
A FIELD EVALUATION OF ADRENAL SUFFICIENCY IN A TOAD-EATING SNAKE (*HETERODON PLATIRHINOS*) WITH PROFOUND ADRENAL ENLARGEMENT

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Abstract.—Genetic resistance to toad toxins exists in many snake lineages but is not restricted to toad-eating species. In contrast, all hognose snakes (Dipsadinae, *Heterodon*) are genetically resistant, and most preferentially prey on toads. Eastern Hognose Snakes (*Heterodon platirhinos*) are toad specialists. Eastern Hognose Snakes have disproportionately large adrenal glands compared to other snakes and are known to exhibit death-feigning behavior when disturbed, which may be related to the stress physiology of the species. The secretory capability of the enlarged adrenal glands of Eastern Hognose Snakes has not been fully investigated, due in part to their cryptic nature and declining populations in many areas. Here we evaluate the adrenal response to stress in eight Eastern Hognose Snakes using a field stress test. Individuals exhibited one of three behaviors upon capture: (1) attempting to flee; (2) active defensive behavior; or (3) death-feigning. Overall change in plasma corticosterone levels (Δ CORT) did not differ significantly between these three behavioral categories, though the limits of our sample size should be considered. Although previous studies document sexual dimorphism in the size of Eastern Hognose Snake adrenal glands, baseline, post-stress, and Δ CORT did not differ significantly between the sexes. Our comparisons showed that Eastern Hognose Snakes have a high increase in CORT compared to other species in the literature. These results imply that snakes with enlarged adrenal glands are capable of secreting exceedingly high levels of the adrenal hormone corticosterone when stressed. Given the role of this hormone in glucose mobilization and ion homeostasis, this may represent an additional mechanism for physiological resistance to toad toxins.

Key Words.—corticosterone; ecophysiology; stress physiology; stress response; toad toxin

INTRODUCTION

Snakes comprise a speciose group of predators, with diets that range from broad to highly specialized. Many of the prominent morphological and physiological features of snakes are associated with prey capture and handling (e.g., venom glands, kinetic skulls, thermoreceptive pit organs), and some have arisen independently in multiple lineages (Savitzky 1980; Jackson et al. 2017). Adrenal enlargement is one such feature, having evolved in several phylogenetically independent lineages of snakes that specialize in consuming toads (Mohammadi et al. 2013).

Many snakes, including numerous taxa that do not prey on toads, have retained genetic resistance to toad toxins that arose in relatively early common ancestors (Mohammadi et al. 2016). The bufadienolide toxins (BDs) of toads function as cardiotoxic steroids, inhibiting ion homeostasis by binding to the membrane transporter Na⁺/K⁺-ATPase (NKA). Contractile cells (such as those in the heart) are strongly affected, and the interruption of ion gradients will lead to death in non-resistant species. In resistant species, mutations in a subunit of this transporter reduces the binding affinity of BDs (Ujvari et al. 2013; Mohammadi et al. 2021). The mutations that result in target-site insensitivity to BDs appear to be retained among many descendant taxa, independent of their preferred

prey, which suggests that the mutations that underlie target-site insensitivity may not carry a heavy performance cost in species that do not consume toads. This phylogenetic pattern suggests that other attributes more tightly coupled to bufophagy may impart essential physiological benefits to specialized toadeaters. In mammals, the corticosteroid aldosterone is a regulator of NKA gene expression and may act to increase such expression and restore cardiac function under pathophysiological conditions (Ikeda et al. 1991). If a larger adrenal gland has a greater capacity to secrete corticosteroids, the release of those hormones may facilitate post-meal recovery in snakes that prey heavily on toads, supplementing genetic resistance with physiological defense.

An analysis of relative adrenal gland size in several members of the colubrid subfamilies Dipsadinae and Natricinae showed that the Eastern Hognose Snake (*Heterodon platirhinos*) had a high and sexually dimorphic adrenal mass relative to snout-vent length (Mohammadi et al. 2013). The adrenal glands of vertebrates are comprised of two main cell types, interrenal cells and chromaffin cells. Interrenal cells produce the corticosteroids corticosterone (CORT) and aldosterone, which are involved in ion regulation and energy mobilization (Capaldo 2023). Chromaffin cells, the dominant cells of the mammalian adrenal medulla, produce the catecholamines epinephrine and norepinephrine, and are involved in the release of certain endorphins (Norris 2020). Histological analysis of adrenal sections from *H. platirhinos* revealed a higher ratio of interrenal cells relative to chromaffin cells, compared to adrenal sections from snake species that do not largely prey on toads (Megen Kepas et al., unpubl. data). In principle, larger adrenal glands with a higher mass of corticosteroid-producing cells should be capable of releasing more CORT, increasing the magnitude of the stress response. Studies of the adrenal physiology of *H. platirhinos* have historically been very challenging, given that this species is difficult to find in abundance, seasonal in its activity, and is experiencing population declines in much of its range (https://explorer.natureserve.org/Taxon/ELEMENT_GLOBAL.2.106140/Heterodon_platirhinos).

Heterodon platirhinos is one of four congeneric species and inhabits southeastern Canada and eastern USA. The species frequently occupies sandy beaches and pine barrens, where they prey primarily on toads (Kroll 1976). More specifically, *H. platirhinos* has shown the ability to specialize on Fowler's Toads (*Anaxyrus fowleri*) on the barrier islands of the



FIGURE 1. Death-feigning response of an Eastern Hognose Snake (*Heterodon platirhinos*) upon capture. (Photographed by Cody D. Godwin).

eastern U.S., where other amphibian prey is not available (Lieto 2018; Vanek and Burke 2020). All North American *Heterodon* exhibit some degree of death-feigning behavior upon disturbance (Fig. 1), and it has been speculated that this extreme response may be related to the stress physiology of the species (Edgren and Edgren 1955; McDonald 1974). Of the North American toadeaters, Western Hognose Snakes (*H. nasicus*) and Mexican Hognose Snakes (*H. kennerlyi*) are less limited in their range of prey, consuming other anurans, as well as lizards, turtle eggs, and small rodents, in addition to toads (Platt 1969; Durso and Mullin 2017). The fourth member of the genus, Southern Hognose Snakes (*H. simus*), also preys heavily on toads (Beane et al. 2014; Myers and Arata 1961), but it is listed as Vulnerable by the International Union for the Conservation of Nature, and Critically Imperiled in most of its native range (https://explorer.natureserve.org/Taxon/ELEMENT_GLOBAL.2.106140/Heterodon_platirhinos). It is therefore a poor candidate for even slightly invasive physiological studies. Given these considerations, we selected *H. platirhinos* as the focal species for a field evaluation of adrenal response to a stressor.

Despite limitations in *H. platirhinos* sampling, the anatomy and behavior of Eastern Hognose Snakes has been a topic of study since the mid-20th Century (Edgren and Edgren 1955; Smith and White 1955). Comparisons of adrenal mass relative to body weight by Smith and White (1955) show *H. platirhinos* as having the largest adrenal to body weight compared to the other species dissected, including *H. nasicus* and several members of the subfamily Natricinae. Subsequent studies (Spaur and Smith 1971) reinforced these findings, revealing sexual dimorphism in adrenal size, with males having larger adrenal glands,

and documenting adrenal hypertrophy in hatchlings. These observations suggest that adrenal enlargement is genetically determined rather than a plastic response to toad consumption.

Early studies focusing on death feigning in hognose snakes have suggested that the death feigning response may be endocrine mediated (Edgren and Edgren 1955; Gehlbach 1970). Neither exogenous epinephrine, acetylcholine, or norepinephrine fully initiated death feigning (Edgren and Edgren 1955), however, though these catecholamines do not represent the full complement of adrenal hormones. More recent research has identified environmental and morphometric factors associated with the onset, duration, and variability of death-feigning behavior in *Heterodon* species (Gerald 2008; Durso and Mullin 2014). Recent descriptions have also revealed death feigning to be more taxonomically ubiquitous than previously documented (Magallón et al., 2021), including species that do not have considerable adrenal enlargement.

Field stress tests have become relatively common in ecophysiology and comparative vertebrate endocrinology, and standard protocols for such tests are well established (Tylan et al. 2020). Given the indications from seminal studies on adrenal hypertrophy, we expected that *H. platirhinos* would respond to a field stress test with high levels of plasma CORT, and that absolute levels of plasma CORT would be higher relative to species without profound adrenal hypertrophy. Here, we report the stress-induced CORT response of *H. platirhinos* and compare our measured plasma CORT levels with those reported for other snake species in studies that applied comparable protocols.

MATERIALS AND METHODS

Sampling.—We sampled blood from three female and five male *H. platirhinos* (Table 1) on a barrier island off of the northeast coast of the U.S. on 20–26 April 2021. The full survey period for the species was from 15 April to 1 May 2021, but snakes were only found within the sampling dates listed above. All snakes were located within a small area near sand dunes surrounded by Common Reed (*Phragmites australis*). When we encountered a snake, we marked the capture location with a flag labeled with the ID number of the snake (Table 1). When the field stress test was complete, we returned individual snakes to their exact point of capture and recovered the flagging. We also recorded GPS coordinates at the precise location of each capture. Due to the vulnerability of this population and the ease of public access, we omit precise location information from this report, per an agreement with the New York State Office of Parks, Recreation, and Historic Preservation.

Stress test.—We captured each snake within 30 s of initial detection, with no extended pursuit. We collected blood samples via cardiocentesis with a 28-gauge needle. Given that plasma CORT will begin to rise during prolonged pursuit and handling, cardiocentesis was performed within 8 min of capture, except for one individual that required 11 min for the first bleed. After the initial blood sample was collected, we placed each snake in a breathable bag made of ripstop nylon and left it undisturbed in the shade for 60 min, prolonging the exposure to a stress stimulus. After 60 min of confinement, we removed the snake from the bag and collected a second (post-

TABLE 1. Individual data for Eastern Hognose Snakes (*Heterodon platirhinos*) collected in New York, USA, 20–26 April 2021 to determine corticosterone hormone levels. Abbreviations are DC = date of capture, Time = time of capture, Bleed = length of time to bleed the snake, SVL = snout-vent length, Tail = tail length, Pre-stress = pre-stress corticosterone hormone levels (ng/ml), and Post-stress = post-stress corticosterone hormone levels (ng/ml).

Snake ID	DC	Time	Bleed	Sex	SVL (cm)	Tail (cm)	Mass (g)	Pre-stress	Post-stress	Behavior
HN1	20 April	0901	8 min	Male	42	10.5	100	14.9	512.8	Feign
HN2	20 April	0921	4 min	Female	58	8.0	160	139.1	376.6	Feign
HN3	20 April	0921	6 min	Male	57	16.0	180	337.7	508.0	Flee
HN4	21 April	1231	7 min	Female	54	10.5	150	78.2	753.5	Flee
HN5	24 April	0920	6 min	Male	54	15.0	180	63.1	367.5	Flee
HN6	24 April	1000	4 min	Female	52	13.5	140	129.0	392.8	Flee
HN7	26 April	1000	3 min	Male	57	11.0	200	38.9	---	Defense
HN8	26 April	1129	11 min	Male	52	15.0	200	460.5	675.3	Feign

stress test) blood sample. We selected the 1-h time window because previous studies show this to be a key time interval for capturing significant stress-induced plasma CORT elevation in squamate reptiles (Cartledge and Jones 2007; Neuman-Lee et al. 2020). We were unable to obtain a post stress bleed from the individual that exhibited active defensive behavior upon capture. This individual (HN #7) appeared emaciated, and we released it without obtaining a post-stress blood sample after three unsuccessful attempts. For each snake, we recorded body mass, snout-to-vent length (SVL), tail length, and sex prior to release.

We centrifuged the whole blood using a Benchmark Scientific mini-centrifuge (Model# BSC1006-B) at maximum speed (6,600 RPM) for 10 min in the field to separate the plasma from the blood cells. After centrifugation, we withdrew the plasma from the top of the fractionated sample using a 1-mL Hamilton syringe. We stored these samples in a freezer at -20°C until transport. We then transported the plasma samples frozen on dry ice to Utah State University, where they were maintained at -20°C for approximately two weeks until the first day of the radioimmunoassay.

Behavior.—Between the time of capture and the initiation of the blood draw, we observed animals and recorded the following behaviors: (1) death-feigning; (2) fleeing, or (3) defending. We defined death-feigning as tonic immobility upon handling, fleeing as struggling to escape during handling, and defense as hood-spreading, striking, and/or hissing.

Corticosterone radioimmunoassay.—We measured plasma CORT following the protocol of French et al. (2017) by assaying plasma samples in duplicate for CORT using a competitive binding radioimmunoassay (corticosterone antibody, no. 07120016; MP Biomedicals, Santa Ana, California, USA). We extracted the hormone from each sample using 30% ethyl acetate:isooctane prior to the assay. Following the assay, we calculated individual recoveries and applied the correction to final concentrations. We ran all samples within the same assay and calculated the intra-assay coefficient of variation (CV), which was 16.4%. Given that all samples were included in the same batch, there was no inter-assay coefficient of variation to report.

Species comparisons.—Because methods for field stress tests in reptiles have become widely standardized in recent years, we were able to identify two additional studies that used a similar confinement method, duration, assay, and units for reporting plasma CORT (ng/ml) in snakes. Four additional studies using different stress test durations were also included in the literature review and their differences are noted (Table 2). Although we were able to locate multiple studies that used confinement stress, we were not able to make direct comparisons of plasma CORT concentrations, given the differences in confinement method and duration, and the differences in manufactured antibodies used in the different assays. Our comparisons of plasma CORT response to stress between species are relative. It should be noted that limitations in the equivalence of our cross-study comparisons also arose from the different uses of individuals for sampling. Lakusic et al. (2020)

TABLE 2. Comparative stress-induced corticosterone hormone levels (CORT) levels for all species reviewed. Means are rounded to the nearest whole number. Abbreviations are n = sample size, ST = stress test time (minutes), Container = type of capture container; Assay = CORT type; RIA - Radioimmunoassay, EIA - Enzyme immunoassay kit, Pre = pre-stress test CORT mean, and Post = post-stress test CORT mean. Species are Eastern Hognose Snake (*Heterodon platirhinos*; HetPla), Children's Python (*Antaresia childreni*; AntChi), Red-sided Garter Snake (*Thamnophis sirtalis parietalis*; ThSiP), Western Terrestrial Garter Snake (*Thamnophis elegans*; ThE), Dice snake (*Natrix tessellata*; NaTe), Cottonmouth (*Agkistrodon piscivorus*; AgPi), and Copperhead (*Agkistrodon contortrix*; AgCon).

Species	Reference	(n)	ST	Container	Assay	Pre (ng/ml)	Post (ng/ml)	ΔCORT	% change	Toad eating
HetPla	Current study	8	60	Cloth bag	RIA	160	510	350	218.8	Specialist
AntChi	Dupoue et al. 2014	16	60	Clear box	RIA	10	12.5	2.5	25.0	None
ThSiP	Moore et al. 2000	36	240	Cloth bag	RIA	60	175	115	191.7	Generalist
ThE	Neuman-Lee et al. 2020	30	60	Cloth bag	RIA	23	339	316	1,373.9	Generalist
NaTe	Lakusic et al. 2020	14	30	Cloth bag	RIA	20	100	80	400.0	Generalist
AgPi	Herr et al. 2017	32	30	Bucket	EIA	9	57	48	533.3	Generalist
AgCon	Owen et al. 2014	29	30	Cloth bag	RIA	7.5	40	32.5	433.3	Generalist

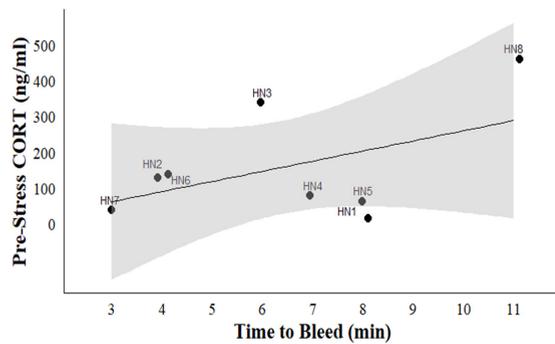


FIGURE 2. Relationship of time to bleed a snake (minutes) to pre-stress corticosterone hormone levels (CORT; ng/ml) for eight (HN numbers) wild-caught Eastern Hognose Snakes (*Heterodon platirhinos*) caught in New York, USA. The 95% confidence limits are shown in grey.

and Moore et al. (2000) used different individuals for their baseline and post-capture stress blood draws, although Lakusic et al. (2020) did obtain repeated blood draws from some individuals.

Statistical analysis.—We used R, version 4.3.3 (R Core Team 2023) for all statistical tests and graphs. We used the tidyverse suite of packages for data organization (Wickham et al. 2019), produced graphs using the package ggplot2 (Wickham et al. 2019), and used the following additional packages for formatting: ggsignif (Ahmann-Eltze and Patil 2021); ggpubr (Kassambara 2023); and patchwork (Pedersen 2024). We performed statistical tests using the base R stats package and the car package (Fox and Weisberg 2019). Data within all groupings passed the Shapiro-Wilk test for normality and were visualized using QQ plots. Levene’s test indicated homogeneity of variance between the groups being compared. We evaluated the relationship between time to bleed and plasma CORT using simple Linear Regression. We used Two-tailed *t*-tests for comparisons of CORT levels (ng/ml) between males and females, and to compare the behavioral groups we called feign and flee. Active defensive behavior was not compared to the other two behaviors due to lack of a post-stress sample. We used a Two-way Analysis of Variance (ANOVA) to analyze the effects of sex and stress condition (pre-stress vs post-stress) on the dependent variable CORT. In all tests, we set significance at $P \leq 0.05$. We used pre-stress and post-stress means from species specific literature studies to calculate Δ CORT and percentage change in CORT for each group. We calculated percentage change using the following equation: Percent Change = ([Post-Stress

CORT Mean - Pre-Stress CORT Mean] / Pre-Stress CORT Mean) $\times 100$.

RESULTS

Field capture.—Across all study dates, the time of capture was between 0901 and 1231, with most captures occurring between 0901 and 1000. The ambient temperature during capture ranged from 11.7° C to 20.8° C. Two males and one female-initiated death-feigning behavior upon capture, two females and two males attempted to flee upon capture, and one male exhibited defense upon capture. The male that displayed active defensive behavior exhibited mild hood spreading, accompanied by hissing and striking upon handling. None of the individuals secreted a substantial amount of musk or feces/uric acid from the cloacal glands upon handling. Snakes did not appear well nourished upon capture and weighed between 100–200 g (Table 1).

Comparisons.—Mean SVL for males = $52.4 \pm$ (standard error) 2.77 cm (range of values, 42–57 cm) and mean SVL for females = 54.7 ± 1.76 cm (range of values, 52–58 cm); mean tail length for males = 13.5 ± 1.14 cm (range of values, 10.5–16 cm) and mean tail length for females = 10.7 ± 1.59 cm (range of values, 8.0–13.5 cm). The mean for female CORT upon capture was $115.4 \pm$ (standard deviation [SD]) 41.28 ng/ml, and for males was 183.0 ± 200.6 ng/ml. The mean post-stress CORT for females was 507.6 ± 213.1 ng/ml, and 515.9 ± 125.8 ng/ml for males. The change in CORT values (Δ CORT) between the pre-stress and post-stress samples was 392.2 ng/ml for females and 296.9 ng/ml for males. Time to bleed and the pre-stress CORT values were not significantly related ($F_{1,6} = 1.77$, $P = 0.232$; Fig. 2). Males and females did not differ in measures of pre-stress CORT ($t = -0.730$, $df = 4$, $P = 0.503$), or post-stress CORT ($t = -0.060$, $df = 3$, $P = 0.956$; Fig. 3). There was also no significant difference in the overall change in CORT (Δ CORT) when compared between the sexes ($t = 0.599$, $df = 3$, $P = 0.591$).

There was no significant difference in CORT between male and female snakes ($F_{1,6} = 0.385$, $P = 0.549$), which is consistent with the previous comparison of means. There was a significant difference in CORT between the pre-stress and post-stress values overall (Fig. 3), indicating that the stress test resulted in a highly significant increase in plasma CORT ($F_{1,6} = 14.317$, $P = 0.004$). There also was no significant interaction between condition

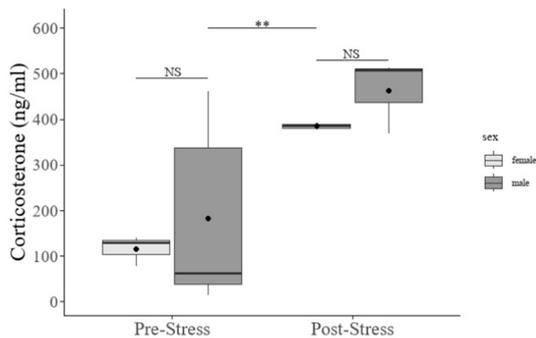


FIGURE 3. Female and male Eastern Hognose Snake (*Heterodon platirhinos*) pre-stress corticosterone hormone levels (CORT; ng/ml) taken upon capture, and post-stress test CORT after one hour of confinement. The lower and upper box limits represent the first and third quartiles, internal bar the median, internal point the mean, and vertical error bars the ± 1 standard deviation around the mean corticosterone level per group. The pre-stress and post-stress groups differ in their corticosterone levels (** $P < 0.010$). Female and male corticosterone values do not differ significantly within the pre-stress and post-stress groups (NS; $P > 0.05$).

(pre-stress vs. post-stress) and sex ($F_{1,6} = 0.279$, $P = 0.609$). In this model, behavior was not included as an independent variable. Exclusion of the individual with the pre-stress bleed time that exceeded 8 min slightly increased the significance values for stress test effect ($F_{1,6} = 17.102$, $P = 0.002$), but did not change the significance for sex, or the interaction between sex and condition.

Behavior and physiological stress.—We grouped together males and females into their behavioral categories feign and flee for behavioral comparisons, and the mean Δ CORT for flee was $353.4 \pm$ (standard deviation) 222 ng/dl, and the mean Δ CORT for feign was 316.7 ± 127 ng/dl (Fig. 4). The mean prestress

CORT (drawn upon capture) was 204.8 ± 230 ng/dl for the behavior feign and mean for the behavior flee was $152.0 \pm$ (SD) 127 ng/dl. There was no significant difference in Δ CORT ($t = -0.242$, $df = 5$, $P = 0.818$) or baseline CORT ($t = 0.393$, $df = 5$, $P = 0.710$) between the behaviors flee and feign (Fig. 4). The pre-stress CORT value for the individual that exhibited active defensive behavior was 38.9 ng/dl, which is below the means of both groups. Given the significant natural variation in CORT levels for the other two behaviors, however, comparing this single value to the group means is contraindicated.

Species literature comparisons.—We compared our findings with six existing studies on stress-induced CORT in snakes, which measured plasma CORT levels in the following species: Children’s Python (*Antaresia childreni*), Red-sided Gartersnake (*Thamnophis sirtalis parietalis*), Western Terrestrial Garter Snake (*Thamnophis elegans*), Dice Snake (*Natrix tessellata*), Cottonmouth (*Agkistrodon piscivorus*), and Eastern Copperhead (*Agkistrodon contortrix*). *Heterodon platirhinos* had the highest Δ CORT, calculated as the mean of the pre-stress values subtracted from the mean of the post-stress values (Table 2), and the highest pre-stress CORT and post-stress CORT compared to the other groups. Natricines had the second-highest Δ CORT, with both *Thamnophis* spp. showing higher Δ CORT than *N. tessellata*. Both species of *Agkistrodon* grouped together, with *A. piscivorus* having higher Δ CORT than *A. contortrix*. *Antaresia childreni* had the lowest Δ CORT, with mean CORT falling far below even the second lowest group. There was variation in the duration of stress tests, sampling protocols, and

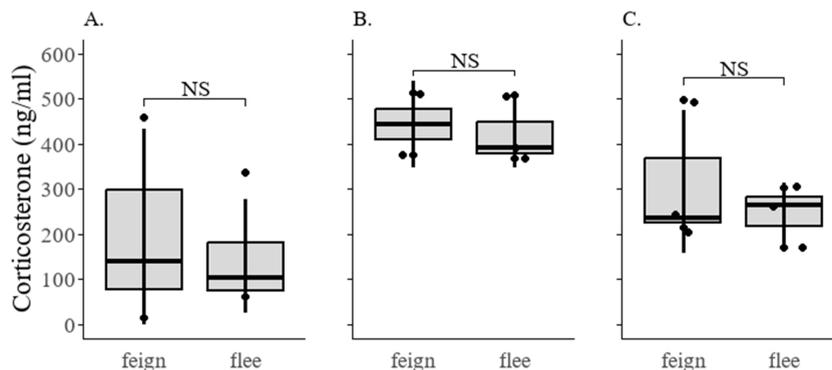


FIGURE 4. Corticosterone hormone levels (CORT; ng/ml) for the behaviors feign ($n = 3$) and flee ($n = 4$) for Eastern Hognose Snakes (*Heterodon platirhinos*) under three conditions: (A) upon capture (pre-stress), (B) following confinement (post-stress), and (C) the change in CORT between the pre-stress and post-stress measurements (Δ CORT). The lower and upper box limits represent the first and third quartiles with black dots showing values within each group, internal bar the median, and vertical error bars the ± 1 standard deviation. Corticosterone values do not differ significantly between behavioral groups (NS; $P > 0.05$).

reporting (Table 2) and should be considered when interpreting these comparisons.

The percentage change in CORT for each of the study groups varied from the overall change in the pre- and post-stress means. Our study species *H. platirhinos*, had one of the lowest percentage changes in CORT at 218.8%, despite having the highest Δ CORT (Table 2). The highest percentage change in CORT occurred in *Thamnophis elegans* (Neuman-Lee et al. 2020), with *A. contortrix* and *A. piscivorous* having the second and third highest Δ CORT (Herr et al. 2017; Lakusic et al. 2020), followed by *N. tessellata* (Lakusic et al. 2020), *H. platirhinos* (this study), *T. sirtalis parietalis* (Moore et al. 2000), and lastly *A. childreni* (Dupoue et al. 2014).

DISCUSSION

Adrenal response to stress.—Our results indicate that *H. platirhinos* responds to field stress tests with a significant increase in blood CORT levels. Although our results show a dramatic increase in CORT after 1 h of confinement, this 1-h window presumably does not capture the full adrenal response over time. Additional studies should seek to establish CORT levels at multiple sampling times (from 30 min to several hours) to determine when CORT levels peak and begin to decline. We limited the number of blood draws in the current study due to the sensitivity of this population, and our interest in following a standard 1-h protocol. It is possible that plasma CORT levels may not have fully peaked or, conversely, may already have begun to drop by the 1-h time point.

The individual with the longest initial bleed time had the highest pre-stress CORT value. There was not a significant correlation, however, between time required to bleed and pre-stress CORT when we examined all pre-stress values and time to bleed. The condition in which the animals were found shortly after emergence may have contributed to longer bleed times, including the one that exceeded our threshold. There was higher variation in individual CORT in the pre-stress samples compared to the post-stress samples. This variation is likely due to the nature of the field study, given that animals were found distributed throughout the field site with unknown but likely diverse pre-capture stressors, rather than housed in a standardized environment prior to the pre-stress test blood draw (Romero 2004).

In addition to hydration potentially affecting time to bleed, it is likely that the hydration state of the snakes in our study may have impacted the plasma

concentration of different solutes, including CORT. In rattlesnakes given supplemental hydration, plasma osmolality decreased, meaning that the particles in the plasma of snakes given supplemental water are more dilute (Capehart et al. 2016). Plasma CORT, however, was not significantly elevated in the snakes that did not receive supplemental hydration, indicating that hydration state and CORT concentration are not direct correlates. The relationship between body condition and CORT has been well documented for reptiles (Moore and Jessop 2003; Wayne and Mason 2008). In one such study, female *T. sirtalis parietalis* with lower body condition scores experienced a higher increase in CORT (Dayger et al. 2013), but this relationship was not seen in a later study (Dayger et al. 2018). Furthermore, CORT and body condition both vary at different times of the year (Zena et al. 2019).

Mohammadi et al. (2013) found that toad-eating snakes with significant adrenal enlargement, including *H. platirhinos*, also exhibit sexual dimorphism, with males having higher adrenal mass relative to SVL. In a functional context, there was no significant difference between the CORT levels of the males and females in our study. Given that our field sampling was opportunistic, males and females were unevenly sampled, with five males and three females collected and studied. This low sample size may have contributed to the lack of significance in CORT values between the sexes. Although the low sample size and uneven sampling limit our ability to project this trend onto the entire population or species, observing both sexes in their natural environment, even at low numbers, likely offers a more realistic view of baseline CORT, compared to a captive study (Sparkman et al. 2014). Sex differences in plasma CORT levels between male and female reptiles during the breeding period have been documented (Cartledge and Jones 2007), and both season and vitellogenic stage influence CORT levels in an all-female species of lizard (Hudson et al. 2019). Sex differences in CORT levels might be observed during different periods of the breeding season in *H. platirhinos*, as documented previously in garter snakes (Dayger et al. 2017). All males and females captured during this study exceeded the minimum sizes reported for sexual maturity reported at this site (Vanek 2016), indicating that all were likely reproductively active.

Elevated CORT levels over extended periods of time may suppress immune function, modulate energy expenditure, or otherwise impact longevity (Lucas and French 2012). *Heterodon platirhinos* is

known to exhibit intense death-feigning behavior when confronted by a potential threat. A possible link between death-feigning and activation of the hypothalamic-pituitary-adrenal (HPA) axis, brought on by acute stress, has been suggested (Edgren 1955; McDonald 1974; Burghardt and Greene 1988). A functional association between this behavior and increased production of corticosteroids, however, has not been demonstrated experimentally. In our study, snakes that simulated death did not have significantly different CORT levels compared to those that initiated other behaviors, although a post stress mean for the behavior called active defense could not be compared. Though the limited sample size almost certainly affected this result, it suggests that death-feigning may not be a sympathetic response that correlates strongly with acute CORT release. While death-feigning has been prominently documented in *Heterodon* over many decades (Edgren 1955), this behavior is widespread among many groups of colubrids (Magallón et al. 2021), although rarely expressed to the same degree as in *Heterodon*. The widespread occurrence of death-feigning across snake taxa suggests that adrenal enlargement and death-feigning might not be highly correlated. The ratio of adrenal size to body size has not been evaluated in many species of death-feigning snakes, nor in other taxa that do not feign death. Given that *H. platirhinos* sampling occurred opportunistically, and only eight snakes were captured during the study period, our results do not provide definitive evidence that CORT and death-feigning behavior are not related in this species.

Species comparisons.—Compared to the six other species of snakes studied using similar test protocols, the *H. platirhinos* in our study had the highest Δ CORT as a result of confinement stress. Absolute CORT values (i.e., baseline CORT and post-stress test CORT) cannot be directly compared across these studies due to differences between assays and the antibodies used therein. Therefore, our species comparisons focus on Δ CORT and percentage change in CORT between the initial blood draw and the post-stress blood draw.

Percentage change in CORT was assessed in addition to pre-stress, post-stress, and overall change. In theory, percentage change allows for comparison in scenarios where baseline concentrations differ. In studies of vertebrate stress that use a stressor, the pre-stress mean for each group indicates environmental stress prior to capture, and the post-stress mean for

each group indicates the increase in CORT brought on by the stressor. Percentage change should then reflect the magnitude of the change in CORT between the pre-stress and post-stress means within our groups. When we ranked species by percentage change in CORT rather than the absolute change in CORT, *H. platirhinos* ranked much lower due to their high average baseline CORT. Romero (2004) has argued that receptor production decreases with chronically high levels of CORT, and that CORT binds to secondary receptors when those expressed in baseline that are normally present in pre-stress conditions are saturated. As a result, comparing the percentage change in CORT levels might be misleading. In European Starlings (*Sturnus vulgaris*), chronic stress decreased the expression of glucocorticoid receptors in a region of the brain (Dickens et al 2009). This downregulation could necessitate the need for higher levels of CORT to initiate stress behaviors. Nonetheless, future studies should address glucocorticoid receptor expression in response to hormone levels in wild reptiles, and we have chosen to report both percentage change in CORT and Δ CORT with these discrepancies in mind.

Previous studies reported the time of the year when the animals were caught, except for *A. childreni*, which were sampled from a captive colony. The *A. childreni* group had the lowest pre-stress, post-stress, percentage change, and Δ CORT values. It is possible that habituation affected the pre-stress CORT values for the *A. childreni* examined in this study, but long-term captivity may in some instances also lead to prolonged elevation in CORT (de Assis et al. 2015). We do not expect that captivity would reduce the ability of the *A. childreni* group to react to a novel stressor, as captive snakes have been shown to maintain their rapid CORT responses when subjected to handling stress (Schuett et al. 2004). There is seasonal and geographical variation in the capture times and locations for the other species we compare to *H. platirhinos*: *T. sirtalis parietalis* (May; Manitoba, Canada); *T. elegans* (March, April, September, October; Cache County, Utah, USA); *N. tessellata* (July; Golem Grad Island, Macedonia); *A. piscivorous* (May-July; Alabama, USA); and *A. contortrix* (May-September; southern Indiana, USA). Large differences in latitude certainly have an effect on seasonal activity patterns (Sperry et al. 2010), however, and all species in our comparisons were observed during their active season.

Toad eating and genetic resistance to toad toxins (i.e., target-site insensitivity) are not tightly coupled

among snakes (Mohammadi et al. 2016). This differs from the reciprocal selection that has driven garter snake (*Thamnophis*) resistance to tetrodotoxin in newts (*Taricha*; Brodie et al. 2002). Genetic resistance to toad toxins is widespread, although not universal, among members of the colubrid subfamilies Natricinae and Dipsadinae. As stated earlier, *H. platirhinos* has both the highest Δ CORT and degree of toad specialization of the species compared here. The difference in Δ CORT between *H. platirhinos* and *T. elegans*, however, is quite small and may be reflective of their similarities in life history and/or adrenal morphology. The two species of *Thamnophis* (Natricinae), which are facultative toadeaters, had the second (*T. elegans*) and third (*T. sirtalis parietalis*) highest Δ CORT, followed by *Natrix tessellata*, a primarily piscivorous natricine, that has been found not to have enlarged adrenals (Mohammadi et al. 2013). In an ecological context, *N. tessellata* is highly aquatic and preys mainly on fish, to such an extent that resource partitioning with its sympatric congener *N. natrix* has been proposed (Hutinec and Mebert 2011). This distinguishes *N. tessellata* from the other natricines in these comparisons. Genetic resistance to toad toxins in vipers is quite common, and *Agkistrodon contortrix* exhibits toxin resistance (Mohammadi et al. 2016), although they are known to prey mainly on small mammals and squamates (Lagesse and Ford 1996; McKnight et al. 2014). Our other *Agkistrodon* species compared here, *A. piscivorous*, is a well-documented toadeater and is certainly presumed to have retained genetic resistance to toad toxins due to the frequency with which it feeds on them (Spencer et al. 2020). In contrast, all sampled species from the family Pythonidae lacked genetic resistance to toad toxins (Mohammadi et al. 2016). Although the python *A. childreni* was not included in the study by Mohammadi et al. (2016), we can infer that this species, which occurs outside the native range of any bufonid, is probably not resistant to toad toxins.

Our literature comparison shows that snakes with higher levels of bufophagy, and especially those with larger adrenal glands, seem to produce higher levels of plasma CORT in a standardized stress test. Importantly, the toad specialist *H. platirhinos*, which possesses an extreme degree of adrenal enlargement, responded to a stress test with the highest increase in plasma CORT measured to date. Although blood CORT levels do not appear to be tightly associated with death-feigning, our small sample size renders that determination preliminary. The extreme adrenal

enlargement seen in the toad specialist *H. platirhinos* translates to high baseline and Δ CORT when the HPA axis is activated by confinement stress, but not to the highest percentage change, given that baseline CORT is already relatively high at the time of capture. In context, our results indicate that snakes with extreme adrenal enlargement have relatively high baseline CORT and post-stress CORT values, but the degree of change from baseline to post-stress is not greatest in this species. The ability to release CORT at high concentrations may prove beneficial when ingesting poisonous toads but may incur potential costs. More importantly, our results imply, but do not conclusively demonstrate, that enlarged adrenals may be functionally associated with a greater capacity for corticosteroid secretion.

Additional studies examining the relationship between adrenal enlargement and the functional capacity of enlarged adrenal glands to release CORT and other adrenal hormones (aldosterone, catecholamines, etc.) are necessary to make any definitive correlation between adrenal hypertrophy and capacity. The snake phylogeny presents a unique framework for these functional studies based on the high degree of diversity, number of extant species, and breadth of literature surrounding adrenal morphology, diet, and stress physiology. Future studies should consider the use of an Adrenocorticotropin (ACTH) challenge to test the full capacity of the adrenal gland to secrete CORT, as it has been shown to produce a robust response in squamates (Klukowski 2011; Dayger 2016; Neuman-Lee 2020). Adrenocorticotropin is the precursor hormone to CORT, and manipulation of the hypothalamus-pituitary-adrenal (HPA) axis may help control for the impacts of the multiple environmental factors that play a role in natural CORT responses in the wild. Further investigation of the relationship between glucocorticoid receptor expression and CORT levels in wild squamates are certainly indicated. We also encourage caution when working with sensitive species and populations such as the *H. platirhinos* population sampled here. The conservation and recovery of wild hognose snakes should take precedence when considering these recommendations.

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Kepas et al.—Adrenal sufficiency of the Eastern Hognose Snake.

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