge tubes for drying	Divide the filtrate of each sample into corresponding 1.5-ml microcentrifuge tubes (Fisherbrand, ThermoFisher Scientific)	N/A
Step 9: Dry down samples	Dry down methanol in all microcentrifuge tubes using a SpeedVac Concentrator (SPD1010; ThermoFisher Scientific) for 2 h at 45°C (113°F)	Dillon *et al*. (2021); Zena *et al*. (2022)
Step 10: Resuspend corticosterone in assay buffer	Divide 400 μl of assay buffer equally among microcentrifuge tubes for each sample, vortex for 2 min, then recombine buffer from all tubes for a single sample into a single microcentrifuge tube	N/A
Step 11: Storage or enzyme immunoassay	Perform enzyme immunoassay immediately or freeze samples at −20°C (−4°F) until the assay is performed	Follow assay kit manufacturer instructions

one shed-skin sample from one anemic individual that did not survive to the end of the study. There was no relationship between shed-skin corticosterone and snake sex or month of collection. When shed-skin levels were compared to the plasma level of blood collected most recently prior to that snake shedding, and to average levels from all blood samples collected since the last shed was collected, plasma and shed-skin corticosterone were not correlated ($P > 0.05$; Figs. 7–8).

Discussion

All of the animals in this research population had previously tested positive for *C. serpentis* on multiple occasions via gastric swab. This was the only captive research population of Louisiana pine snakes; therefore we did not have any *Cryptosporidium*-negative snakes available for this study.

We collected monthly blood samples and shed skin produced over the course of 1 yr from captive Louisiana pine snakes to quantify plasma and shed-skin corticosterone and to evaluate any possible relationships with physiologic variables and stressors. The only significant relationship we found between plasma corticosterone and other variables evaluated was an inverse correlation between plasma corticosterone and suspected heterophilia—the one snake showing suspected heterophilia had significantly lower plasma corticosterone compared to the others. However, as only one snake exhibited suspected heterophilia, this finding, while statistically significant, may not be biologically significant. Further studies using a larger sample size are needed to determine if this is a true relationship. In fact, we expected plasma corticosterone and heterophil levels to be positively correlated, as both typically increase in the presence of a stressor (Dunlap and Wingfield, 1995; Romero and Wikelski, 2001; de Kloet *et al*., 2005; Cartledge and Jones, 2007; Sheriff *et al*., 2011; Cockrem, 2013; Sparkman *et al*., 2014; Fabrício-Neto *et al*., 2019). Chronic disease may lead to an inverse correlation; however, the single individual exhibiting suspect heterophilia showed no evidence of other active disease processes based on health evaluations. Additionally, the suspected heterophilia noted was less than 0.2×10^3 cells/μl higher than the maximum

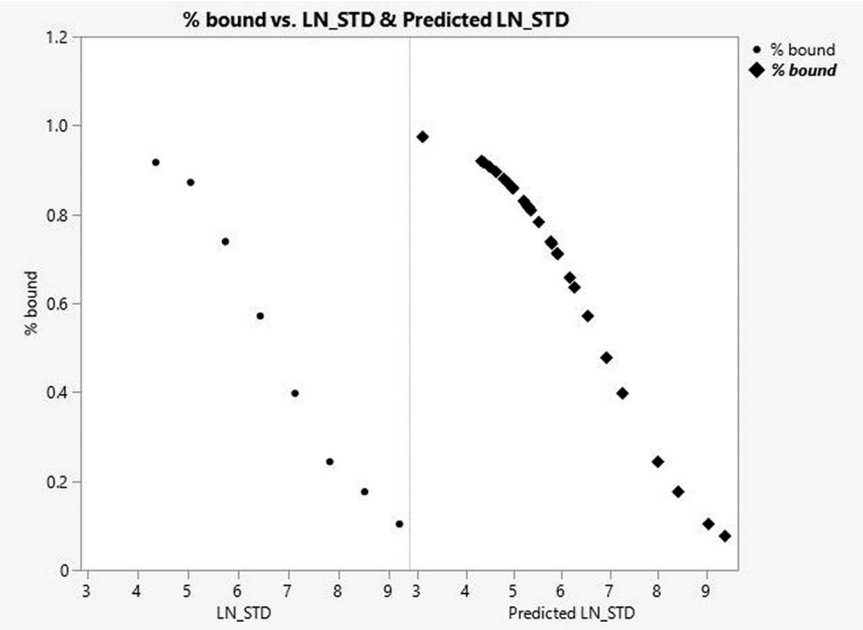

Figure 2. Parallelism of natural logarithm transformed corticosterone standards (LN_STD, points) and measured shed-skin corticosterone (predicted LN_STD, diamonds) in Louisiana pine snakes (*Pituophis ruthveni*).

levels reported by Giori *et al.* (2020) and would not be considered clinically significant. The study by Giori *et al.* (2020) represents the only published hematologic data for this species. However, only 11 snakes were included in that study, which is an insufficient number to determine true reference intervals or ranges for Louisiana pine snakes. Therefore, it is quite possible that our interpretation of the white blood cell changes may not be significant because of a lack of white blood cell data on a larger population of pine snakes. We must also consider the possibility of lymphatic contamination and the impact that this can have on hematologic values reported. Grossly, investigators did not observe visible lymphatic contamination; however, there is a lack of objective methods to measure lymphatic contamination, and it is possible that the white blood cell values were affected if lymphatic contamination was present (Heatley and Russell, 2019).

No other statistically significant relationship between plasma corticosterone and factors such as health parameters, sex, sampling month, or stressor exposure was identified in this study. A small sample size and high within-individual variability in plasma corticosterone in this population likely contributed to the lack of correlations. As plasma corticosterone concentrations may change rapidly as a consequence of several independent and interrelated factors (including stress, disease, reproductive status, time of day, and environmental parameters), it is possible that variability across individuals was due to an unknown factor we did not account for (Moore *et al.*, 1991, 2001; Sapolsky, 1992; Romero, 2002; Preest *et al.*, 2005; Reeder and Kramer, 2005; Sheriff *et al.*, 2011; Cockrem, 2013; Anderson *et al.*, 2014; Dayger and Lutterschmidt, 2017; Lind *et al.*, 2018; Racic *et al.*, 2020). This high variability highlights the challenges of using plasma corticosterone concentrations in reptiles as a reliable marker of physiologic stress (especially if only measured at a single time point), and the perhaps unrealistic goal of establishing species-specific baseline reference ranges for this hormone.

Table 2. CBC results for each Louisiana pine snake (*Pituophis ruthveni*) performed 1–2 months prior to the study. Values that are higher or lower than those reported in the population examined by Giori *et al.* (2020) are in bold. Please note that Snakes 2 and 16 died prior to completion of the study.

Snake ID (sex)	PCV, %	Total white blood cell count, $\times 10^3$ cells/μl	Absolute heterophil count, $\times 10^3$ cells/μl	Absolute lymphocyte count, $\times 10^3$ cells/μl	Absolute monocyte count, $\times 10^3$ cells/μl	Absolute basophil count, $\times 10^3$ cells/μl	TS, g/dl	H:L ratio
2 (F)	**28 (L)**	6.3	2.1	**1.38 (L)**	**2.6 (H)**	0.1	**7.8 (H)**	1.50
4 (M)	39	15.5	**2.9 (H)**	9.46	**3.1 (H)**	0.0	**9.8 (H)**	0.30
5 (M)	**50 (H)**	8.5	2.5	**2.13 (L)**	**3.3 (H)**	**0.6 (H)**	**7.9 (H)**	1.20
6 (M)	40	13.3	2.5	8.88	**1.7 (H)**	0.1	6.9	0.30
12 (M)	**48 (H)**	9.3	1.9	5.19	**2.1 (H)**	0.0	**9.0 (H)**	0.40
16 (M)	38	**24.3 (H)**	1.7	**17.7 (H)**	**4.4 (H)**	**0.5 (H)**	**11.3 (H)**	0.10
17 (F)	39	**22.0 (H)**	1.3	**20.02 (H)**	0.4	0.2	**9.8 (H)**	0.06
18 (F)	39	13	0.8	11.18	1.0	0.0	**11.5 (H)**	0.07

CBC, complete blood count; F, female; M, male; H, values that are higher than the maximum value reported in Giori *et al.* (2020); L, values that are lower than the minimum value reported in Giori *et al.* (2020); PCV, packed cell volume; TS, total solids; H:L, heterophil to lymphocyte.

Table 3. Gross endoscopic findings and histopathology results for esophageal and/or gastric biopsies from Louisiana pine snakes (*Pituophis ruthveni*) collected 1–2 months prior to the study. Please note that Snakes 2 and 16 died prior to completion of the study.

Snake ID (sex)	Gross endoscopic findings	Histologic findings (esophageal biopsy)	Histologic findings (gastric biopsy)
2 (F)	Ulcer present in cranial esophagus, and esophageal lymphoid nodules appeared prominent.	Moderate subacute to chronic multifocal heterophilic and lymphoplasmacytic esophagitis.	NAS
4 (M)	NAS	NAS	NAS
5 (M)	Esophageal lymphoid nodules appeared prominent. Markedly thickened gastric mucosa.	Minimal lymphoid hyperplasia.	NAS
6 (M)	NAS	NAS	NAS
12 (M)	Moderately thickened gastric folds intermittently present.	NAS	NAS
16 (M)	Moderately thickened gastric folds intermittently present.	NAS	Small numbers of scattered lymphocytes and rare granulocytes present in the lamina propria.
17 (F)	Markedly thickened gastric folds diffusely present.	NAS	Marked proliferative gastritis with intraluminal and intraepithelial apicomplexan protozoa, consistent with presumptive cryptosporidiosis. Acid-fast staining did not highlight the presumptive *Cryptosporidium* sp.
18 (F)	Esophageal lymphoid nodules appeared prominent. A discolored tan, slightly thickened and roughened region of the gastric mucosa was sharply demarcated from surrounding normal tissue.	Marked subacute lymphocytic and granulocytic esophagitis.	NAS

F, female; NAS, no abnormalities seen; M, male.

For shed skin, we identified a positive correlation between corticosterone and the H:L ratio from a single time point. The clinical significance of this relationship is somewhat unclear because this blood sample was obtained 6 wk prior to the start of the study, though it seems likely that shed-skin corticosterone reflected circulating corticosterone from a time period that included this blood sample. In addition, Louisiana pine snakes are a primarily lymphocytic species, which makes interpretation of H:L ratios more challenging (Giori *et al*., 2020). Nonetheless, future investigations using serial measurements of H:L ratios and shed-skin corticosterone should be pursued to define this relationship further, as H:L ratios are thought to correlate with physiologic stress in a number of other species (Dunlap and Wingfield, 1995, Romero and Wikelski, 2001; de Kloet *et al*., 2005; Cartledge and Jones, 2007; Sheriff *et al*., 2011; Cockrem, 2013; Sparkman *et al*., 2014; Fabrício-Neto *et al*., 2019).

To our knowledge, this is only the second study to quantify shed-skin corticosterone in snakes. The previous study investigated shed-skin corticosterone levels in captive African house snakes and Eastern Massasauga rattlesnakes (*Sistrurus catenatus catenatus*; Berkvens *et al*., 2013). A consistent finding between this investigation and the previous study is a lack of correlation between exposure to stressors and shed-skin corticosterone concentrations (Berkvens *et al*., 2013). It is logical to presume that exposure to stress will have some effect on corticosterone accumulation and concentrations in keratinized tissue in snakes, as it does in other species; however, further investigations need to be pursued to understand the nature of this complex relationship (Berkvens *et al*., 2013; Baxter-Gilbert *et al*., 2014; Matas *et al*., 2016; Carbajal *et al*., 2018; Hamilton *et al*., 2018; Gormally and Romero, 2020; Dillon *et al*., 2021). It is possible that short-term exposure to stressors only causes a brief elevation in corticosterone that is not reflected in a long-term tissue such as shed skin. An additional consideration is that the stressors in our study were incidental and we did not apply experimental stressors as in the case of Berkvens *et al.* (2013); therefore a direct comparison between that study and ours cannot be made.

Husbandry conditions may have contributed to some of the lack of trends that might have been expected in plasma and shed skin corticosterone. These snakes were housed individually and maintained at a constant temperature, humidity, and light cycle, disallowing for normal brumation behavior and visual reproductive cues, which may have contributed to suppression of normal reproductive hormonal cycles that can influence seasonal corticosterone concentrations (Sapolsky, 1992; Shine and Mason, 2001; Romero, 2002; Reeder and Kramer, 2005; Sheriff *et al*., 2011; Cockrem, 2013). Additionally, we did not collect blood samples at the same time of day during each sampling session (although all occurred during the day period of the light cycle), which may have influenced circulating corticosterone levels. Circadian variation in corticosterone levels has been demonstrated in some,

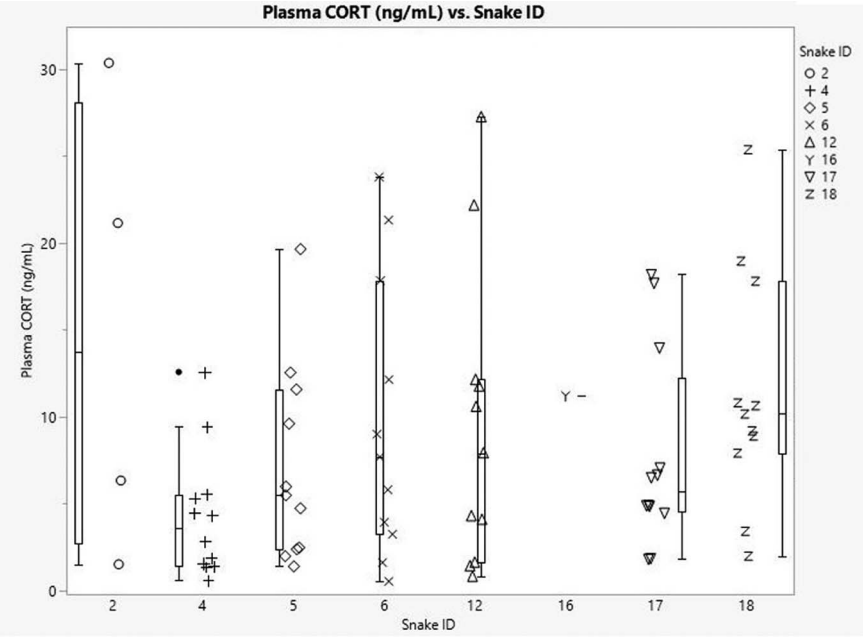

Figure 3. All plasma corticosterone (CORT) values measured from each Louisiana pine snake (*Pituophis ruthveni*) over the course of 1 yr in captivity with averages, interquartile ranges, and minimum and maximum values displayed for each individual using box-and-whisker plots. Each monthly sample is represented by an individual data point for each snake. Please note that Snakes 2 and 16 died prior to completion of the study.

but not all, species of reptiles (Chan and Callard, 1972; Lance and Lauren, 1984; Tyrell and Cree, 1998). Finally, as snakes had been housed in captivity with a consistent daily husbandry protocol (daily cleaning and weekly feedings) for 3 yr prior to the study, we did not consider husbandry to be a stressor. However, even these routine procedures may have affected plasma corticosterone, and husbandry-associated stressors should be considered in future research.

Another limitation of this study was a small sample size. These snakes were the only group of captive *P. ruthveni* in a research colony; therefore we could not control for sample size. We began our study with only eight snakes, and two died during the course of the study, one before it produced any shed skin samples. Even in the surviving snakes, three snakes only produced one or two shed skins over the course of the year. The incidence of different pathologies was also low, and often only one or two individuals would exhibit abnormalities that would be expected to correlate with plasma or shed-skin corticosterone levels. For example, although corticosterone levels are expected to rise in the presence of clinical disease, only two snakes developed illness of any kind over the course of the investigation (Romero and Wikelski, 2001; Hunt *et al.*,

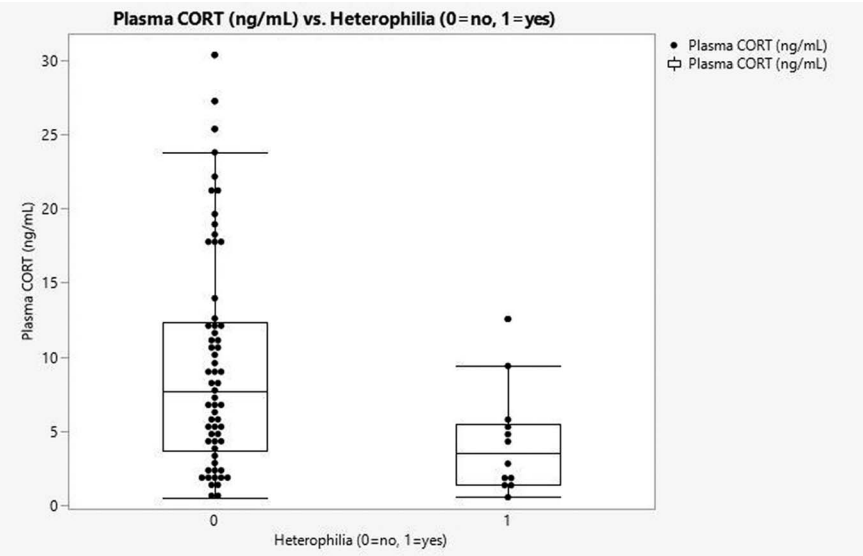

Figure 4. Heterophilia was inversely correlated with plasma corticosterone (CORT) levels ($P = 0.024$) in captive Louisiana pine snakes (*Pituophis ruthveni*). Box-and-whisker plots display interquartile range, median, minimum, and maximum values (excluding outliers).

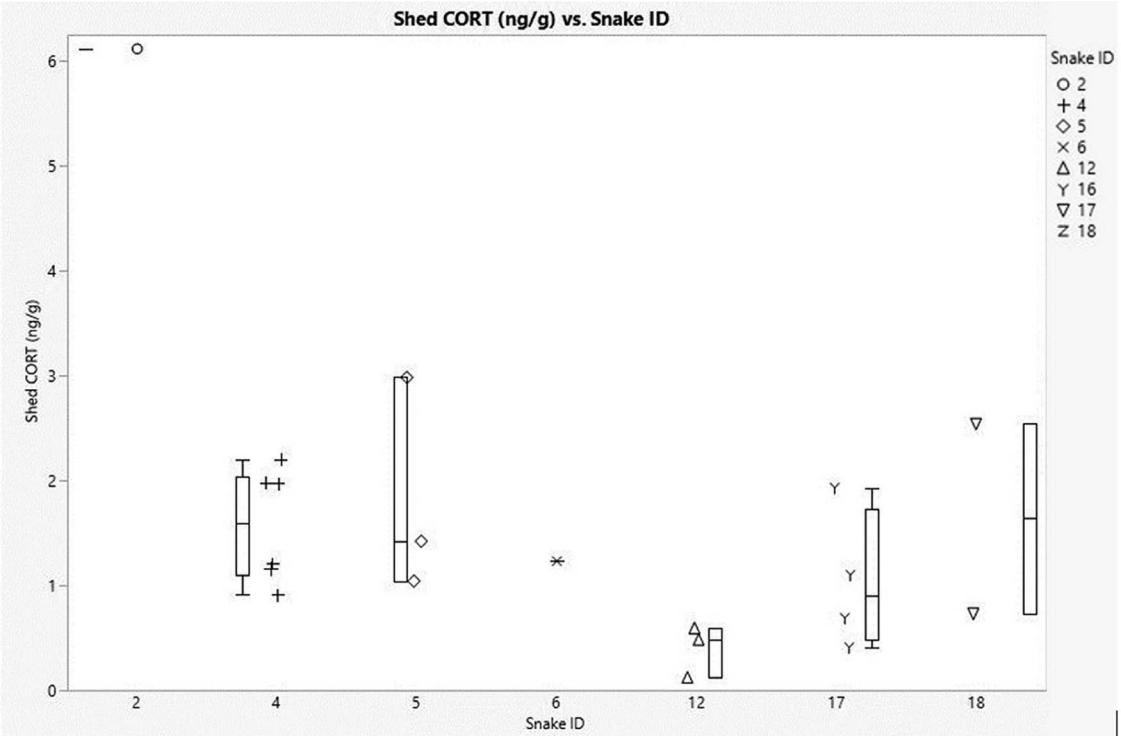

Figure 5. All shed-skin corticosterone (CORT) values measured from each Louisiana pine snake (*Pituophis ruthveni*) ($n = 7$) over the course of 1 yr in captivity with averages, interquartile ranges, and minimum and maximum value displayed for each individual using box-and-whisker plots. Note that Snake 2 produced only one shed-skin sample and died prior to completion of the study.

2012; de Assis *et al*., 2015; Lind *et al*., 2018). One of these individuals died 5 months into the investigation and exhibited shed-skin corticosterone levels that were higher than levels exhibited by any other snake over the course of the entire study. Although this is intriguing evidence that shed-skin corticosterone may reflect snake health, this was the only snake from which shed skin was collected that became clinically ill during this investigation, so additional research is necessary to confirm this link.

Additionally, although all members of the population were considered to be subclinically infected with *C. serpentis* based on previous positive tests, only one snake had a positive PCR result from biopsies obtained at the beginning of the study. It is unclear if the negative PCR results from the other

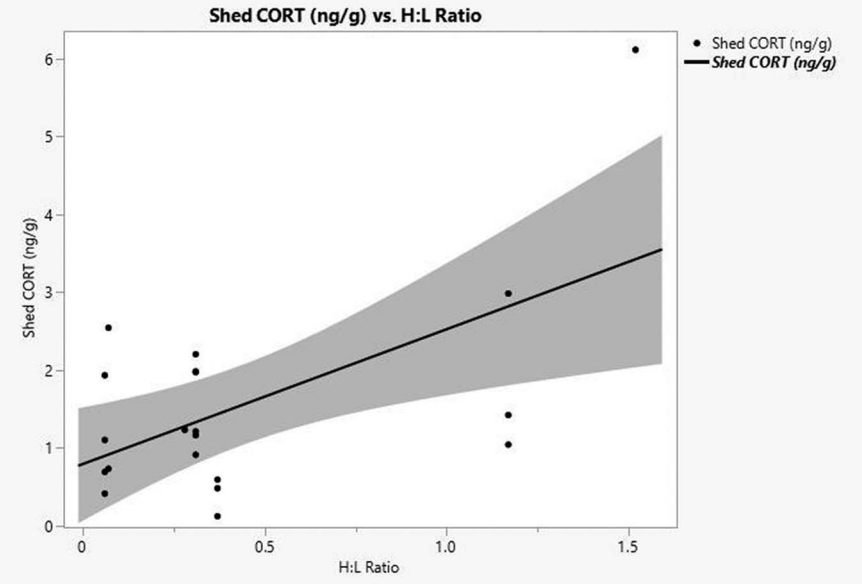

Figure 6. Heterophil-to-lymphocyte (H:L) ratio was positively correlated with shed skin corticosterone (CORT) levels in captive Louisiana pine snakes (*Pituophis ruthveni*), with a slope of 1.73 ng/g per H:L unit ($P = 0.007$). The shading represents confidence intervals as calculated from the linear regression determining the line of fit.

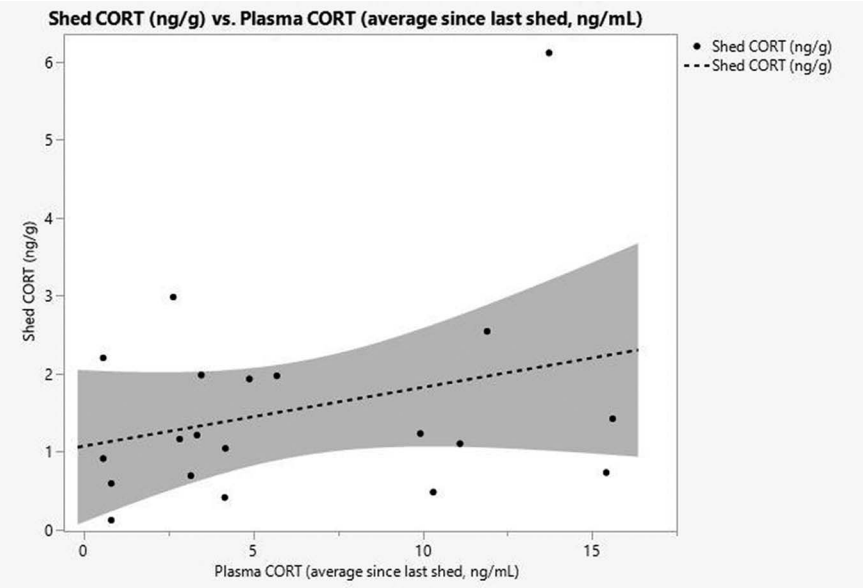

Figure 7. Shed-skin corticosterone (CORT) levels correlated to plasma corticosterone levels of blood from the same individual Louisiana pine snake (*Pituophis ruthveni*) collected most recently prior to shedding ($r = 0.29$, $P = 0.21$). The shading represents confidence intervals as calculated from the linear regression determining the line of fit.

seven individuals indicate that the infection had cleared in these snakes or indicate that *C. serpentis* was simply not causing active disease in those snakes at the time biopsies were obtained. Additionally, as only one gastric biopsy was collected in snakes that did not exhibit gross abnormalities during endoscopic examination, it is possible these may have yielded false-negative PCR results because of insufficient sampling, representing a further limitation of this study. Future investigations with a larger population and defined cohorts of healthy and ill individuals should be pursued to determine what correlations may exist between plasma and shed-skin corticosterone and overall health, and to help define the utility of plasma and shed-skin corticosterone levels. We also did not test this population for *Cryptosporidium varanii* during the course of this investigation; all had previously tested positive for *C. serpentis* and negative for *C. varanii* through PCR performed on fecal samples and gastric swabs. Both *C. serpentis* and *C. varanii* have been documented in Louisiana pine snakes and present with similar site predilections as found in other snake species (Xiao *et al*., 2004; Bogan, 2019). Gastric biopsies are not the preferred sample type for detection of *C. varanii*, as this parasite is enterotropic, not gastrotropic

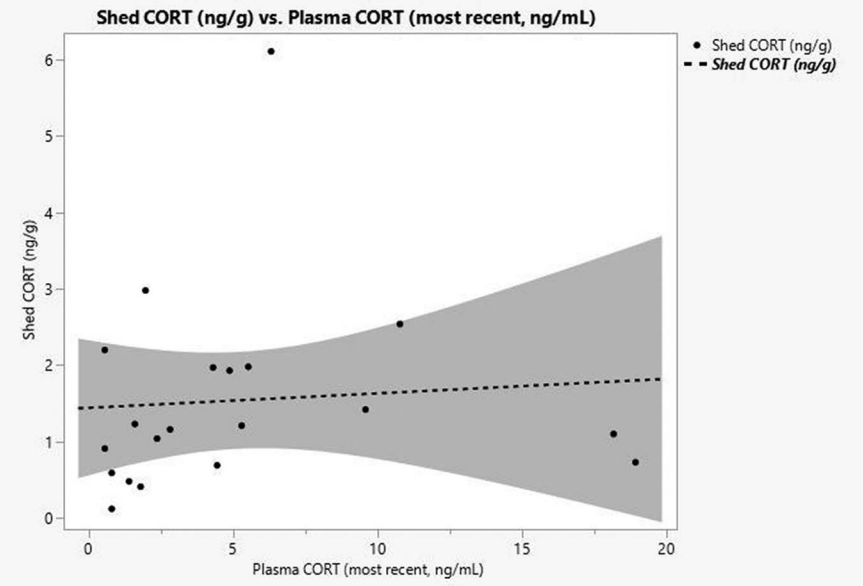

Figure 8. Shed-skin corticosterone (CORT) levels correlated to average of plasma corticosterone levels from the same individual Louisiana pine snake (*Pituophis ruthveni*) collected since the last shed ($r = 0.08$, $P = 0.74$). The shading represents confidence intervals as calculated from the linear regression determining the line of fit.

(Bogan, 2019). We only performed qPCR for *C. serpentis* in this investigation; however, it is possible that some snakes may have been subclinical carriers for *C. varanii*, which could have influenced measured corticosterone levels.

The extraction technique and commercial enzyme immunoassay used in this investigation was successful for quantification of corticosterone in the shed skin of Louisiana pine snakes. This technique could represent a method for routine measurement of long-term corticosterone levels in this species without the need for invasive sampling; however, these results are preliminary and further validation needs to be performed to ensure that corticosterone levels are representative of physiologic stress. For example, shed-skin corticosterone levels quantified via EIA can be compared with those measured utilizing other techniques such as high-performance liquid chromatography (HPLC). Additionally, biological and physiological validation should be performed by comparing shed-skin corticosterone levels before and after applying known stressors and pharmacological hypothalamic-pituitary-adrenal axis regulators (such as injections of exogenous adrenocorticotropin hormone; Cyr and Romero, 2008).

Using shed-skin corticosterone to assess stress exposure in snakes is appealing because samples can be collected in a noninvasive way and because of the longer temporal window of keratinized tissues compared to plasma (Sheriff *et al*., 2011; Berkvens *et al*., 2013; Palme, 2019; Gormally and Romero, 2020). However, the results of our study, as well as previous work, indicate that corticosterone measured in shed skin may not be a reliable means of monitoring stress in snakes (Berkvens *et al*., 2013). Our study suggests that on the extreme end, shed-skin corticosterone may reflect snake health, as one of the snakes that died during the study had much higher shed-skin corticosterone than was found in any other sample. However, the findings of this study are preliminary, and this result needs to be confirmed in future studies using a larger sample size. The ability to assess corticosterone from the shed skin of threatened and endangered snake species reliably would be a valuable tool in conservation efforts and in the management of captive breeding populations, and further research into this subject should be pursued. Studies investigating the physiologic stress response and its relationship to reproduction and overall health in various reptile species have highlighted the complex nature of these relationships, and further species-specific work is needed to improve our understanding and be able to apply this knowledge to conservation efforts, population management, and veterinary medicine (Moore *et al*., 1991, 2001; Romero and Wikelski, 2001; Preest *et al*., 2005; Hunt *et al*., 2012; Anderson *et al*., 2014; de Assis *et al*., 2015; Dayger and Lutterschmidt, 2017; Flower *et al*., 2018; Lind *et al*., 2018).

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