Stressed Snakes Strike First: Hormone Levels and Defensive Behavior in Free Ranging Cottonmouths (*Agkistrodon piscivorus*).

¹MARK W. HERR, ²SEAN P. GRAHAM, ^{1,3}TRACY LANGKILDE

¹Department of Biology, The Pennsylvania State University

²Department of Biology, Geology, and Physical Sciences, Sul Ross State University

³Intercollege Graduate Degree Program in Ecology, and The Center for Brain, Behavior and Cognition, The Pennsylvania State University

Mark W. Herr (Corresponding author)

208 Mueller Lab, The Pennsylvania State University, University Park, PA 16802 USA

Email: mwherr@gmail.com; Phone: 814 867 2252; Fax: 814 865 9131

29 October 2016

Revised manuscript for consideration in General and Comparative Endocrinology

Abstract

Stress is believed to be an important factor mediating animal behavior. Here we explore the relationship between concentrations of a stress hormone and defensive behavior of a snake. The cottonmouth (*Agkistrodon piscivorus*) is an abundant, large-bodied pitviper that is well known for its intense defensive behaviors. The defensive behaviors and hormonal ecology of cottonmouths have been studied extensively, but the interaction between these is not well understood. We conducted field trials, recording the snake's behavior and obtaining blood samples to quantify plasma CORT concentrations, both upon first encountering a snake and after a 30 minute standardized confinement stressor. We found that snakes with elevated levels of baseline CORT at first encounter were more likely to strike than exhibit a threat display when approached in the field. However, this behavior was not related to the magnitude of the snake's CORT increase following confinement, suggesting that more stress-prone snakes are not more defensive. Post-stressor antipredator behavior was also not related to any of our CORT measures. This study suggests that baseline CORT levels can be important correlates of defensive behavior. If this is a causative relationship, environmental challenges that increase baseline stress levels of populations may elevate cottonmouth defensive behavior. This would increase costs associated with defensive behavior (energetic, lost opportunity, etc.) and have important consequences for animal-human interactions.

Keywords: physiology, behavior, snake, defense, stress, corticosterone

Introduction

Predation is one of the most intense selective pressures acting on wildlife (Harvey and Greenwood, 1978). Prey can behaviorally mitigate the risk of predation by avoiding detection by and encounters with predators (Turner and Montgomery, 2003), for example by adopting crypsis (Cooper et al., 2008) or reducing activity when predators are active (Festa-Bianchet, 1988). Prey can also engage in behaviors that reduce their probability of being killed once they come into contact with a predator, for example by fleeing or directly confronting predators (Swaigood et al., 1999). While it may seem counterproductive for prey to confront their predators, some species are capable of seriously injuring or even killing their predators (reviewed in Mukherjee and Heithaus, 2013) due to their brute strength (Auffenberg, 1981), spines or other weaponry (Quick, 1953), or chemical defenses (Brodie III and Brodie Jr, 1990; Brugger, 1989). To reduce the costs (to both predator and prey) of physical interactions or fruitless pursuits, prey may showcase their defenses or ability to escape (Smith, 1975; Tilson and Norton, 1981).

While the paramount functional importance of antipredator behaviors is recognized, the underlying proximate mechanisms that mediate them are poorly understood. Encountering a predator is often a highly stressful event (Sheriff et al., 2009), and most vertebrates respond by activating the physiological stress response via the hypothalamic-pituitary-adrenal (HPA) axis (Wingfield and Romero, 2001). Activation of the HPA axis results in the secretion of glucocorticoid hormones, such as corticosterone (CORT), the levels of which are frequently used as a direct proxy for the amount of stress an animal is experiencing (Bonier et al., 2009).

CORT elevation has been implicated in the modulation of processes that promote immediate survival in the face of a threat, including antipredator behavior (reviewed in Sapolsky et al., 2000).

Research examining the relationship between CORT and antipredator behavior has, however, produced contradictory results, showing that this relationship may vary with species and context. CORT can facilitate antipredator behavior, as evidenced by fence lizards (*Sceloporus undulatus*) that typically display aversive learning and modify their antipredator escape behavior (increase flight initiation distance, decrease hiding duration) after repeated predator encounters; but fail to display such learning when CORT responses are chemically inhibited (Thaker et al., 2010). Similarly, tree lizards (*Urosaurus ornatus*) treated with exogenous CORT patches flee from predators sooner and display more pursuit-deterrence signals than untreated lizards (Thaker et al., 2009). CORT can suppress antipredator behavior, as evidenced by aposematically-colored newts (*Taricha granulosa*) displaying their brightly colored ventral surfaces for less time if they had previously received doses of exogenous CORT (Neuman-Lee et al., 2015). And CORT may have no effect on some antipredator behavior, as suggested by exogenous CORT having no impact on locomotor activity of salamanders (*Plethodon shermani*), a proxy for antipredator behavior (Wack et al., 2013). The effect of CORT on antipredator behavior has also been shown to differ between populations of fence lizards (*S. undulatus*), depending on whether they were collected at sites either with or without invasive predatory ants (Trompeter and Langkilde, 2011).

Several important questions therefore remain. For example, CORT levels typically begin to rise only several minutes after an initial predator encounter (Romero and Reed, 2005), but such encounters themselves can be over in a matter of seconds. How, then, does CORT influence antipredator behavior? The initial response to a threat is believed to be mediated directly by rapid (within seconds) secretion of epinephrine and norepinephrine (Wingfield et al., 1998). Baseline circulating levels of CORT, present before encountering a stressor, can prime this “fight or flight” threat response (Sapolsky et al., 2000). The priming effects of CORT are widely accepted, however, research examining this functional role of CORT on antipredator behavior in nature is rare (but see Langkilde et al., 2011; Thaker et al., 2009). This is particularly striking as 1) interactions with predators are an archetypal stressor used in discussions of stress biology (e.g. Sapolsky et al., 2000) and 2) discussion of the vertebrate stress response revolves around its evolutionary implications in nature and thus field studies on free living animals are critical to test hypotheses derived from lab studies.

The cottonmouth (*Agkistrodon piscivorus*) serves as an excellent model organism for the examination of these questions. Cottonmouths are venomous, large bodied, semi-aquatic pitvipers endemic to the southeastern United States (Ernst and Ernst, 2003). Across their range, these snakes are notorious for displaying aggressive antipredator behavior towards humans (although their reputation is exaggerated; Gibbons et al., 2002). For this reason, and because they display a suite of quantifiable antipredator behaviors, cottonmouth defensive behavior has been studied extensively (Glaudas and Gibbons, 2005; Glaudas and Winne, 2007; Glaudas et al., 2006; Hoss and Clark, 2014; Roth and Johnson, 2004). Concurrent with the behavioral research,

the endocrinology of cottonmouths has also been subject to recent extensive study (Bailey et al., 2009; Graham et al., 2011; Graham et al., 2008; Hoss et al., 2014). While there has been substantial research separately examining the antipredator behavior and field endocrinology in this species, potential connections between these two areas have remained unexplored. In this study, we examine the relationship between circulating CORT levels and antipredator behavior in cottonmouths.

Specifically, we tested 1) how antipredator behavior exhibited upon first encountering a threat is related to a) baseline CORT levels and b) the magnitude of the CORT response to a standardized stressor; 2) whether antipredator behavior following a standardized stressor is related to a) post-stressor CORT levels and b) the magnitude of the CORT response to the stressor.

Methods

Study Sites

Field work was conducted at three sites in Alabama, USA: Conecuh National Forest, Covington Co. and Escambia Co.; Piney Woods Creek, Crenshaw Co., and Tuskegee National Forest, Macon Co. Samples were collected over an 8 week period from mid-May to mid-July 2013.

Cottonmouths are semi-aquatic, and we therefore focused our searching in and around wetlands such as creeks, ponds, beaver marshes, and cypress swamps. The three field sites

were all similar in that they included a diversity of wetland habitats that were sampled for cottonmouths.

Behavioral Assays and Blood Sample

We measured CORT levels and antipredator intensity of cottonmouths (n=32) both during an initial encounter and following 30 minutes of captivity stress (timeline of sampling protocol: Fig. 1). We conducted visual searches of appropriate habitat as we either waded through wetlands or walked along the water's edge. Once a cottonmouth was located, we immediately initiated our behavioral assay. All snakes were visible to the observer without manipulating habitat, and therefore vulnerable to predatory interactions. To ensure we captured the full suite of antipredator behavior, we conducted both passive and contact observations. The observer approached and stood 1 meter from the snake for 15 seconds and recorded any antipredator behaviors that the snake displayed over this period. The observer then grasped the snake at mid-body using tongs for a further 15 seconds and recorded any behaviors that the snake displayed while being restrained. If the snake began to flee from the observer during the passive observation phase (before being grasped with the tongs) then that phase was considered over; the snake was grasped with tongs to prevent escape and observed for 15 seconds while restrained. The snake was then secured in a clear plastic restraining tube and an initial blood sample was collected from the caudal sinus. We collected at least 500 ul of blood (range: 500-1000ul) using heparinized, 26 gauge tuberculin syringes. The total time from initial encounter until completion of blood collection was recorded (bleed time, average = 4 minutes; range = 2 - 7 minutes) and included as a covariate in analyses. Omitting all samples with a bleed

time longer than 5 minutes (within which plasma CORT levels are reflective of baseline in Crotalids, Taylor et al., 2004) or 3 minutes (the traditional cutoff for vertebrates, Romero and Reed, 2005) did not qualitatively change our results.

After the initial behavioral assay and blood sample, the snake was placed in a 5 gallon plastic bucket to induce capture stress (Holding et al., 2014). The snake was held in the bucket until 30 minutes had passed from first seeing the snake (Fig. 1), at which time the snake was removed from the bucket and the second “post-stressor” behavior assay was conducted. This assay mirrored the first: the snake was placed on the ground 1 meter away from the observer and observed for 15 seconds at which time the observer grasped the snake mid-body with snake tongs for a further 15 seconds; all behaviors were recorded. After the post-stressor behavioral assay was completed, a post-stressor blood sample was collected in the same way as the first. The snake was then measured, weighed, sexed, and released at the point of capture. Sampled snakes (n=32) had an average snout-vent-length of 61.5 cm (range: 29.5-99cm), an average mass of 318.6 g (range: 30-1100g), and were comprised of 9 juveniles, 13 adult females, and 10 adult males (Table 2). Adults (n=23) and juveniles (n=9) were differentiated on the basis of size, with snakes with an SVL of under 50 cm being considered to be juveniles. All snakes received unique scale clippings before release in order to ensure that each snake was sampled only once.

We scored behavior using an intensity scale for this species adapted from Glaudas (2004). Behavior is listed here in order of increasing intensity. Snakes that failed to respond and

remained in crypsis during the behavioral assays were scored as “no response”. Snakes that fled during the behavioral assays but engaged in no other antipredator behaviors were scored as “flee”. Snakes that exhibited tongue flicks, gaping (snakes exposing the white lining of the inside of their mouths), and tail vibration were categorized as exhibiting “threat behavior” (as per Glaudas, 2004; Table 1). Snakes that struck at or bit the tongs were scored as “striking”. Snakes that engaged in multiple categories of behaviors received the score of the most intense behavior that they displayed (e.g., cottonmouths that exhibited both threat behavior and struck were scored as striking only).

Hormonal Assays

We stored all blood samples on ice in the field for a maximum of 8 hours (Taylor and Schuett, 2004). Samples were returned to our field lab and centrifuged, and plasma was drawn off and immediately frozen (-20 C) until assays were performed.

Plasma CORT concentrations were measured using commercially available enzyme immunoassay (EIA) kits (Corticosterone High Sensitivity EIA Kits, Immunodiagnostic Systems Ltd., Fountain Hills, AZ, USA). We validated these kits for use in *A. piscivorus* by assessing parallelism and quantitative recovery. Plasma was initially diluted 1:8.3 with assay buffer so that samples fell within the detectable range of the assay’s standard curve. A previous study determined average CORT recovery for these kits ranged between 100% and 106% (Trompeter and Langkilde, 2011). Each sample was run in duplicate using instructions provided with the kits. Coefficients of variation were calculated from the values of control samples provided in the

kits run in quadruplicate on 2 plates: the mean intraassay coefficient of variation within the two kits was 2.03% (range = 2.01–2.06%), and the interassay coefficient of variation between the two kits was 0.03%.

Statistical Analysis

To capture the maximal behavioral repertoire of the snakes, we combined the observations from the passive and the contact observations. During initial observations, only one snake failed to respond and only one snake fled without threatening or striking; no snakes exhibited these behavioral responses during the second, post-stressor behavioral assay. We therefore omitted these two behavioral categories from analyses, though retaining them did not qualitatively alter our results. In addition to the 2 animals that failed to threaten or strike, we were unable to obtain bleed time data for 4 animals so these were not included in analyses including bleed time as a covariate (2 juveniles and 2 adult females; $n = 28$). Snake mass and time to obtain a blood sample were log transformed to meet assumptions of normality. We calculated CORT reactivity as post-stressor CORT minus baseline CORT. Dividing this value by baseline CORT concentrations to account for the increase relative to initial levels (fold increase) did not qualitatively change our results.

We tested to see whether 30 minutes of captivity was an effective stressor by comparing initial CORT concentration with CORT concentration following the captivity stressor using a repeated measures ANOVA. We tested for a relationship between CORT concentrations and antipredator behavior (strike or threat without striking) using four separate nominal logistic regressions to

test for effects of baseline CORT and CORT reactivity (post-stressor CORT minus baseline CORT) on initial antipredator behavior, and effects of post-stressor CORT and CORT reactivity on post-stressor antipredator behavior. Behavioral category (threat or strike) was the dependent variable, CORT concentrations and sex were included as independent variables, and mass was included as a covariate. Elapsed time to obtain the blood sample (bleed time) was included as a covariate for analyses involving baseline CORT concentrations (baseline CORT and CORT reactivity). Baseline CORT concentrations do not exhibit circadian rhythms in this species (Trompeter and Langkilde, 2011), and time of day was thus not included in any analyses.

We tested for the effects of sex, mass and bleed time on baseline CORT using a linear regression with baseline CORT as the dependent variable and sex, mass and bleed time as independent variables. We included interaction terms between sex, mass, and bleed time in the initial model and subsequently reanalyzed the data after removing the non-significant interaction terms. We also tested for the effect of snake body condition on baseline CORT using a linear regression with baseline CORT as the dependent variable and the residuals of a regression between log mass and log SVL as the independent variable.

We compared antipredator behavior after 30 minutes of captivity stress versus at initial encounter using an ordinal logistic regression with behavior (threat vs strike) as the dependent variable, sampling period (initial vs post-stressor) and sex as fixed factors, individual identity as a random factor to account for repeated measurements on the same individual, and mass as a covariate. CORT concentration and mass data were log-transformed to meet assumptions of

parametric tests. All statistical analyses were performed using JMP (version 7.0, SAS Institute Inc., Cary NC) with $\alpha = 0.05$.

This research was approved by the Institutional Animal Care and Use Committees at Penn State University (#35780) and Auburn University (#2011-1922), and animal collection was permitted by the respective states.

Results

Initial defensive behavior was significantly related to baseline CORT concentrations; snakes that struck had higher baseline CORT than snakes that only exhibited threat behavior without striking (baseline CORT: $\chi^2_1=5.70$, $P=0.02$; Fig. 2a). Initial defensive behavior did not significantly vary with sex (sex: $\chi^2_2=2.63$, $P=0.27$; Table 2). Initial defensive behavior did vary with mass (mass: $\chi^2_1=$, $P=0.01$), with larger snakes being more likely to strike than smaller snakes (Fig. 3a), irrespective of sex (sex * mass: $\chi^2_2=0.01$, $P=0.99$). There was no effect of sex or mass on baseline CORT concentrations (sex: $F_{2,23}=1.79$, $P=0.19$; mass: $F_1=0.17$, $P=0.68$; sex * mass: $F_{2,19}=0.48$, $P=0.63$; Table 2; Fig. 4a). The time it took to obtain a blood sample was positively related to baseline CORT concentrations (bleed time: $F_{1,23}=5.15$, $P=0.03$), irrespective of sex (bleed time * sex: $F_{2,19}=0.77$, $P=0.48$; Fig. 4b). Bleed time was also related to whether snakes threatened or struck on initial encounter (bleed time: $\chi^2_1=10.59$, $P=0.001$; Fig. 3b), irrespective of sex (bleed time * sex: $\chi^2_2=0.76$, $P=0.69$). The higher baseline CORT concentrations of these striking snakes (Fig. 2a) is not, however, explained by longer handling times, as snakes that initially struck took less, not more, time to bleed (Fig. 3b). We found no relationship between

261 snake body condition and baseline CORT levels (residuals of regression of log mass and log SVL:
 262 $\chi^2_1=0.10$, $P=0.65$).

263

264 Whether a snake exhibited threat behavior only or struck upon initial encounter was not
 265 related to CORT reactivity (change in CORT from baseline to post-stressor concentrations: χ^2_1 -
 266 $=0.22$, $P=0.64$; sex: $\chi^2_2=3.27$, $P=0.19$; mass: $\chi^2_1=3.63$, $P=0.06$; bleed time: $\chi^2_1=6.94$, $P=0.01$).

267 CORT levels were elevated following 30 minutes of exposure to a standardized captivity stressor
 268 (repeated measures ANOVA: $F_{1,24} = 13.98$, $P = 0.001$; see positive reactivity values in Fig 2b,d).

269

270 Antipredator behavior following 30 minutes of captivity stress was not related to either post-
 271 stressor CORT concentrations (post stressor CORT: $\chi^2_1<0.01$, $P=0.96$; Fig. 2b) or CORT reactivity
 272 (change in CORT from baseline to post-stressor concentrations: $\chi^2_1=0.003$, $P=0.95$; Fig. 2d).

273 Post-stressor antipredator behavior was not affected by the snake's sex or mass, or bleed time
 274 (CORT reactivity only) (post-stressor CORT: sex: $\chi^2_2=0.54$, $P=0.76$; mass: $\chi^2_1=0.74$, $P=0.39$; CORT
 275 reactivity: sex: $\chi^2_2=0.55$, $P=0.76$; mass: $\chi^2_1=0.41$, $P=0.52$; bleed time: $\chi^2_1<0.01$, $P=0.95$).

276

277 There was no significant difference in the intensity of antipredator behavior (threat vs strike)
 278 after 30 minutes of captivity stress versus at initial encounter (sampling period: $\chi^2_1<0.01$,
 279 $P>0.99$; Table 1). Two of the snakes displayed more intense antipredator behavior following the
 280 captivity stressor (threatening initially, but striking after confinement), six displayed less intense
 281 antipredator behavior (going from strike to threat), and 22 snakes did not change their behavior

(threatened both before and after the stressor, or struck both before and after the stressor).

This was not affected by the snakes sex or mass (sex: $\chi^2_1=0.84$, $P=0.66$; mass: $\chi^2_1=1.46$, $P=0.23$).

Discussion

We found that cottonmouth snakes with higher baseline corticosterone concentrations were more likely to strike during an initial encounter with a potential predator (human) than snakes with lower baseline CORT levels, which were more likely to engage in threat displays without striking. Additionally, we found that larger snakes were more likely to strike than were smaller snakes. However, these more defensive snakes did not exhibit a greater CORT response to a standardized captivity stressor. CORT levels were elevated following 30 minutes of captivity stress, but the antipredator behavior of these snakes after being released from short-term captivity was not related to either baseline or post-stressor CORT concentrations. Similarly, antipredator behavior was not more intense after being exposed to captivity stress than on first encounter.

Studies that have examined the relationship between CORT and behavior have focused on the role of elevated CORT following an acute stressor (Thaker et al., 2009), with less attention given to the potential role of baseline CORT in determining the behavioral response to an initial encounter (but see Berger et al., 2007). Mean baseline CORT for snakes in this study (Table 2), correspond to previous studies on this species (Graham et al., 2008; Bailey et al., 2009). We found that variation within baseline CORT concentrations was related to the initial antipredator behavioral response of cottonmouth snakes. There are several potential explanations for the

higher baseline CORT levels of snakes that exhibited more intense antipredator behavior (striking versus threat displays). Firstly, baseline CORT may have permissive effects on an animal's initial behavioral response to a threat, potentially by priming the defensive response of the snake (Sapolsky et al., 2000). Previous studies have shown that animals living in predator rich landscapes tend to maintain higher baseline glucocorticoid concentrations (Graham et al., 2012; Sheriff et al., 2011). These higher baselines would prime the animal for rapid, intense antipredator actions (such as striking) when the need for such behaviors outweighs the costs of maintaining high CORT. Secondly, apparent higher baseline CORT levels could instead reflect recent predator encounters or result from other general physiological stressors such as infection, injury, or malnutrition (Sapolsky et al., 2000). Upon examination, however, we found no relationship between snake body condition and baseline CORT levels. Injury, infection, or other physiological stressors might instead be driving elevated CORT levels in these snakes. Snakes in such weakened states would likely be more vulnerable to attack and thus benefit from the CORT-associated diversion of resources towards antipredator defenses. Interestingly, we found that larger snakes were more likely to strike than smaller snakes. Larger snakes may be more willing to engage in this inherently dangerous behavior, as striking requires physically contacting the predator, because they are less vulnerable to such predators than are smaller snakes.

We did not, however, find a relationship between initial behavior and the snake's CORT reactivity (how much their CORT increases from baseline in response to a stressor). This supports the notion that the CORT response is unlikely to play a direct role in mediating an

animal's initial antipredator response, and suggests that snakes that are more prone to stress (more stress reactive) are not also more defensive. The intensity of antipredator behavior is therefore not likely related to a high-reactivity-phenotype in this species (Boyce and Ellis, 2005). CORT levels and behavior were not related following 30 minutes of captivity stress, challenging a widely held view that snakes that have been stressed by humans (by being cornered, chased, etc.) are more likely to strike (Ahmed, 2008; Johnson and Main, 2016; Plowman et al., 1995). Together, these results suggest that baseline and stress reactivity of CORT serve different functions in determining behavioral responses to threats.

Studies of this sort are scarce in ectotherms such as reptiles (but see Thaker et al., 2009). This is particularly unfortunate because there are differences in energetic requirements between ectotherms and endotherms (Walsberg, 2003). In mammals, CORT has a primary function in energy mobilization, and high CORT levels have been implicated in energetic costs in reptiles as well (Cote et al., 2010). While variations in energy flux have profound impacts on the fitness of endotherms (McEwen and Wingfield, 2003), ectotherms such as reptiles are comparatively far more flexible regarding energetic homeostasis (Pough, 1980). In light of these differences, studies on the adaptive significance of CORT using ectotherm models are important as they provide a critical platform to test hypotheses derived from studies conducted on endotherms. Theoretical frameworks on the vertebrate stress response have been developed from a literature that is focused strongly on endotherm (bird and mammal) models (McEwen and Wingfield, 2003), and it is therefore imperative that we critically examine such conclusions in systems that function with very different energetic requirements (Walsberg, 2003).

348

349 Understanding the relationship between glucocorticoids and antipredator (and other)
350 behaviors is important not only for the evolutionary insights that it offers, but also in the
351 context of contemporary ecosystem change. Animals often display elevated glucocorticoid
352 concentrations in disturbed environments (Graham et al., 2012; Strasser and Heath, 2013;
353 Tennessen et al., 2014; Wingfield, 2013). Our results therefore suggest that snakes inhabiting a
354 compromised ecosystem (and exhibiting associated higher average baseline CORT levels) may
355 be more likely to strike during initial predator encounters rather than relying on deterrent
356 threat signaling. In addition to the fitness costs associated with unnecessarily elevating
357 antipredator behavior (Persons et al., 2002), the results of this study could have important
358 implications with regards to human-wildlife conflict and global public health. Globally,
359 snakebite is a major public health problem in the developing world, estimated to result in
360 between 25,000 to 125,000 deaths annually (Gutierrez et al., 2013) and as many as 400,000
361 amputations each year (Williams et al., 2010), particularly within rural, agricultural settings
362 where permanent injury can result in greater economic and social costs (Kasturiratne et al.,
363 2008). If human encounters with snakes within disturbed, high stress environments are more
364 likely to result in striking and associated envenomation, steps to mitigate environmental
365 stressors could potentially reduce the burden of snakebite.

366

367 By contrast, there is some evidence that animals within disturbed environments have lower
368 baseline CORT concentrations, possibly due to selection to avoid costs of elevated CORT (e.g.
369 copperheads (*Agkistrodon contortrix*) on roads with high traffic, Owen et al., 2014). In the

context of our study, suppressed baseline CORT levels may result in cottonmouth snakes failing to appropriately mount antipredator behaviors. This may be of particular concern if the novel environmental threat is an invasive predator that is not deterred by threat displays due to a lack of co-evolutionary history (Sih et al., 2010).

Further research needs to determine whether the positive relationship between baseline CORT concentrations and intensity of antipredator behavior observed in the present study is indeed causative. This could be explored through the use of experimentally manipulated snakes treated with endogenous CORT or HPA blockers. Additionally, future studies should examine whether elevated baseline CORT is associated with elevated antipredator responsiveness in other species, and the potential costs of this for the affected species and the organisms with which they interact, including humans. To this end, future studies should explore the possibility of greater snake-bite incidence within disturbed, high-stress environments and the potential causative role of environmental stress. These findings will have important implications for understanding resilience of native species to ecosystem change, and predicting associated changes in species interactions and human-wildlife conflict.

Acknowledgements

The authors would like to thank the Solon Dixon Forestry Education Center for logistical support, G. McCormick and C. Thawley for assistance running CORT assays, N. Freidenfelds for assistance with the figure preparation and manuscript formatting, and R. Clark and E. Taylor for

assistance planning the project. This research was funded by a Summer Discovery Grant from the Penn State Office of Undergraduate Education (to MWH), and the National Science Foundation (IOS-1051367 to TL). Funders had no influence on the design of the study.

References

- Ahmed, S.M., 2008. Emergency treatment of a snake bite: Pearls from literature. *Journal of Emergencies, Trauma and Shock* 1, 97-105.
- Auffenberg, W., 1981. *The Behavioral Ecology of the Komodo Monitor*. University Press of Florida.
- Bailey, F.C., Cobb, V.A., Rainwater, T.R., Worrall, T., Klukowski, M., 2009. Adrenocortical effects of human encounters on free-ranging cottonmouths (*Agkistrodon piscivorus*). *Journal of Herpetology* 43, 260-266.
- Berger, S., Wikelski, M., Romero, L.M., Kalko, E.K., Rodl, T., 2007. Behavioral and physiological adjustments to new predators in an endemic island species, the Galapagos marine iguana. *Hormones and Behavior* 52, 653-663.
- Bonier, F., Martin, P.R., Moore, I.T., Wingfield, J.C., 2009. Do baseline glucocorticoids predict fitness? *Trends in Ecology & Evolution* 24, 634-642.
- Boyce, W.T., Ellis, B.J., 2005. Biological sensitivity to context: I. An evolutionary–developmental theory of the origins and functions of stress reactivity. *Development and Psychopathology* 17, 271-301.

- 414 Brodie III, E.D., Brodie Jr, E.D., 1990. Tetrodotoxin resistance in garter snakes: an evolutionary
415 response of predators to dangerous prey. *Evolution* 44, 651-659.
- 416 Brugger, K.E., 1989. Red-tailed hawk dies with coral snake in talons. *Copeia* 1989, 508-510.
- 417 Cooper, W.E., Caldwell, J.P., Vitt, L.J., 2008. Effective crypsis and its maintenance by immobility
418 in *Craugastor* frogs. *Copeia* 2008, 527-532.
- 419 Cote, J., Clobert, J., Poloni, L.M., Haussy, C., Meylan, S., 2010. Food deprivation modifies
420 corticosterone-dependent behavioural shifts in the common lizard. *General and*
421 *Comparative Endocrinology* 166, 142-151.
- 422 Ernst, C., Ernst, E., 2003. Snakes of the United States and Canada. Washington, DC: Smithsonian
423 Books.
- 424 Festa-Bianchet, M., 1988. Seasonal range selection in bighorn sheep: conflicts between forage
425 quality, forage quantity, and predator avoidance. *Oecologia* 75, 580-586.
- 426 Gibbons, J.W., Dorcas, M.E., Guyer, C., 2002. Defensive behavior of cottonmouths (*Agkistrodon*
427 *piscivorus*) toward humans. *Copeia* 2002, 195-198.
- 428 Glaudas, X., 2004. Do cottonmouths (*Agkistrodon piscivorus*) habituate to human
429 confrontations? *Southeastern Naturalist* 3, 129-138.
- 430 Glaudas, X., Gibbons, J.W., 2005. Do thermal cues influence the defensive strike of
431 cottonmouths (*Agkistrodon piscivorus*)? *Amphibia Reptilia* 26, 264-267.
- 432 Glaudas, X., Winne, C.T., 2007. Do warning displays predict striking behavior in a viperid snake,
433 the cottonmouth (*Agkistrodon piscivorus*)? *Canadian Journal of Zoology* 85, 574-578.
- 434 Glaudas, X., Winne, C.T., Fedewa, L.A., 2006. Ontogeny of anti-predator behavioral habituation
435 in cottonmouths (*Agkistrodon piscivorus*). *Ethology* 112, 608-615.

- 436 Graham, S.P., Earley, R.L., Guyer, C., Mendonca, M.T., 2011. Innate immune performance and
437 steroid hormone profiles of pregnant versus nonpregnant cottonmouth snakes
438 (*Agkistrodon piscivorus*). General and Comparative Endocrinology 174, 348-353.
- 439 Graham, S.P., Earley, R.L., Hoss, S.K., Schuett, G.W., Grober, M.S., 2008. The reproductive
440 biology of male cottonmouths (*Agkistrodon piscivorus*): do plasma steroid hormones
441 predict the mating season? General and Comparative Endocrinology 159, 226-235.
- 442 Graham, S.P., Freidenfelds, N.A., McCormick, G.L., Langkilde, T., 2012. The impacts of invaders:
443 basal and acute stress glucocorticoid profiles and immune function in native lizards
444 threatened by invasive ants. General and Comparative Endocrinology 176, 400-408.
- 445 Gutierrez, J.M., Warrell, D.A., Williams, D.J., Jensen, S., Brown, N., Calvete, J.J., Harrison, R.A.,
446 Global Snakebite, I., 2013. The need for full integration of snakebite envenoming within a
447 global strategy to combat the neglected tropical diseases: the way forward. PLoS
448 Neglected Tropical Diseases 7, e2162.
- 449 Harvey, P.H., Greenwood, P.J., 1978. Anti-predator defence strategies: some evolutionary
450 problems. Behavioural Ecology, 129-151.
- 451 Holding, M.L., Frazier, J.A., Dorr, S.W., Henningsen, S.N., Moore, I.T., Taylor, E.N., 2014.
452 Physiological and behavioral effects of repeated handling and short-distance
453 translocations on free-ranging Northern Pacific rattlesnakes (*Crotalus oreganus*
454 *oreganus*). Journal of Herpetology 48, 233-239.
- 455 Hoss, S.K., Clark, R.W., 2014. Mother cottonmouths (*Agkistrodon piscivorus*) alter their
456 antipredator behavior in the presence of neonates. Ethology 120, 933-941.

- 457 Hoss, S.K., Garcia, M.J., Earley, R.L., Clark, R.W., 2014. Fine-scale hormonal patterns associated
458 with birth and maternal care in the cottonmouth (*Agkistrodon piscivorus*), a North
459 American pitviper snake. *General and Comparative Endocrinology* 208, 85-93.
- 460 Johnson, S.A., Main, M.B., 2016. Dealing with Venomous Snakes in Florida School Yards.
461 Institute of Food and Agricultural Sciences (IFAS) Extension, University of Florida.
- 462 Kasturiratne, A., Wickremasinghe, A.R., de Silva, N., Gunawardena, N.K., Pathmeswaran, A.,
463 Premaratna, R., Savioli, L., Lalloo, D.G., de Silva, H.J., 2008. The global burden of
464 snakebite: a literature analysis and modelling based on regional estimates of envenoming
465 and deaths. *PLoS Medicine* 5, e218.
- 466 Langkilde, T., Freidenfelds, N.A., Robbins, T.R., 2011. Stress and invasion: elevated levels of
467 corticosterone may facilitate survival-enhancing behavior of native lizards. *Frontiers in*
468 *Endocrinology Conference Abstract: NASCE 2011: The inaugural meeting of the North*
469 *American Society for Comparative Endocrinology*. doi:
470 10.3389/conf.fendo.2011.04.00099
- 471 McEwen, B.S., Wingfield, J.C., 2003. The concept of allostasis in biology and biomedicine.
472 *Hormones and Behavior* 43, 2-15.
- 473 Mukherjee, S., Heithaus, M.R., 2013. Dangerous prey and daring predators: A review. *Biological*
474 *Reviews of the Cambridge Philosophical Society* 88, 550-563.
- 475 Neuman-Lee, L.A., Stokes, A.N., Greenfield, S., Hopkins, G.R., Brodie, E.D., Jr., French, S.S., 2015.
476 The role of corticosterone and toxicity in the antipredator behavior of the Rough-skinned
477 Newt (*Taricha granulosa*). *General and Comparative Endocrinology* 213, 59-64.

- Owen, D.A., Carter, E.T., Holding, M.L., Islam, K., Moore, I.T., 2014. Roads are associated with a blunted stress response in a North American pit viper. *General and Comparative Endocrinology* 202, 87-92.
- Persons, M.H., Walker, S.E., Rypstra, A.L., 2002. Fitness costs and benefits of antipredator behavior mediated by chemotactile cues in the wolf spider *Pardosa milvina* (Araneae: Lycosidae). *Behavioral Ecology* 13, 386-392.
- Plowman, D.M., Reynolds, T.L., Joyce, S.M., 1995. Poisonous snakebite in Utah. *Western Journal of Medicine* 163, 547-551.
- Pough, F.H., 1980. The advantages of ectothermy for tetrapods. *American Naturalist* 115, 92-112.
- Quick, H.F., 1953. Occurrence of porcupine quills in carnivorous mammals. *Journal of Mammalogy* 34, 256-259.
- Romero, L.M., Reed, J.M., 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? *Comparative Biochemistry and Physiology A: Molecular and Integrative Physiology* 140, 73-79.
- Roth, E.D., Johnson, J.A., 2004. Size-based variation in antipredator behavior within a snake (*Agkistrodon piscivorus*) population. *Behavioral Ecology* 15, 365-370.
- Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews* 21, 55-89.
- Sheriff, M.J., Krebs, C.J., Boonstra, R., 2009. The sensitive hare: sublethal effects of predator stress on reproduction in snowshoe hares. *Journal of Animal Ecology* 78, 1249-1258.

- 500 Sheriff, M.J., Krebs, C.J., Boonstra, R., 2011. From process to pattern: how fluctuating predation
501 risk impacts the stress axis of snowshoe hares during the 10-year cycle. *Oecologia* 166,
502 593-605.
- 503 Sih, A., Bolnick, D.I., Luttbeg, B., Orrock, J.L., Peacor, S.D., Pintor, L.M., Preisser, E., Rehage, J.S.,
504 Vonesh, J.R., 2010. Predator-prey naïveté, antipredator behavior, and the ecology of
505 predator invasions. *Oikos* 119, 610-621.
- 506 Smith, S.M., 1975. Innate recognition of coral snake pattern by a possible avian predator.
507 *Science* 187, 759-760.
- 508 Strasser, E.H., Heath, J.A., 2013. Reproductive failure of a human-tolerant species, the American
509 kestrel, is associated with stress and human disturbance. *Journal of Applied Ecology* 50,
510 912-919.
- 511 Swaisgood, R.R., Owings, D.H., Rowe, M.P., 1999. Conflict and assessment in a predator–prey
512 system: ground squirrels versus rattlesnakes. *Animal Behaviour* 57, 1033-1044.
- 513 Taylor, E., Schuett, G., 2004. Effect of temperature and storage duration on the stability of
514 steroid hormones in blood samples from western diamond-backed rattlesnakes (*Crotalus*
515 *atrox*). *Herpetological Review* 35, 14-16.
- 516 Taylor, E.N., DeNardo, D.F., Jennings, D.H., 2004. Seasonal steroid hormone levels and their
517 relation to reproduction in the Western Diamond-backed Rattlesnake, *Crotalus atrox*
518 (Serpentes: Viperidae). *General and Comparative Endocrinology* 136, 328-337.
- 519 Tennessen, J.B., Parks, S.E., Langkilde, T., 2014. Traffic noise causes physiological stress and
520 impairs breeding migration behaviour in frogs. *Conservation Physiology* 2, cou032.

- 521 Thaker, M., Lima, S.L., Hews, D.K., 2009. Acute corticosterone elevation enhances antipredator
522 behaviors in male tree lizard morphs. *Hormones and Behavior* 56, 51-57.
- 523 Thaker, M., Vanak, A.T., Lima, S.L., Hews, D.K., 2010. Stress and aversive learning in a wild
524 vertebrate: the role of corticosterone in mediating escape from a novel stressor. *The*
525 *American Naturalist* 175, 50-60.
- 526 Tilson, R.L., Norton, P.M., 1981. Alarm duetting and pursuit deterrence in an African antelope.
527 *The American Naturalist* 118, 455-462.
- 528 Trompeter, W.P., Langkilde, T., 2011. Invader danger: lizards faced with novel predators exhibit
529 an altered behavioral response to stress. *Hormones and Behavior* 60, 152-158.
- 530 Turner, A.M., Montgomery, S.L., 2003. Spatial and temporal scales of predator avoidance:
531 experiments with fish and snails. *Ecology* 84, 616-622.
- 532 Wack, C.L., Ratay, M.K., Woodley, S.K., 2013. Effects of corticosterone on locomotory activity in
533 red-legged salamanders. *Herpetologica* 69, 118-126.
- 534 Walsberg, G.E., 2003. How useful is energy balance as a overall index of stress in animals?
535 *Hormones and Behavior* 43, 16-17.
- 536 Williams, D., Gutiérrez, J.M., Harrison, R., Warrell, D.A., White, J., Winkel, K.D.,
537 Gopalakrishnakone, P., on Toxinology, I.S., Group, G.S.B.I.W., 2010. The Global Snake Bite
538 Initiative: an antidote for snake bite. *The lancet* 375, 89-91.
- 539 Wingfield, J.C., 2013. Ecological processes and the ecology of stress: the impacts of abiotic
540 environmental factors. *Functional Ecology* 27, 37-44.

- 541 Wingfield, J.C., Maney, D.L., Breuner, C.W., Jacobs, J.D., Lynn, S., Ramenofsky, M., Richardson,
542 R.D., 1998. Ecological bases of hormone—behavior interactions: the “emergency life
543 history stage”. *American Zoologist* 38, 191-206.
- 544 Wingfield, J.C., Romero, L.M., 2000. Adrenocortical responses to stress and their modulation in
545 free-living vertebrates, in: McEwen, B.S. (Ed.), *Handbook of Physiology, Section 7: The*
546 *Endocrine System, Volume 4: Coping With The Environment: Neural and Endocrine*
547 *Mechanisms*, Oxford University Press, Oxford, pp. 211–236.

Table 1. The frequency of threat behaviors exhibited by juvenile, adult female, and adult male cottonmouths. Behavior tallies are shown as initial behavioral • post-stressor behavior.

Sex / Stage	Sample Size	# Exhibit Threat Behavior		
		Tongue Flick	Gape	Vibrate
Juvenile	9	7 • 8	2 • 2	2 • 1
Adult Female	13	10 • 12	6 • 3	10 • 3
Adult Male	10	7 • 10	3 • 2	4 • 3
Total	32	24 • 30	12 • 7	16 • 7

Table 2: Antipredator behavior, mass, and CORT concentrations (baseline, post-stressor, and reactivity) of juvenile, adult female, and adult male snakes. Behavior is shown as initial behavioral • post-stressor behavior. Two snakes failed to threat or strike and are not represented in the initial behavior counts.

Sex / Stage	Sample Size	# Threat (No Strike)	# Strike	Mean Mass (g)	Mean CORT Concentration (ng/ml)			Bleed time (sec)
					Baseline	Post-stressor	Reactivity	
Juvenile	9	5 • 7	3 • 2	66.67	7.76	77.74	70.04	232.14
Adult Female	13	9 • 10	4 • 3	389.62	10.78	48.12	39.70	211.00
Adult Male	10	5 • 8	4 • 2	453.00	6.76	65.83	59.07	272.00
Total	32	19 • 25	11 • 7	318.59	8.68	61.99	54.20	238.07

Figure Legends:

Figure 1: Timeline of the experiment showing timing of procedures from first encountering a snake to obtaining a post-stressor blood sample.

Figure 2: The antipredator behavior exhibited by cottonmouth snakes when first encountered in the field (left panels; n=26) and their baseline (A) and reactivity (change in CORT from baseline to post-stressor concentrations; B) plasma corticosterone (CORT) concentrations. The antipredator behavior of these snakes after being exposed to a confinement stressor (right panels) and their post-stressor (C; n=32) and reactivity (D; n=28) plasma corticosterone (CORT) concentrations. Data are mean CORT values ± 1 standard error.

Figure 3: The relationship between antipredator behavior of cottonmouth snakes when first encountered in the field and mass (A; n=30) and time to bleed (B; n=26).

Figure 4: The relationship between baseline plasma corticosterone (CORT) concentrations and mass (A) and time to bleed (B) for juvenile (square symbols, dotted line, n=9), adult female (triangle symbols, solid line, n=13), and adult male (diamond symbols, dashed line, n=10) cottonmouth snakes. Lines are best fit trend lines.

Figure 1.

Figure 2.

Figure 3.

Figure 4.