Plasma corticosterone mediates metabolic rate in Cottonmouth snakes

By

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ABSTRACT

The use of glucocorticoid hormones has become a powerful technique to assess the physiological and behavioral responses to stressful environmental changes. Glucocorticoids are metabolic hormones secreted by the hypothalamus–pituitary–adrenal axis that trigger an increase in glucose metabolism and available energy to cope with a stressor. Stressors originate from environmental, physiological, and behavioral pressures and produce immediate and delayed hormone responses that influence an individual's metabolic response. The objective of this study was to test the relationship between metabolism and corticosterone (CORT; primary glucocorticoid in reptiles) by comparing baseline and stress-induced changes in CORT with standard metabolic rates in Cottonmouth snakes. I assessed the relationship between stress and metabolism by relating mass-adjusted metabolic rates and residuals from a linear model relating CORT to environmental variables that could potentially influence the stress response. Covariates influencing CORT varied between the baseline and elevated models, with the baseline model having more environmental factors influencing the level of plasma CORT (Julian date, sex, shedding status, and time of day) than stress-induced (body temperature). Both baseline and elevated CORT levels showed a positive relationship with individual metabolic rate. Accounting for factors that influence individual variation in the stress response can provide more information into the potential for natural selection to shape hormonal response resulting from different metabolic phenotypes.

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1. Introduction

Glucocorticoids (GCs) play an important role in managing metabolism at both the baseline level (a stable, homeostatic state) and increased acute stressed level (elevation in hormones in response to a stressor) by regulating glucose levels to meet energetic needs in vertebrates (Johnson et al., 1992; Moore and Jessop, 2003; Thomas et al., 2017). Glucocorticoids are metabolic hormones released by the hypothalamus–pituitary–adrenal axis (HPA) as part of the stress response that create a chain reaction to stabilize bodily function and maintain homeostasis (Wingfield et al., 1998; Sapolsky et al., 2000; McEwen and Wingfield, 2003; Romero, 2004; MacDougall-Shackleton et al., 2019). Stressors can originate from a variety of abiotic and biotic factors (e.g., temperature changes, food shortages, reproductive demands, social status or anthropogenic disturbances) requiring an alteration in behavior and physiology (Sapolsky et al., 2000; Romero, 2004). Stress responses can influence metabolism to provide immediate energy to overcome environmental challenges, including predator avoidance/escape efforts and increased locomotor performance (Sapolsky et al., 2000; Miles et al., 2007). Additionally, altered GC levels can ultimately leverage effects on immunity levels and reproductive physiology (DeNardo and Licht, 1993; Sapolsky et al., 2000; Saino et al., 2003; Moore and Jessop, 2003; Patterson et al., 2014).

Allostasis, or the hormonal fluctuation that initiates a physiological or behavior change to maintain physiological stability, can be described by three different levels of response (McEwan and Wingfield 2003): (1) allostatic responses to predictable stressors (e.g., circadian and circannual rhythms) which alters hormones within an anticipated range, (2) allostatic load (unpredictable stressors) leading to the adjustment of hormone levels outside the usual range, and (3) allostatic overload, where the organism is unable to cope with a stressor through the increased production of GCs despite behavioral or physiological changes to increase available

energy. Most environmental and physiological stressors are considered unpredictable and are associated with allostatic load, which requires an amplified hormonal response to mobilize glucose (energy) needed to maintain bodily functions until the stressor has passed (McEwen and Wingfield, 2003; Romero et al., 2009; McEwen and Wingfield, 2010). The organism reaches a state of allostatic overload when they are unable to acquire the energy needed to cope with the stressor resulting in a negative energy balance (McEwen and Wingfield, 2003).

The magnified hormonal response is accomplished through the permissive and stimulatory GC signaling activities on metabolism via the combined effects of glucagon, catecholamines (epinephrine and norepinephrine), and growth hormones (Fujiwara et al., 1996; Sapolsky et al., 2000; Romero, 2004). GCs can take several minutes to fully circulate through the body (stimulatory effects) while glucagon and epinephrine (adrenaline) act immediately to increase glucose levels via glycogenolysis and gluconeogenesis (permissive effects; Sapolsky et al., 2000; Munck and Náray-Fejes-Tóth, 1995). Permissive and stimulatory effects often work together in response to a stressor. Permissive effects of GCs are often associated with baseline levels occurring without a stressor present and react first to magnify the stress response when faced with a stressor (Sapolsky et al., 2000). Stimulatory effects elevate GC concentrations in response to a stressor as part of the first wave of hormonal response to increase glucose levels (Sapolsky et al., 2000). Elevated glucose compensates for energetic deficiencies associated with the stressor (e.g., Ensminger et al., 2018; Vijayan et al., 1997; Gangloff et al., 2017).

Studies evaluating the upregulation of GC production typically compare baseline plasma GC levels to stress-induced levels. Differences in these GC levels reflect different types of receptors, Type 1 (baseline) and Type 2 (stress-induced), based on the affinity of the receptor type to bind with GCs, beginning with GCs saturating Type 1 receptors before binding with Type 2 receptors (Romero, 2004; Sapolsky et al., 2000; Dupoué et al., 2014). Evaluating the GC

concentrations associated with the two receptor types (Type 1 and Type 2) can provide insight to potential fitness consequences, especially when individuals are faced with chronic stressors leading to inhibition (negative feedback) of the HPA from sustained high GC production, reducing or eliminating the ability of GCs to turn off the HPA system, and altering the ability for the HPA to respond to future stressors (Sapolsky et al., 2000; Wingfield and Romero, 2001; Romero, 2004; but also see Boonstra, 2013).

Maintaining internal physiological balances (e.g., hormonal, thermal) during predicted and unpredicted events varies among individuals as a function of exposure to varying environments and individual phenotypes (i.e., metabolic rate; McEwen and Wingfield, 2010). Glucocorticoids (metabolic hormones) regulate balance through processes associated with intermediate metabolism (e.g., gluconeogenesis, glycogenolysis) to increase available energy (Guillette et al., 1995; Mommsen et al., 1999). The increase in energy created through intermediate metabolism can be measured as increased oxygen consumption (a measure of whole-animal metabolism; Guillette et al., 1995; DuRant et al., 2008). Whole-animal metabolism has been used by previous studies to assess the energetic demands of increased GCs (e.g., Buttemer et al., 1991; Miles et al., 2007; Preest and Cree, 2008; Wack et al., 2012; Haase et al., 2016; Jimeno et al., 2017) but has generally found mixed effects across species and within vertebrate taxa.

I hypothesize there is a relationship between standard metabolic rates (SMR) and corticosterone (CORT) levels in cottonmouth snakes, *Agkistrodon piscivorous*. Cottonmouths, a common species in the Southeastern United States, demonstrate a measurable response in stress-induced CORT after capture and confinement (Graham, 2006; Herr et al., 2017). I predicted a positive relationship between CORT levels and SMR, where individuals with higher CORT levels (both baseline and stress-induced) would also have a higher metabolic phenotype. The

metabolic phenotype is based on an underlying genetic component (Maciak and Konarzewski, 2010; Busheuv et al., 2011). Assessing the relationship between standard metabolic rate and CORT response provides more insight into the complex relationship between hormonal response and energic needs of individuals when placed in an unforeseen, stressful environment.

2. Materials and Methods

2.1. Field Collection and Husbandry

I collected Cottonmouth snakes (n = 44) opportunistically from May to October 2019 from a back-water slough of the Cumberland River in Cheatham County, Tennessee. Snakes were captured using tongs and tubed to collect blood samples from the caudal tail vein. Date and time of day (24-hr clock) were recorded. Snakes that I could not be bleed or that appeared gravid were released. For each blood sample, I drew 100-200 μL of blood using a 1 mL heparinized syringe with a 25-gauge needle. I collected baseline blood samples within an average of 3.2 min ± 1.2 (range 1.47 – 8.00 min; Romero and Reed, 2005) of initial contact before snakes were placed in a cloth bag and left in a dry and shaded spot near the capture location. Confinement within a cloth bag was used to experimentally induce a stress response, permitting the measurement of elevated CORT as a function of exposure to a standardized acute stressor (Romero and Wikelski, 2002). I collected a second blood sample approximately one hour after the initial sample. Body temperature was taken at the time of each blood draw using a Schultheis thermometer inserted 2 cm into the cloaca (Jessop et al., 2016). Samples were stored in 1.5 mL microcentrifuge tubes on ice immediately following collection and were transported back to the lab within 5 hours. Whole blood was centrifuged, and plasma stored at -80°C until assayed. Snakes successfully bled were housed in individual cages in the APSU Animal Care Facility with a temperature gradient of 24-30°C provided by an under-tank heat pad across one-third of

the cage floor. Snakes were provided water *ad libitum*, a size-appropriate hide box, and were maintained on a 12:12 hour photoperiod (light:dark).

2.2. CORT Assays

I used enzyme-linked immunosorbent assay (ELISA) corticosterone assay kits (900-097, Enzo Life Sciences Inc., Farmingdale, NY) to analyze the baseline and elevated (stress-induced) plasma samples. Plasma samples were thawed on ice and brought to room temperature before aliquoting 20 µL plasma into microcentrifuge tubes. A Steroid Displacement Reagent (SDR) was prepared to manufacture's specifications in a 1:100 solution of 10 mL of reagent to 990 mL of deionized water. SDR (20 µL) was added to each microcentrifuge tube and incubated for 10 minutes at room temperature before adding 560 µL assay buffer to create a 30-fold dilution.

Standards and diluted plasma samples were pipetted into 96-well microplates before adding conjugate and antibody solutions. Plates were covered and incubated at room temperature for 2 hours. Wells were rinsed three times with a buffered saline solution to remove excess reagents before adding *p*-nitrophenyl phosphate substrate and incubating for 60 min. The addition of a trisodium phosphate solution arrested the enzyme reaction, and the residual color was read by a spectrometer (Molecular Devices Optimax microplate reader) at 405 nm. I created a standard 4-parameter logistic curve using manufacture-provided stock solutions to determine plasma CORT concentrations in samples (Iacchetta et al., 2018; Klukowski, 2011). Intra- and inter- assay variations were 2.8% and 4.6% respectively.

2.3. Respirometry

Snakes were housed for seven days prior to undergoing metabolic measurements to ensure a post-absorptive digestive state. The metabolic measurement for ectotherms (standard

metabolic rate, SMR) is a measurement taken at a constant temperature when the animal is in a post-absorptive, resting state (Hulbert and Else, 2004). Because ectotherms are unable to mediate their body temperature through metabolic means used by endothermic animals (basal metabolic rate), they are physiologically reliant on the ability to access different temperatures to thermoregulate (Bogert, 1949; Angilletta, 2009). I measured metabolic rates using an open-flow respirometry system to quantify rates of oxygen consumption (VO_2; Withers, 2001; Lighton, 2008). Individuals were weighed and placed in a size-appropriate cylindrical plexiglass chambers varying in size from 1.2 L to 8.4 L. Chambers were placed in a Percival Scientific incubator programmed to maintain a constant temperature of 25°C and 12:12 hour (light:dark) photoperiod during the trials. Using a Sable Systems MUX flow multiplexer, four snakes were measured sequentially for 30 minutes, each with a 15-minute baseline between chambers. Trials were run for 72 hours, resulting in 24 sampling periods per snake.

An Ametek R-1 flow controller pumped room air through a Drierite drying column and air flow was divided among five lines containing Sierra Smart Trak Mass Flow Controllers. Air flow through metabolic chambers was maintained at a constant rate between 15 to 160 mL/min based on the size of the snake (Spencer et al., 2020). Excurrent chamber air was then scrubbed of water vapor and CO_2 using Drierite and Ascarite II drying columns. Air flow passed through a mass flow meter serving as a check on the flow rate before entering the Sable Systems FC-10 oxygen analyzer. A laptop computer recorded O_2 concentration and chamber flow rates every 3 seconds. The most level 15 minutes of each 30-minute sample period was used to calculate the rate of gas exchange (Withers, 1977) using LabAnalyst (Warthog Systems, http://warthog.ucr.edu). The mean of the three lowest 15-minute measures during the trial period was considered to be the standard metabolic rate (SMR) in place of using the single lowest measurement to avoid including periods of apnea (Heatwole, 1977).

2.4. Statistical analyses

The relationship between body mass and SMR was assessed using a linear regression. The SMR estimate for each individual was adjusted to remove the allometric influence of body mass on oxygen consumption; each point (individual) was adjusted along the common allometric regression slope to the observed mean population body mass (Fig. 1; Adjusted-SMR = SMR estimate – (common slope) * (body mass – mean body mass)). I accounted for the effects of mass allometrically (i.e., mass-adjusted) as the relationship was not isometric (i.e., mass-specific). A linear regression was fit between baseline CORT and elevated CORT levels to assess the relationship between sample types. A paired t-test was used to analyze the natural log-transformed baseline and elevated CORT samples.

I used separate general linear models for the baseline and elevated (stress-induced) CORT responses to account for the potential for individual and environmental covariates to influence the sample types differently (Vitousek et al., 2019). I included in the model predictor variables determined *a priori* to be important to produce a model with the most explanatory power among individuals. Linear models included natural log-transformed CORT concentration as the response variable with the predictor variables of Julian date (Tyrrell and Cree, 1998; Romero and Remage-Healey, 2000; Romero, 2002; Palacios et al., 2012), time of day (Chan and Callard, 1972; Pancak and Taylor, 1983; Thurmond et al., 1986), cloacal temperature (Tyrrell and Cree 1998; Preet and Cree, 2008; Sykes and Klukowski, 2009; Telemeco and Addis, 2014; Jessop et al., 2016), sex (Moore and Jessop, 2003; Sykes and Klukowski, 2009; Vitousek et al., 2019)), body mass (Moore et at., 2001; Palacios et al., 2012; Vitousek et al., 2019, Francis et al., 2018), and shedding state (shedding or not; Jørgensen and Larsen, 1964; Atkinson et al., 2011). The baseline model also included length of time to collect the sample as a predictor variable (Romero and Reed, 2005). Continuous predictor variables were scaled and centered to account

for different magnitudes among variables. The GLM analysis allows for the influence of each predictor variable to be assessed after accounting for the influence of each of the other predictors. The residual for each point indicates the difference between the observed value for each individual and the predicted CORT value from the full GLM. Using the residual value standardizes each CORT value allowing for additional among-individual analyses. Residuals from the CORT and covariate models were analyzed with mass-adjusted SMR using linear regression. Analyses were performed using R (v3.6.1; R Core Team 2019).

3. Results

3.1. Metabolic Rate

I measured metabolic rates and plasma CORT for 44 cottonmouth snakes (*Agkistrodon piscivorus*). There was an even sex ratio (22 females : 22 males) and the mean body mass was 366.9 ± 274.9 g (range = 74 – 1,535 g). Mean raw standard metabolic rate was 8.6 ± 5.6 O_2 mL hr^{-1} (range = 2.2 – 26.6 O_2 mL hr^{-1}). There was a positive relationship between body mass and standard metabolic rate (Fig. 1; slope = 0.018 $F_{1,42} = 190.7$, R = 0.82, P < 0.001).

3.2. Plasma CORT

I found a difference between baseline and stress-induced (elevated) CORT levels. Mean baseline CORT was 14.8 ± 12.9 ng mL^{-1} (range = 0.8-50.7 ng mL^{-}) and mean elevated CORT level was 71.1 ± 58.2 ng mL^{-1} (range = 13.3 – 234.7 ng mL^{-}). The mean time to complete the blood draw was 3.2 ±1.2 min (range = 1.5-8.0 min), and the elevated blood sample was collected a mean of 64.9 min ± 4.5 (range = 59 – 79 min) after the baseline sample. There was considerable individual variation within each sample type, and a positive relationship between the observed CORT values in the different sample types (Fig. 2; $F_{1,41} = 16.5$, R = 0.28, p <

0.001). There was a difference between natural log-transformed baseline CORT level and natural log-transformed elevated CORT level (Fig 3.; t= -11.57, df = 43, $p < 0.001$).

3.3. Generalized Linear Models

The baseline global model showed that covariates had a significant effect on baseline CORT (Table 1; baseline $R^2 = 0.39$; $F_{7,36} = 3.26$, $p = 0.01$) including: Julian date (Fig. 4A; $\beta = -0.34 \pm 0.15$, $p = 0.03$), sex (Fig. 4C; $\beta = -0.66 \pm 0.31$, $p = 0.04$), shedding status (Fig 4D; $\beta = -1.03 \pm 0.50$, $p = 0.04$), time of day (Fig 4B; $\beta = 0.32 \pm 0.15$, $p = 0.05$), body mass ($\beta = 0.23 \pm 0.15$, $p = 0.11$), blood draw length ($\beta = -0.21 \pm 0.15$, $p = 0.17$), and body temperature ($\beta = -0.11 \pm 0.16$, $p = 0.48$). Covariates did not have a significant effect on elevated CORT samples in the global model (elevated $R^2 = 0.24$; $F_{6,37} = 1.92$, $p = 0.10$). The only covariate to have an influential effect was body temperature (Table 1, Fig. 4E; $\beta = 0.27 \pm 0.12$, $p = 0.03$). The other covariates did not appear influential in the elevated model (Table 1): time of day ($\beta = 0.21 \pm 0.12$, $p = 0.08$), sex ($\beta = 0.20 \pm 0.24$, $p = 0.41$), Julian date ($\beta = -0.04 \pm 0.13$, $p = 0.78$), shedding status ($\beta = -0.09 \pm 0.38$, $p = 0.81$), and mass ($\beta = -0.01 \pm 0.13$, $p = 0.92$).

3.4. Adjusted SMR and CORT Residuals

Both baseline and stress-induced CORT concentrations increased with mass-adjusted SMR. There was a positive relationship between baseline CORT residuals and mass-adjusted SMR (Fig. 5A; $F_{1,42} = 3.93$, $R^2 = 0.09$, $p = 0.05$) and between elevated CORT residuals and mass-adjusted SMR (Fig 5B; $F_{1,42} = 4.49$, $R^2 = 0.10$, $p = 0.04$).

4. Discussion

My results support the hypothesis that there is a relationship between circulating plasma CORT concentration and standard metabolic rate in Cottonmouth snakes. Model residuals for baseline CORT were correlated with mass-adjusted SMR, suggesting individuals with lower circulating levels of GCs also had lower-energy metabolic phenotypes (after accounting for environmental covariates). A relationship between lower CORT values and lower metabolic rate would indicate a lower level of circulating GC needed to maintain a homeostatic state for coping with minor changes in the environment via glycogenolysis and gluconeogenesis (McEwen and Wingfield, 2003). The 'CORT-fitness hypothesis' (Bonier et al., 2009) predicts that high baseline CORT levels should have a negative effect on physiological parameters associated with the fitness of individuals ultimately impairing the health and condition of the population (e.g., thermoregulation, energetic demand, reproductive success). The negative relationship between baseline CORT and physiological parameters that results in decreased fitness occurs when two conditions are met: (1) there is a positive relationship between baseline CORT and environmental stressors and (2) environmental stressors negatively affect fitness (Bonier et al., 2009).

Individuals with lower CORT production and a lower metabolism (relative to conspecifics) require less energy to maintain homeostasis, which could have a fitness advantage during normal environmental fluctuations (Fig. 5A; Bonier et al., 2009; Biro and Stamps, 2010). Lower metabolism can also reduce the costs of maintaining homeostasis during prolonged stressful events (Biro and Stamps, 2010; Wingfield, 2013). The advantage of lower metabolism would be evident during chronic stressors when foraging opportunities could be restricted (Biro and Stamps, 2010). In the event of inclement weather or rapid environmental changes that prevent small endothermic animals from foraging, a higher GC response resulting in a higher

metabolic rate would help meet energic demands (Biro and Stamps, 2010; Wingfield, 2013). Ectotherms exposed to environmental stressors will undergo metabolic depression (reduced SMR) to save energy stores, however a higher metabolic rate during times of food scarcities could result in the depletion of available fat stores leading to starvation (Guppy and Withers, 1999). When baseline GC levels increase to cope with a stressor, hierarchical tradeoffs are created among fitness traits (e.g., survival, reproductive effort) and the energy and resources required to overcome a stressor, ultimately impacting individual's cumulative fitness and survival (Pease and Bull, 1988; Dufty et al., 2002; Lancaster et al., 2008; Bonier et al., 2009).

The relationship between GC and metabolism can unfold one of several ways depending on behavior, life history, and physiology of the individual. The "pace-of-life" syndrome hypothesizes that covariation among individual behavior, life history, and physiology lead to a "slow" or "fast" pace-of-life strategy (Ricklefs and Wikelski, 2002). In a review by Royauté et al (2018), there was limited overall support for "pace-of-life" hypothesis, however, both metabolism and hormone levels were linked with behaviors supporting the idea that individuals with higher metabolism and hormone expression have bold personalities as part of a "fast" pace-of-life. My results showing that individuals with a higher CORT production also exhibited a higher metabolic phenotype can be partially applied to this theory providing insight into the interactions between physiology and behavior (Fig. 5).

In cottonmouths, the difference between baseline CORT and elevated CORT suggests a hormonal coping response from a homeostatic to a stress-induced state likely as a function of the HPA pathway responding to the stress-inducing confinement treatment, mobilizing GCs and elevating blood glucose (Fig. 3; Romero, 2004). Despite the differences between the baseline and elevated samples, some individuals can take a longer to demonstrate stress responses due to a negative feedback loop within the HPA axis resulting from chronic stress (Romero, 2004). Under

normal conditions, negative feedback of GCs suspend the HPA axis at the hypothalamus after a threat has passed. However, if exposed for an extended period of the time, the negative feedback loop is also initiated, inhibiting the release of GCs to future stressors (Romero, 2004). Juveniles and smaller individuals are more likely to be impacted by the negative feedback due to increased susceptibility to predation and limited resources that cause routine chronic stress, lowering GC response levels (Sapolsky et al., 1986; Barton et al., 1987; Moore et al., 2000; Eskew et al., 2009).

The factors underpinning the stress response are complex and a myriad of environmental and individual covariates influencing CORT production and response levels (Landys et al., 2006; Hau et al., 2016; Schoenle et al., 2018; Vitousek et al., 2019). Ecdysis (shedding) in cottonmouths may act as an acute stressor elevating baseline CORT production due to impaired vision causing a feeling of vulnerability to predation, or there may be a decrease in circulating CORT as seen in my results possibly due to allocating more energy to shorten the shedding process (Table 1, Fig. 4D; Brown, 1956; King and Turmo, 1997). Many vertebrate species exhibit circadian rhythms in GC production based on physiology, behavior, and environmental conditions (e.g., Chan and Callard, 1972; Pancak and Taylor, 1982; Thurmond et al., 1986). Cottonmouths primarily forage nocturnally, maintaining lower activity levels during the day, which may temporally influence baseline GC production and lower GC levels in the morning (Fig. 4B; Lillywhite et al., 2002; Lillywhite and Brischoux, 2012). Observed seasonal influences on CORT secretion found in this study can be attributed to sampling the entirety of the active season from Spring breeding until returning to hibernacula in the Fall as shown to be influential in previous research (Fig. 4A; Tyrrell and Cree, 1998; Romero and Remage-Healey, 2000; Romero, 2002; Palacios et al., 2012). Visibly gravid females were not included in this study, however the influence of sex on GC concentrations can be influenced by different factors

(reproductive condition, needs, investment, and success of the species of interest) influencing samples taken during the reproductive season (Jessop, 2001; Vitousek et al., 2018).

Accounting for the natural individual variation in GC production has allowed us to build on previous research focused on inducing changes in GC levels (typically by administering exogenous GCs) to see the effects of metabolism (e.g., DuRant et al., 2008; Meylan et al., 2010). While previous studies show that GC production influences metabolism, they did not account for variation in GC phenotypes that result from abiotic and biotic factors influencing activation, an individual's capacity to synthesize GC based on stimulation from releasing hormones (corticotropin and adrenocorticotropin), or the ability for circulating GC (once synthesized) to be effective (Wingfield, 2013; Boonstra, 2013; Breuner et al., 2013; Hau et al., 2016). It is this natural variation within and among individuals (if repeatable) that allows the potential for natural selection to occur through heredity (Lessells and Boag, 1987; Ellis et al., 2006; Wolak et al., 2012; Hau et al., 2016).

The relationship between GCs and metabolism are recognizably complex and have led to some inconsistencies in disentangling origins and mechanisms (e.g., stimulation from a stressor, energetic demand to maintain homeostasis) of the stress response process. The traditional rational is the HPA axis releases GCs to increase blood glucose, one of several components that fabricates an individual's metabolic phenotype (Wingfield et al., 1998; McEwen and Wingfield, 2003; Romero, 2004). The alternative viewpoint takes the perspective that metabolism drives GC levels due to the energic demand required to produce and stimulate hormones (Haase et al., 2016). More likely, both ways of thinking play a role in the response system and further conveys the lack of knowledge about the mechanisms that mobilize in a stress response.

Overall, my findings suggest a positive relationship between GCs and metabolic rate at a homeostatic and stress-induced state. Baseline and elevated CORT were influenced by different

covariates: Julian date, sex, shedding status, time of day, and body temperature. Individual variation found in CORT production was influenced by these different selective pressures and vary between sample types, possibly due to environmental or genetic differences affecting the phenotype of individuals. Future research should determine if variations in CORT production are consistent within individual. Such repeatable measures of CORT production would provide more insight into the potential for natural selection to act on variations in stress response.

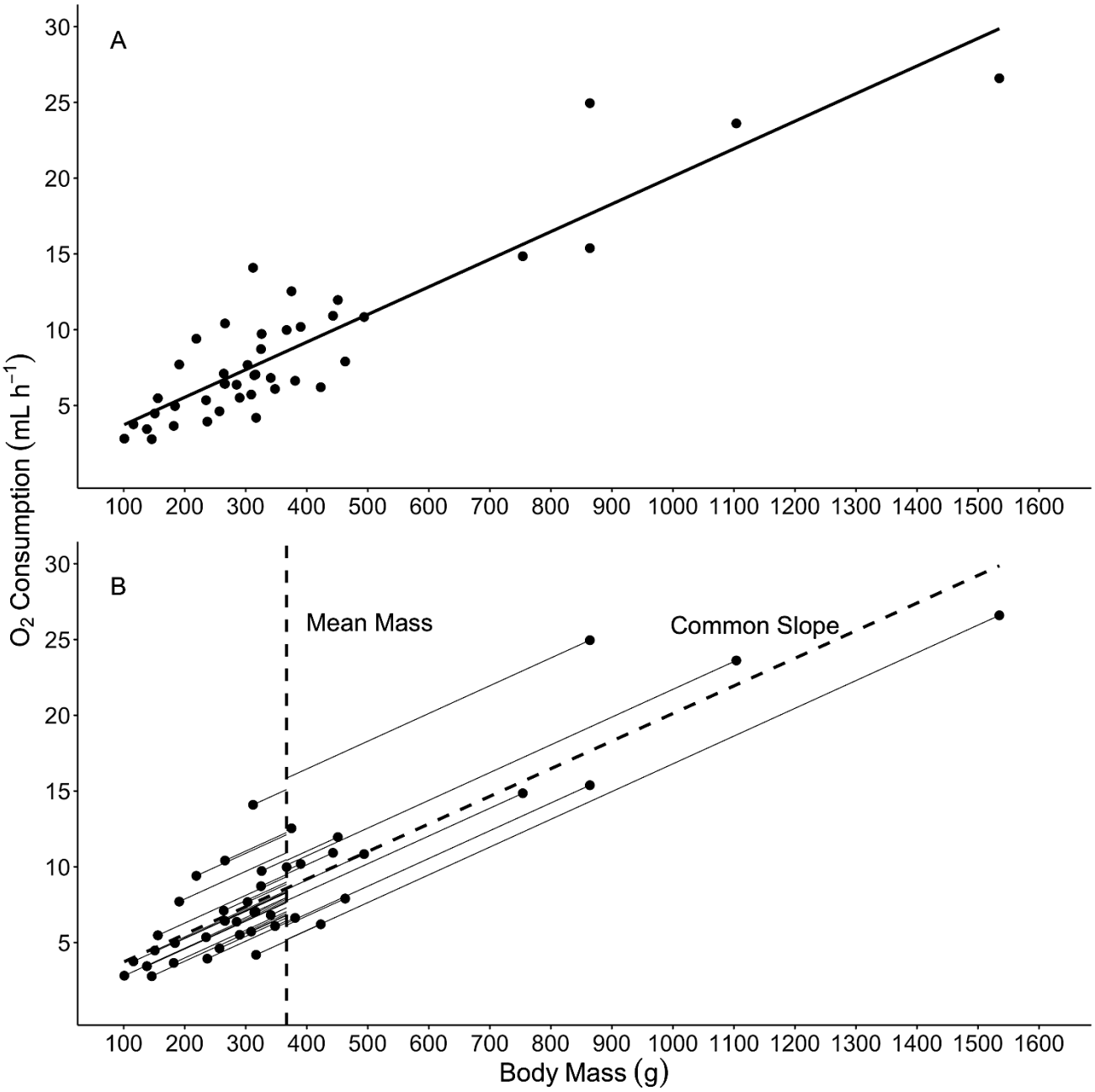

Fig. 1. Relationship between body mass (g) and estimated-SMR (mL O_2 h^{-1}).
(A) Positive allometric relationship between body mass (g) and estimated-SMR (mL O_2 h^{-1}) in 44 Cottonmouth snakes (*Agkistrodon piscivorus*) at 25°C, (B) Mass-adjusted standard metabolic rate (SMR; mL O_2 h^{-1}) was calculated by adjusting body mass using the common allometric slope (m = 0.018; fitted dashed line) and shifting each SMR value to the mean of body mass (vertical dashed line).

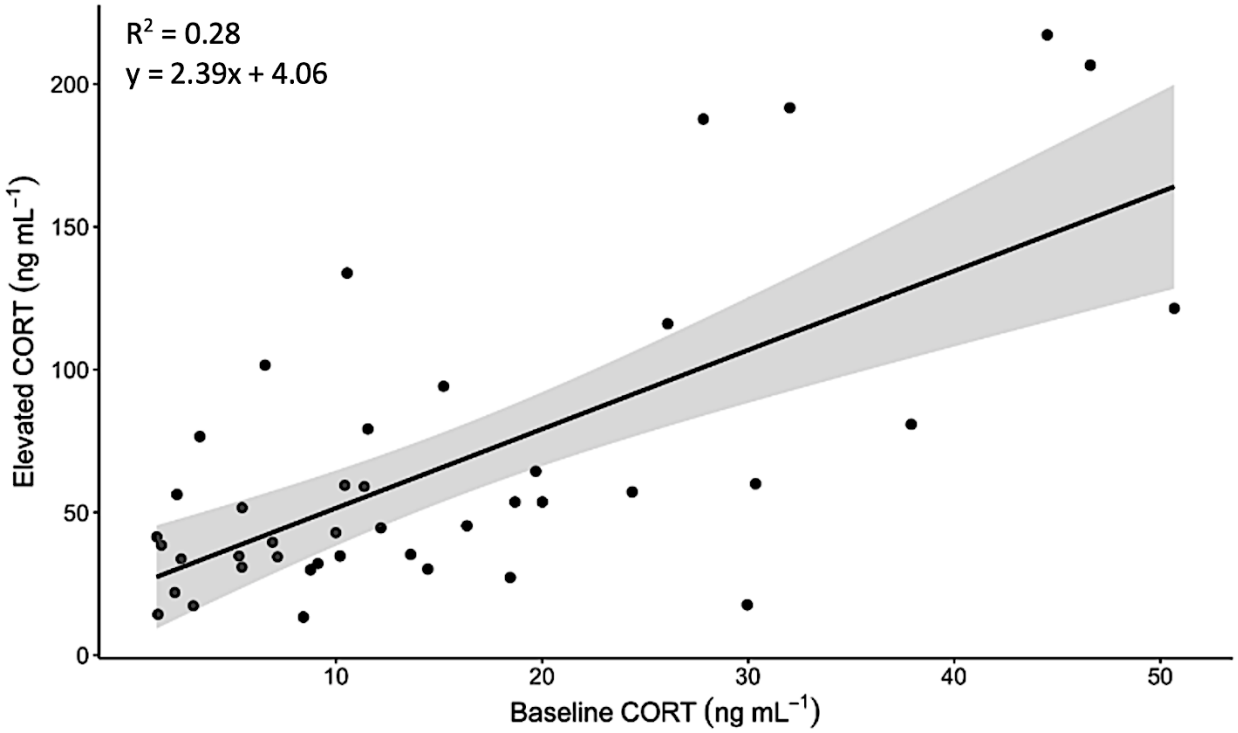

Fig. 2. Relationship between baseline and elevated (stress-induced) corticosterone. Relationship between baseline and elevated (stress-induced) corticosterone (CORT) in 44 Cottonmouth snakes (*Agkistrodon piscivorus*). Individuals with high baseline CORT levels also tended to have high elevated CORT levels ($F_{1,41} = 16.5$, $R^2 = 0.28$, $p < 0.001$, shaded region signifies 95% confidence intervals).

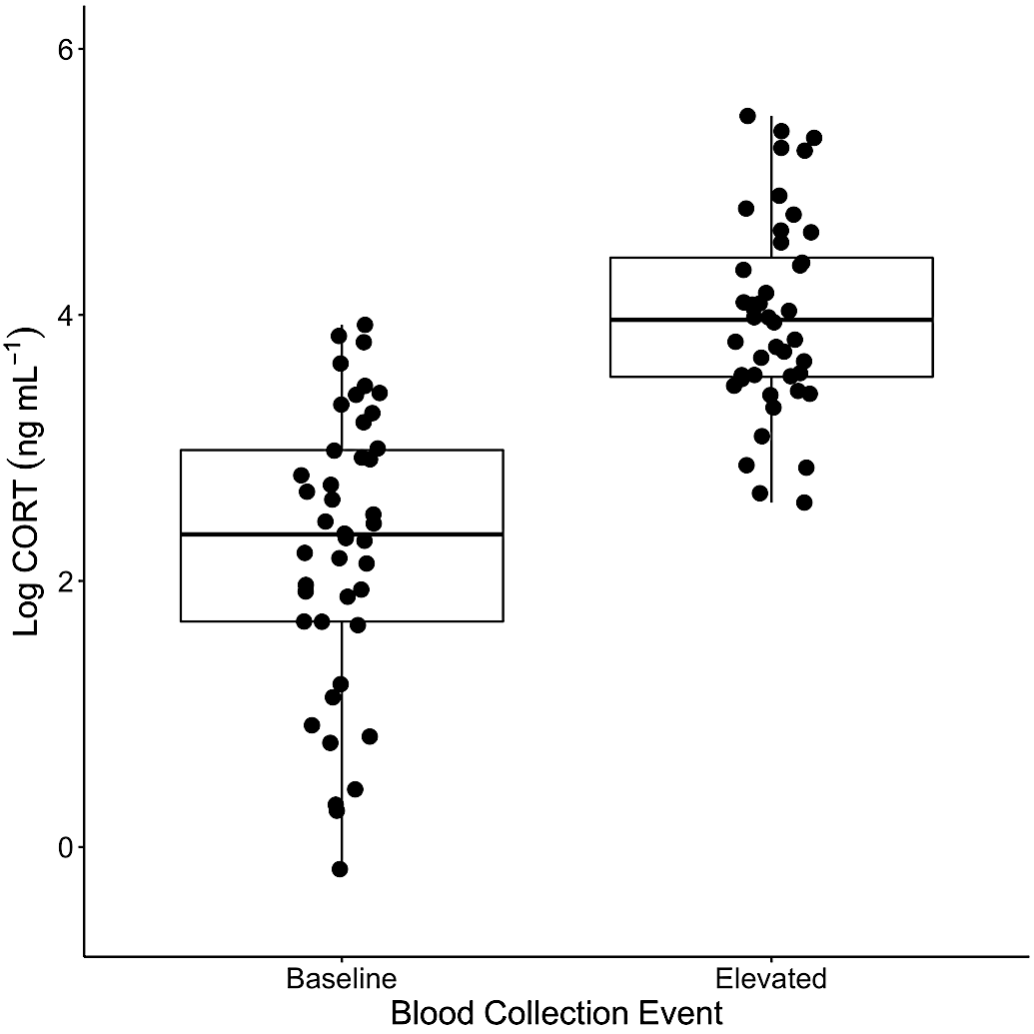

Fig. 3. Natural log corticosterone concentrations for each sample event.
Differences in natural log corticosterone (CORT) concentrations between sample collection events (baseline and elevated) for cottonmouth snakes ($t = -11.57$, $df = 43$, $p < 0.001$). Baseline measurements occurred within an average of 3.2 ±1.2 min (range = 1.5-8.0 min) of collection, while elevated (stress-induced) measurements occurred after one hour of collection.

Table 1. Corticosterone covariate coefficients and standard errors. Coefficients (β) and standard errors (SE) for each covariate included in the global linear models for baseline and elevated corticosterone levels.

Sources of Variation	β	SE	t-value	p-value
Baseline				
Date	-0.34	0.15	-2.23	0.03*
Sex	-0.66	0.31	-2.12	0.04*
Shedding State	-1.03	0.50	-2.07	0.04*
Time of Day	0.32	0.15	2.05	0.05*
Mass (g)	0.23	0.15	1.61	0.11
Blood Draw Length (min)	-0.21	0.15	-1.40	0.17
Body Temperature (°C)	-0.11	0.16	-0.71	0.48
Elevated				
Body Temperature (°C)	0.27	0.12	2.22	0.03*
Time of Day	0.21	0.12	1.80	0.08
Sex	0.20	0.24	0.83	0.41
Date	-0.04	0.13	-0.28	0.78
Shedding State	-0.09	0.38	-0.25	0.81
Mass (g)	-0.01	0.11	-0.10	0.91

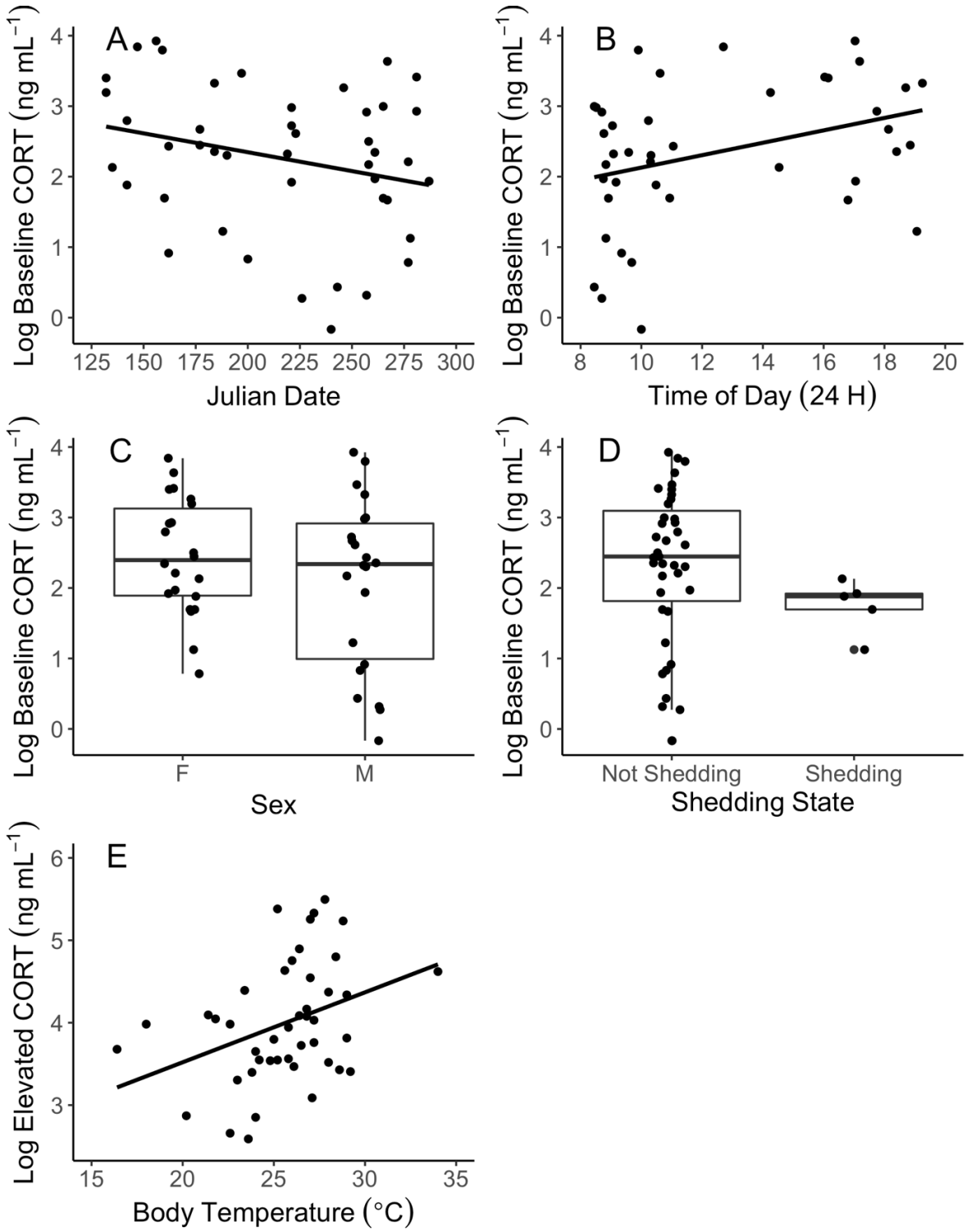

Fig. 4 Plots of significant model covariates with natural log corticosterone values. Significant model covariates plotted against natural log corticosterone (CORT) values for cottonmouth snakes; (A) Julian date (β = -0.34 ± 0.15, p = 0.03), (B) time of day (β = 0.32 ± 0.15, p = 0.05, (C) sex (M = males, F = females; β = -0.66 ± 0.31, p = 0.04), (D) shedding status (β = -1.03 ± 0.50, p = 0.04), (E) body temperature (β = 0.27 ± 0.12, p = 0.03).

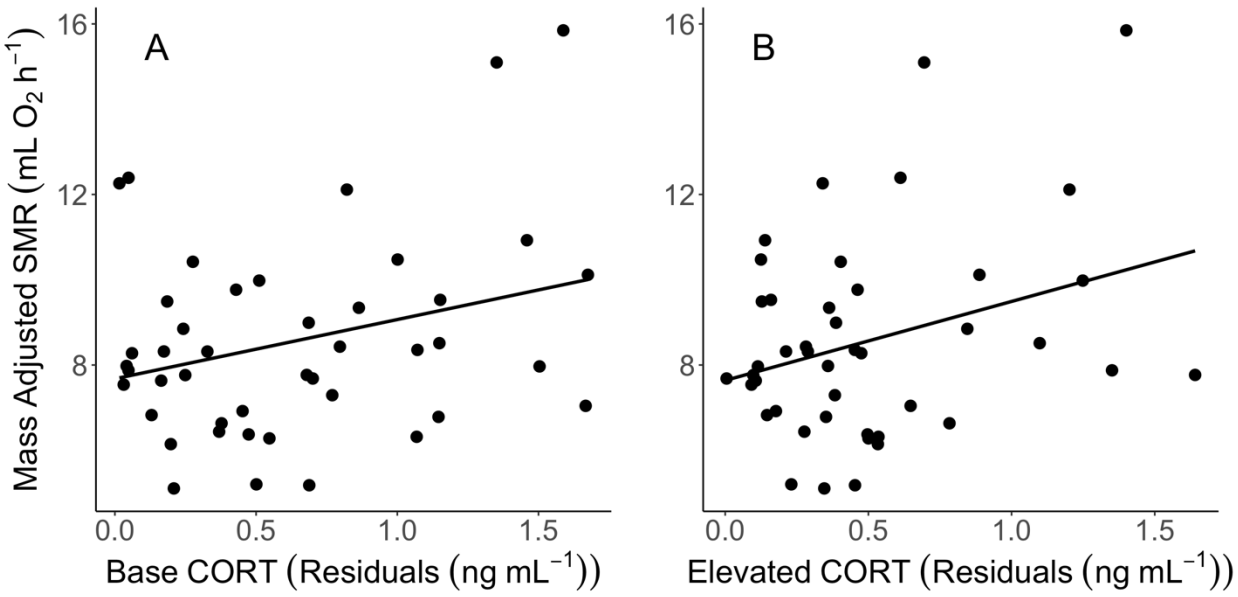

Fig. 5. Linear models between mass-adjusted standard metabolic rate and residual corticosterone values.

Linear models showing a positive relationship between mass-adjusted standard metabolic rate with (A) residual baseline corticosterone values (CORT; $R^2 = 0.09$, $p = 0.05$) and (B) residual elevated (stress-induced) CORT values ($R^2 = 0.10$, $p = 0.04$) in 44 Cottonmouth snakes *(Agkistrodon piscivorus).*

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