Fear-based aggression and its relationship to corticosterone responsiveness in three species of python

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ABSTRACT

It has long been known that even closely related species can vary in their antipredator behavior, and in the last two decades there has been mounting interest in how these differences might relate to the hormonal stress response. We tested the relationship between fear-based aggression, a form of antipredator behavior, and plasma corticosterone levels in three species of python [Children's Python (*Antaresia childreni*), Ball Python (*Python regius*), Bismarck Ring Python (*Bothrochilus boa*)]. We recorded the amount of striking in response to perturbation before and after a controlled, stressful confinement. We also measured plasma corticosterone levels prior to confinement, after confinement, and after confinement plus an adrenocorticotropin hormone (ACTH) injection, the later to induce a maximal corticosterone response. We performed among species analyses using two mixed models, and we determined between individual variance within each species to estimate repeatability. Bismarck Ring Pythons struck more than either Ball Pythons or Children's Pythons, and Ball Pythons had a suppressed corticosterone response compared to Children's and Bismarck Ring Pythons. Thus, mean species fear-based aggression correlated with species level differences in corticosterone profile. We also found evidence suggesting behaviors are repeatable within individuals. Our results point to a need for further exploration of aggression, anti-predator behavior, and corticosterone profile.

1. Introduction

Stable behavioral tendencies, sometimes called temperament, have long been a topic of interest to biologists (Sih et al., 2004). While such behavioral tendencies are often applied to differences among individuals within a population, they are also appropriate for comparisons among populations or species (Sih et al., 2004; Réale et al., 2007; Coutant et al., 2018). Some of the most fruitful research in this area has focused on antipredator behavior, which often exhibits stable, interspecific differences (Edmunds, 1974; Greene, 1988; Randall et al., 1995; Lingle et al., 2005; Žagar et al., 2015). These species level differences, however, are a product of variation in behavioral tendencies among individuals (Bell et al., 2009). Several behavioral components have been used to map individual differences, with a bold-shy continuum one of the most commonly applied to antipredator behavior (Réale et al., 2007). While the total number of species examined thus far is small, studies in laboratory mice (Koolhaas et al., 1999), other mammals (Réale et al., 2007), birds (Cockrem et al., 2009), and fish (Bensky et al., 2017) have supplied evidence of individual differences along the bold-shy continuum, with aggression often used as a proxy for boldness.

In vertebrates, antipredator behavior is often expressed concurrently with the hormonal stress response, with an increase in glucocorticoids as a result of stimulation of the hypothalamo-pituitary-adrenal (HPA) axis (Romero and Butler, 2007; Hawlena and Schmitz, 2010;). As individuals can also exhibit stable and distinct glucocorticoid patterns in response to a stressor, one research goal has been the development of models that link antipredator behavior with the glucocorticoid response, in particular focusing on baseline concentrations of glucocorticoid hormones and the sensitivity of the hypothalamic-pituitary adrenal (HPA) axis (Øverli et al., 2006; Koolhaas et al., 2010; Cockrem, 2013). The suite of linked behavioral and hormonal responses, often referred to as coping styles, are modeled along a proactive–reactive continuum. Although coping styles were developed to model individual differences, selection experiments have generated populations that differ in average coping style (Øverli et al., 2006), and they have been explored using species level comparisons (Pottinger, 2010; Lendvai et al., 2013).

Proactive individuals are typically characterized as having active behavioral responses with below average baseline glucocorticoid concentrations and a blunted sensitivity of their HPA axis response (Koolhaas et al., 1999). It should be noted that while aggressive behaviors (e.g., biting, charging) are often used as characterize proactive individuals, flight is often also categorized as an active response (Koolhaas et al., 2010). Reactive individuals are typically characterized as passive in their behavioral response (e.g., freezing, hiding) and have average or higher baseline concentrations of glucocorticoid and sensitive HPA axis responses (i.e., a higher glucocorticoid response to a stressor). As most research in this area has focused on endotherms (Montiglio et al, 2012; Cockrem et al., 2017), with fish (Øverli et al., 2004; Bensky et al., 2017) representing the focus of research in ectotherms, it is risky to generalize these relationships to other taxa. Only a handful of studies have explored these relationships in squamate reptiles (e.g., Claunch et al., 2017; Holding et al., 2014; Mell et al., 2016; Moore et al., 2000; Thaker et al., 2009; Trompeter and Langkilde, 2011).

Squamate reptiles display great variation in antipredator behavior, with snakes arguably displaying some of the most elaborate defenses (Johnson, 1975; Greene, 1988). This diversity, combined with the widespread presence of morphological adjuncts (e.g., frills, hoods, and rattles) or chemical deterrents (e.g., cloacal discharge, venom systems), increases the complexity of any attempt to map behavior to a single continuum of variance. Some studies have also shown squamates capable of complex responses to variable environments (Trompeter and Langkilde, 2011), and there is increasing awareness of the role of development in determining antipredator responses (Landová et al., 2013). Thus, although much work has been done on the ecological relationships driving antipredator behaviors in squamates (Greene, 1988), there have been few efforts to understand its relationship to the hormonal stress response.

In lizards, the relationship between corticosterone, the primary glucocorticoid of reptiles, and antipredator behavior remain unclear. Elevated corticosterone changes male Tree Lizard (*Urosaurus ornatus*) antipredator behavior quantitatively, but not qualitatively (Thaker et al., 2009) and enhances both moving and hiding responses in two populations of Eastern Fence Lizards (*Sceloporus undulatus*) (Trompeter and Langkilde, 2011). However, other studies have found little covariance between aggression and corticosterone concentrations (Mell et al., 2016: European Common Lizards; Rodríguez-Prieto et al., 2010: Iberian Wall Lizards). Interestingly, one study found a density-dependent sex difference in corticosterone response to a stressor (Mugabo et al., 2016: European Common Lizards).

In snakes, early studies of antipredator behavior focused on species-level analysis of correlations between behavior and habitat (Greene, 1979; Scudder and Burghardt, 1983). Using strike tests to measure aggression, several studies found significant differences even among closely related species of garter snake (Herzog and Burghardt, 1986; Herzog et al., 1989). Additionally, in Mexican Garter Snakes (*Thamnophis melanogaster*), aggression was found to be stable in individuals across the first year of life (Herzog and Burghardt, 1988). Subsequent studies have confirmed individual differences in antipredator behavior in the Common Garter Snake (Garland, 1988) and Northern Common Boa (*Boa imperator*) (Šimková et al, 2017).

Only two studies have examined the relationship between antipredator behavior and corticosterone in snakes, both using North American vipers. The tendency to strike in Cottonmouths (*Agkistrodon piscivorus*) increased with higher baseline levels of corticosterone, but striking did not increase with corticosterone in response to confinement (Herr et al. (2017). However, there was no relationship between antipredator behavior and corticosterone when Southern Pacific Rattlesnakes (*Crotalus helleri*) were treated with slow-release corticosterone implants (Claunch et al. (2017). A better understanding of the relationship between antipredator behavior and corticosterone in squamates requires further study across species with different antipredator responses.

Accordingly, we examined the relationship between antipredator behavior and the corticosterone stress response in three species of pythons. We formulated three hypotheses: 1) there are species-level differences in mean python antipredator behavioral responses, 2) there are species-level differences in mean corticosterone profiles, and 3) antipredator behavior and corticosterone profile are correlated.

To test these hypotheses, we selected three species of python that anecdotally show a range of antipredator behaviors: Ball Pythons (*Python regius*), Children's Pythons (*Antaresia childreni*), and Bismarck Ring Pythons (*Bothrochilus boa*). Ball Pythons are the most imported python in the United States in part due to their docile behavior, and, when threatened, they typically coil into a protective ball; striking behavior is uncommon. There is no published literature on antipredator behavior in either Children's Pythons nor Bismarck Ring Pythons, but neither are known to ball when threatened. However, they may differ in fear-based aggression, with handlers of Bismarck Ring Pythons frequently reporting striking behavior. Thus, these species represent an increasing gradient of fear-based aggressive striking behavior. Based on these behaviors, we predicted that Ball Pythons would have higher baseline corticosterone concentrations and a more responsive HPA axis, while Bismarck Ring Pythons would have lower baseline corticosterone concentrations and a blunted HPA response. We used a simple experimental design where we evaluated fear-based striking responses and collected blood samples for plasma corticosterone concentrations before and after controlled confinement, and then analyzed the data for species-level variation.

2. Materials and methods

2.1. Subjects and housing

We used captive colonies of pythons maintained at Arizona State University (ASU) during the fall of 2012. While captivity may influence natural behavior, this possibility did not compromise the validity of our study since we were examining the relationship between behavior and hormone levels, which is of interest whether or not captive behavior and hormone levels are different from that of free-ranging individuals. Children's Pythons are small, thin-bodied snakes that live in rocky areas of northwestern Australia; Ball Pythons are medium length, heavy-bodied snakes that live in equatorial Africa; Bismarck Ring Pythons are long, muscular snakes that live on the Bismarck Archipelago off the northeast coast of Papua New Guinea (Wilson and Swan, 2008). Phylogenetically, these species are in separate sub-groups within the Pythonidae (Reynolds et al., 2014).

Animal rooms were maintained at 27 °C under a 12:12 h photoperiod. All animals were provided supplemental heat using a subsurface heating element (Flexwatt, Flexwatt Corp.) below one end of each cage. Water was provided *ad libitum*, and animals were fed small meals 5 days prior to each hormonal trial so as to avoid the fully fasted and satiated states. At the end of the trials, snakes were weighed (± 1 g) and snout-vent length (SVL, ± 1 mm) was measured (Table 1). All procedures were approved by the ASU Institutional Animal Care and Use Committee (protocol #08-967R).

Table 1
Behavioral results from administration of strike tests to three species of python at baseline and post-stress. The increase in striking behavior in Children's Pythons (*Antaresia childreni*) was driven by a single individual that struck 24 times. All values are given as mean ± SEM.

Python Species	Baseline				Post-Stressing			
	n	Strikes (#)	Range	ICC	n	Strikes (#)	Range	ICC
P. regius	8	0	–	–	8	0	–	–
A. childreni	11	0.4 ± 0.2	0–4	95.5	8	3.0 ± 2.8	0–24	98.1
B. boa	17	5.0 ± 1.8	0–14	94.4	8	5.0 ± 2.1	0–16	70.3

2.2. Administration of strike tests

To quantify fear-based aggression in each species, as well as to obtain individual metrics of repeatability, we performed three strike tests on all animals in the colony (total n = 46 snakes; Children's Pythons = 10 males and 12 females; Ball Pythons = 6 males and 10 females; Bismarck Ring Pythons = 4 males and 4 females). Strike tests were modified from Herzog and Burghardt (1986). The snake was removed from its cage and immediately placed in a clean container, which was then closed. The snake was allowed 5 min to acclimate to the container, and then the container was reopened and the snake tapped on the head with the foam-wrapped end of a dowel (25.4 mm). The snake was tapped at a consistent rate (~1.5 taps/sec) for 1 min, and the number of strikes was recorded. If the head was inaccessible (e. g., Ball Pythons exhibiting balling behavior), then exposed parts of the body nearest the head were tapped instead. A strike was defined as an obvious, aggressive, forward movement towards the foam end of the dowel, regardless of whether it resulted in a bite.

In order to determine the relationship between antipredator behavior and the corticosterone profile, we administered the same strike tests following a modified confinement procedure (see below). As some pythons in the colony were being used for a concurrent reproductive experiment, we used a subset of individuals (total n = 24; Children's Python = 3 males and 5 females; Ball Pythons = 4 males and 4 females; Bismarck Ring Pythons = 4 males and 4 females) for this aspect of the study. As no previous data on corticosterone responsiveness exists for these species, to ensure that we induced a measurable response, we modified the capture stress method of Moore et al. (2000). In that protocol, wild caught Red-sided Garter Snakes (*Thamnophis sirtalis parietalis)* were placed into a cloth sack for 4 h. In contrast to garter snakes, however, pythons are sit-and-wait predators that often prefer enclosed spaces, which possibly limits the effectiveness of a cloth sac in stimulating a corticosterone response. By placing the snake into a cloth sack, and then placing the sac into a plastic container on a plate shaker (M6825, Barnstead/Thermodyne) set to 800 rpm, we exposed the snakes to a constant disturbance for 1 h. Immediately following this procedure, we removed the snake from the shaker and sack and placed it into a clean container, closed the lid for 5 min, and administered a post-confinement strike test. Averages for each snake were calculated from three trials, which were separated by 1–27 days.

2.3. Determination of corticosterone profile

To develop a hormonal profile of each species, the same individuals that were used for the initial behavioral tests were used again to measure corticosterone at baseline, post-confinement (see above methodology), and following confinement plus an injection of adrenocorticotropic hormone (ACTH, 300 μl at 85 IU/ml, time to inject < 10 s), the latter to determine maximum corticosterone response. A minimum of 1 week separated blood collections, and after collecting a blood sample (see description below) the python was placed back in its cage and monitored for any signs of distress over the next two days.

We quantified corticosterone concentrations in each individual by collecting blood (0.6–0.7 ml) via cardiocentesis. All blood samples were drawn within 90 ± 15 s (mean ± SEM) of initializing restraint. Blood was stored on ice until processing, which was done within 5 h of the individual's first collection. It was centrifuged at 10,000 rpm at −4 °C, and the plasma was aliquoted into 0.5 ml centrifuge tubes and stored at −20 °C until corticosterone was assayed. Total plasma concentrations of corticosterone were quantified using commercial competitive enzyme-linked immunoassay kits (ELISA; Assay Designs Inc.) as described in Fokidis et al. (2009). The assay was validated for use in pythons by demonstrating no difference between the slopes of a curve produced by serial plasma dilution (2–16 fold) and a standard curve performed in triplicate (all p ≥ 0.387). Samples were prediluted two-fold with assay buffer according to manufacturer's instructions. All samples were

assayed in duplicate, and samples from conspecifics were assayed on the same plate in a random distribution. The optical density of assay wells was measured at 405 nm with a microplate absorbance plate reader (Opsys MR, Dynex Technologies). Total plasma corticosterone concentrations were calculated via interpolation from the standard curve on the respective plate using GraphPad Prism (V4, GraphPad Software Inc.). The sensitivity of the assay calculated from two standard deviations from a zero standard, ranged from 9.1 to 26.7 pg/ml and the mean intra-assay and inter-assay coefficients of variation were 11.1% and 18.6%, respectively (n = 3 plates, 117 samples).

2.4. Data analysis

We performed all analyses in the R statistical environment (R Core Team, 2016), and for all three models we used the lme4 package (Bates et al., 2015). To analyze striking behavior, we applied a mixed model with the number of strikes modeled as a negative binomial distribution to account for any zero inflation from pythons that did not respond. The model initially included seven independent variables: the confinement treatment (fixed factor with two levels: pre-confinement, post-confinement), species (fixed factor with three levels), a species by treatment interaction term, sex (fixed factor with two levels), mass (continuous variable), time of day (fixed factor with two levels: morning, afternoon), and day of treatment (continuous variable). Individual was included as a random variable to account for repeated measurements. Using the Akaike information criterion (AIC), we removed variables (except individual) from the model until the lowest AIC value was reached.

To analyze corticosterone, we applied a linear mixed model with eight initial independent variables: confinement treatment (fixed factor with three levels: pre-confinement, post-confinement, ACTH induced maximum), species (fixed factor with three levels), time of treatment (fixed factor with two levels: morning, afternoon), a species by treatment interaction term, sex (fixed factor with two levels), mass (continuous variable), day of treatment (continuous variable), and the amount of time (s) taken to draw blood (continuous variable). We combined the baseline concentrations for each species taken during the confinement treatment and the ACTH treatment after a t-test showed no difference (Children's: t_7 = 1.32, p = 0.23; Bismarck ring: t_7 = 0.45, p = 0.66; ball: t_7 = 0.90, p = 0.40; power analysis showed test had a 68% chance of detecting a mean difference of 10 mg/mL). Individual was included as a random variable to account for repeated measurements.

To analyze the covariance between striking behavior and corticosterone concentrations, we applied a bivariate mixed model both among species and among individuals. In this model, both striking behavior and corticosterone concentration were dependent variables, confinement treatment was an independent fixed effect, and species and individual were independent random effects. We also analyzed differences in strike behavior directly by dividing the Bismarck Ring Python counts into two groups: those that had struck at least once during the trials and those that had not struck at all. We then applied a t-test to the two groups between their baseline concentrations of corticosterone and the percent arcsine transformed corticosterone response.

We partitioned the within species variance for both models (striking behavior and corticosterone concentrations) into variance between individuals and variance within individuals by calculating the intraclass correlation coefficient (ICC) in two ways. First by applying the package ICC (Wolak et al., 2012) to our behavioral model, and second through direct calculation or the raw data using the equation ICC = (variance among individuals)/(variance among individuals + variance within individuals) (Lessells and Boag, 1987). We applied this equation to the species baseline behavioral strike tests (n = 46) and the post-confinement behavioral strike tests (n = 24).

Finally, we analyzed the corticosterone response to confinement as a percentage of each individual's maximum response as a result of

ACTH treatment: % response = ([B]$_{\text{post-confinement}}$ − [B]$_{\text{pre-confinement}}$)/ ([B]$_{\text{post-ACTH}}$ − [B]$_{\text{pre-ACTH}}$) × 100. We arcsine transformed these percentages to attain a normal distribution and performed an ANOVA between species. For two Bismarck Ring Pythons that had slightly higher corticosterone responses to confinement than that resulting from ACTH injections (a difference of 7.9 ng/mL and 2.3 ng/mL), we inverted the equation to maintain the highest corticosterone concentration in the denominator. Post-hoc analyses were done using Tukey honestly significant difference (HSD) test.

3. Results

There were qualitative and quantitative differences in antipredator behavior across species (Table 1). Ball Pythons hid their head between their coils and never struck. Children's Pythons typically attempted to flee, although a few strikes were not uncommon. Initially, four out of the eleven (36.4%) struck at least once, and post-confinement one of the eight individuals (12.5%) repeatedly struck with a high frequency in each of the three trials (20, 23, and 24 times). Bismarck Ring Pythons rarely attempted to flee, instead remaining in a coiled position ready to strike during the trials. Initially, six of the eight (75%) tested struck at least once, and five of the eight (62.5%) struck post-confinement.

Our final behavioral model was: Strikes = Treatment + Species + Treatment × Species + Individual, with Individual as a random variable. The Treatment × Species interaction term allowed each species to vary in the slope of its response to the confinement treatment. The variables of sex, mass, time of day, and the day of treatment did not improve the explanatory power of the model. Within the final model, the treatment was also not significant (coefficient = 0.315, z value = 0.311, $P = 0.756$), but treatment did have a significant interaction, driven by the increased striking behavior of Children's Pythons over their baseline striking behavior (coefficient = 2.312, z value = 2.128, $P = 0.033$; Table 1). Within the model, both baseline and post-treatment striking were average between species, so that the striking behavior of Children's Pythons was not significantly different than that of Ball Pythons (coefficient = 1.403, z value = 1.024, $P = 0.306$), but that of Bismarck Ring Pythons was significantly different from both ball and Children's Pythons (coefficient = 6.081, z value = 4.004, $P < 0.001$; Table 1).

We determined corticosterone concentrations for all three species at baseline, post-confinement, and after ACTH injection plus confinement. For Ball Pythons, baseline concentrations increased from 91 ± 2 ng/mL to 100 ± 2 ng/mL after confinement. In Children's Pythons, baseline concentrations increased from 96 ± 5 ng/mL to 111 ± 2 ng/mL. For Bismarck Ring Pythons, baseline concentrations increased from 100 ± 3 ng/mL to 116 ± 3 ng/mL. Following ACTH injection, corticosterone concentrations were 123 ± 2 ng/mL for Ball Pythons, 121 ± 3 ng/mL for Children's Pythons, and 121 ± 1 ng/mL for Bismarck Ring Pythons.

Our final corticosterone model was: [B] = Treatment + Species + Treatment × Species + Individual, with Individual as a random variable. The variables of sex, mass, time of day, day of treatment, and minutes to complete blood draw did not improve the explanatory power of the model. Within the final model, all species showed a significant increase in corticosterone due to confinement treatment ($F_{2, 42} = 225$, $P < 0.001$); species was also significant ($F_{2, 21} = 3.5$, $P = 0.048$), and the interaction effect was significant ($F_{4, 42} = 9.31$, $P < 0.001$). Analyses of the interaction between treatment and species showed that it was driven by Ball Pythons, which had significantly different baseline ($t_{3,42} = -3.77$, $P < 0.001$) and post-confinement ($t_{3,42} = -5.67$, $P = 0.024$) concentrations of corticosterone than both Children's and Bismarck Ring Pythons.

The bivariate model showed that the relationship between striking behavior and corticosterone concentrations among species was highly correlated (0.965) but only moderate correlation among individuals (0.444). This correlation among individuals was greatly increased when

Ball Pythons, which did not strike, were removed from the model (0.952). The t-tests applied within Bismarck Ring Pythons between those that struck and those that did not showed no difference between baseline concentrations ($t_6 = 0.741$, $P = 0.346$) nor between the percentage of corticosterone response ($t_6 = 0.935$, $P = 0.386$) (Fig. 3).

We calculated each species' ICC based on our model and using raw data at baseline and post-confinement (Table 1). For both models, a moderate amount of variance was due to variance among individuals: ICC = 0.595 for striking behavior and 0.646 for corticosterone concentrations. For baseline calculations using raw data, no Ball Pythons struck; four of the eleven Children's Pythons struck (min = 1, max = 4); and six of the eight Bismarck Ring Pythons struck (min = 1, max = 14). This meant that we were unable to calculate the ICC for Ball Pythons, but the baseline ICC for Children's Pythons was 95.5 and for Bismarck Ring Pythons was 94.4, showing high individual repeatability in both species. Post-confinement, the ICC shifted upwards for Children's Pythons to 98.1 and downwards for Bismarck Ring Pythons to 70.3.

ANOVA analyses of baseline corticosterone concentrations among species was significant ($F_{2, 42} = 4.38$, $P = 0.018$), and post-hoc comparisons showed that Bismarck Ring Pythons had higher baseline concentrations than either Children's Pythons ($P < 0.05$) and Ball Pythons ($P < 0.05$) (Fig. 1). ANOVA analyses of the corticosterone response, as arcsine percent, among species was also significant ($F_{2, 21} = 11.42$, $P < 0.001$), and post-hoc comparisons showed that compared to Ball Pythons, both Bismarck Ring Pythons ($P < 0.05$) and Children's Pythons ($P < 0.05$) had significantly greater corticosterone responses.

4. Discussion

This is the first study to explore the relationship between antipredator behavior and the steroid corticosterone response in pythons, expanding on previous work on garter snakes (Moore et al., 2000) and vipers (Claunch et al., 2017; Herr et al., 2017). In alignment with previous studies (Johnson, 1975; Greene, 1988; Herzog and Burghardt, 1986), our results support our first hypothesis that there are species-level differences in antipredator behavior (Table 1; Fig. 3). Each species displayed a characteristic set of antipredator behaviors, with Ball Pythons remaining coiled with their head tucked beneath the coils, Children's Pythons attempting to flee and sometimes striking, and Bismarck Ring Pythons holding a steady strike position, striking frequently, and only rarely attempting to flee. The species in this study thus provided a rough continuum of fear-based aggression, with Ball Pythons as the least aggressive, Bismarck Ring Pythons as the most aggressive, and Children's Pythons in-between.

We also found evidence for individual repeatability in both behavioral and hormone models (Table 1). The intraclass correlation coefficient, the fraction of variance that can be assigned to individuals, was moderately high in both models (ICC = 0.595 for striking behavior; ICC = 0.646 for corticosterone concentrations). The behavioral values increased when we calculated ICC using raw strike numbers for Children's Pythons and Bismarck Ring Pythons, but, as no Ball Pythons struck at any time during the trials, we were unable to estimate behavioral repeatability for them. However, it is apparent that the lack of striking behavior is highly repeatable, regardless of our reluctance to perform a calculation using zeroes. The consistency in striking behavior was not affected by the length of time between trials, suggesting that no learning occurred, which is consistent with previous studies on snakes (Arnold and Bennett, 1984; Brodie and Russell, 1999; Citadini and Navas, 2013). Herzog and Burghardt (1988), for example, found a high repeatability in striking behavior in Mexican Garter Snakes (*Thamnophis melanogaster*). Interestingly, exposure to confinement decreased the ICC from 94.4 to 70.7 in Bismarck Ring Pythons (Table 1), suggesting that confinement increased the variability of individual behavior in this species. In the pre-confinement strike test, six of the eight Bismarck Ring Pythons struck at least once, but the largest difference between
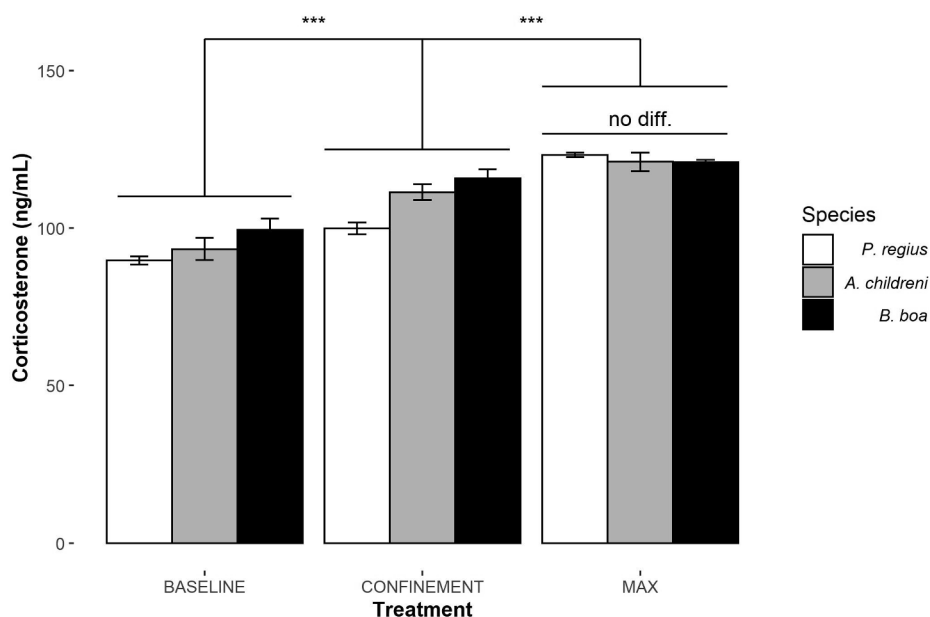
Fig. 1. Plasma concentrations of corticosterone (ng/mL) in three species of python (Ball Pythons: *P. regius*; Children's Pythons: *A. childreni*; Bismarck Ring Pythons: *B. boa*) at baseline, post-confinement, and following injection of adrenocorticotropin hormone (ACTH) plus confinement designed to produce a maximum (MAX) corticosterone response. Asterisks indicate significance in our model of $P < 0.001$.
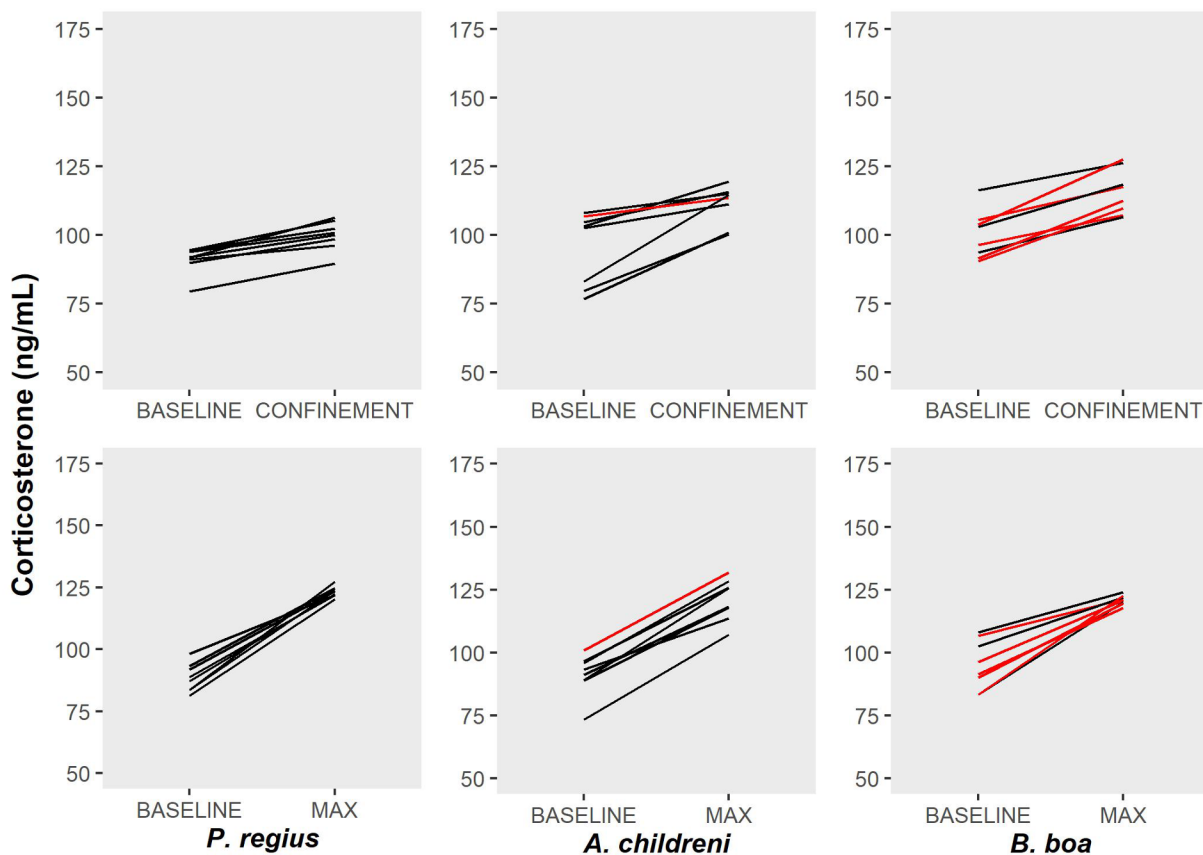

Fig. 2. Individual hormonal responses and striking behavior (red = pythons that struck at least once) in three species of python (Ball Pythons: *P. regius*; Children's Pythons: *A. childreni*; Bismarck Ring Pythons: *B. boa*) during two treatments: confinement and injection of adrenocorticotropin hormone (ACTH) plus confinement designed to produce a maximal corticosterone concentrations.

minimum and maximum number of strikes was two. After confinement, this difference increased to eight, although the same individuals, except one, struck. However, both individual averages and the average strikes for the species (pre-confinement: 5.54, post-confinement: 5.25), were stable. Thus, the mean number of strikes pre- and post-confinement are the same across multiple events, but exposure to confinement increases the probability of extreme values, both high and low. One

interpretation is that the mean and variance in the number of strikes is ontogenetically set, and exposure to a stressor, like our confinement procedure, induces greater variability. Many animals exhibit consistent individual variation, or personality (Wolf and Weissing, 2010), and species-level differences in antipredator behavior are well established in snakes (Johnson, 1975; Greene, 1988; Herzog et al., 1989). Future research on snakes that focuses on the forces driving individual
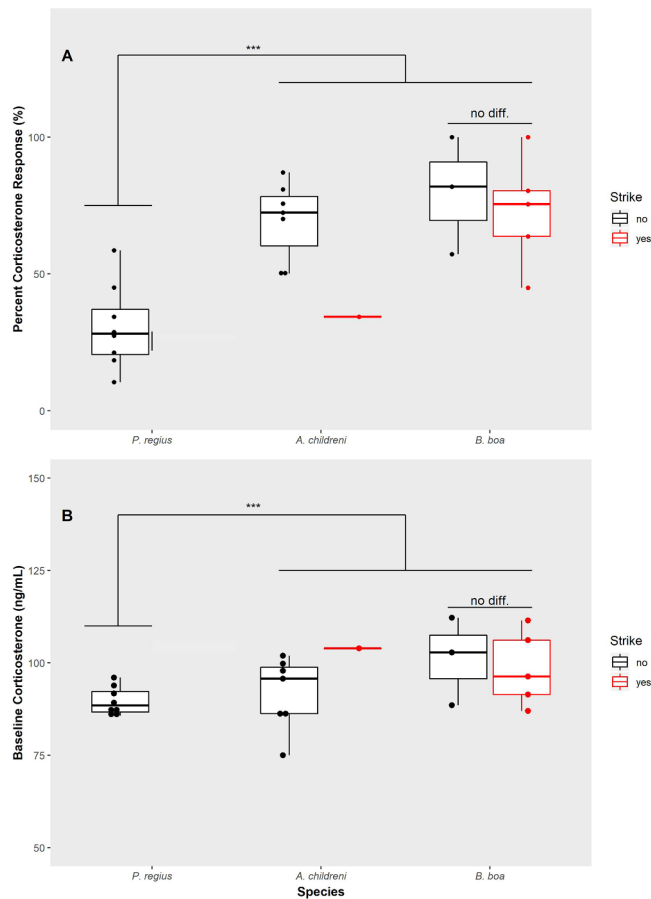
Fig. 3. Hormonal responses and striking behavior (red = pythons that struck at least once) in three species of python (Ball Pythons: *P. regius*; Children's Pythons: *A. childreni*; Bismarck Ring Pythons: *B. boa*). A) Percent of maximum corticosterone response, B) baseline concentration of corticosterone (ng/mL). The middle bar represents the median, the lower and upper hinges represent the first and third quartiles, respectively, and the whiskers extend to minimum and maximum values.

variation, such as density-dependent adaptation (Maynard Smith, 1982), environmental variation coupled with incomplete phenotype matching (Nettle, 2006), and developmental bet-hedging (Brockmann, 2001) would be beneficial.

Our second hypothesis, that species would differ in their corticosterone profile was also supported. Our corticosterone concentrations were well within the ranges presented in previous studies on snakes (Duggan, 1981; Bailey et al., 2009; Claunch et al., 2017). The modified confinement treatment increased corticosterone concentrations above baseline, on average, 13% following confinement and 27% following confinement and ACTH injection. These are moderate values compared to other snake studies, which have found handling approximately doubles corticosterone concentrations in snakes (Moore et al., 2000; Mathies et al, 2001; Schuett et al., 2004; Holding et al., 2014). We also found that Ball Pythons had both lower concentrations of corticosterone at baseline and post-confinement, and that they had a muted corticosterone response in comparison to both Children's Pythons and Bismarck Ring Pythons (Table 1; Figs. 2, 3). However, there was no difference in the maximum corticosterone response following ACTH injection among species (Table 1, Fig. 1), suggesting that the potential of the HPA axes in conserved. While the corticosterone responses following confinement were lower than the maximum response, it is possible that one hour provided sufficient time for at least partial recovery from peak response as a result of negative feedback of the HPA axis. Our results add to the evidence for species-level differences in corticosterone profiles in snakes.

Our third hypothesis, that there would be correlations between behavior and corticosterone profile, was supported, but the trend was opposite to our predictions. We predicted Ball Pythons, which were the least aggressive species in our study, had the lowest average baseline concentrations of corticosterone and the least responsive HPA axis (Fig. 1). Thus, Ball Pythons were exhibiting the corticosterone profiles of proactive individuals according to the paradigm we applied. As energy mobilization is influenced both by glucocorticoid hormones and temperature, it would not be surprising if the adaptive calculus of an ectothermic snake's corticosterone stress response during a predation encounter cannot be inferred from the responses of endothermic animals. Both the 'reactive scope model' developed by McEwen and Wingfield (2010) and the 'reactive scope model' developed by Romero et al. (2009) emphasize that the fitness value of the hormonal stress response depends on the energetic demands of the organism. Furthermore, previous studies in snakes have shown that antipredator behavior may depend on temperature, although the patterns vary across species. For example, while early studies suggested that snake are more aggressive when cold (Arnold and Bennett, 1984), Keogh and DeSerto (1994) found that three colubrid species were more passive when cold, increasing aggressive displays, including strikes, with higher temperature. As the performance of muscle tissue, and its sensitivity to temperature, may vary across ectothermic species (Johnston and Gleeson, 1987; Johnston and Johnston, 1991), it would not be surprising if generalizations were difficult. Indeed, Brodie and Russell (1999) did not find an effect of temperature on individual behavior in Northwestern Garter Snakes (*T. ordinoides*), but Claunch et al. (2017) found that temperature, but not elevated corticosterone, affected individual antipredator behavior in Southern Pacific Rattlesnakes (*Crotalus atrox*). Such energetic considerations may also provide a link to research on corticosterone and locomotor activity (DeNardo and Sinervo, 1994; Miles et al., 2007).

Our comparison within Bismarck Ring Pythons between those that struck and those that did not strike showed no difference in baseline corticosterone concentrations and HPA axis responsiveness (Fig. 2). Although only a single data point, it is interesting that the single Children's Python that struck also had the highest corticosterone concentrations (Fig. 2). While Herr et al. (2017) found that cottonmouths (*Agkistrodon piscivorus*) with higher corticosterone concentrations were more likely to strike, Claunch et al. (2017) found no effect of corticosterone implants on antipredator behavior in male Pacific Rattlesnakes, although they did find a correlation with testosterone.

Although the sample size of our study limits the strength of our conclusions, this study provides valuable comparative data for the study of the relationship between antipredator behavior and the corticosterone stress response in snakes.

CRediT authorship contribution statement

J. Alex Brashears: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **H. Bobby Fokidis:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - review & editing. **Dale F. DeNardo:** Writing - review & editing, Funding acquisition.

Acknowledgment

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