Adrenocortical Effects of Human Encounters on Free-Ranging Cottonmouths (*Agkistrodon piscivorus*)

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ABSTRACT.—The increased encroachment of humans into natural areas is typically viewed as stressful for many wildlife species. A common stress response of many animals, including snakes, is the elevated release of the adrenal hormone, corticosterone. To test whether human encounters elicited a stress response in snakes, we monitored the levels of circulating corticosterone in free-ranging Cottonmouths (*Agkistrodon piscivorus*) during staged interactions. When exposed to a high-level disturbance (i.e., capture and confinement in a bag) for 30 min, Cottonmouths exhibited a significant corticosterone stress response as predicted. This response was four times that of the control treatment (i.e., immediately bled snakes) and shows that Cottonmouths exhibit strong corticosterone responses to confinement. Conversely, blood corticosterone values for low-level disturbance (i.e., nearby human presence for 30 min) did not differ significantly from the control treatment. The lack of a strong stress response to low-level disturbance indicates that Cottonmouths possess a seemingly adaptive mechanism of not being overly alarmed by the mere presence of a potential predator. This suggests that the occasional foot-path encounters humans commonly have with snakes may not be stressful for some snake species.

Glucocorticosteroids (GCs) are involved in vertebrate adaptation to stress (Greenberg and Wingfield, 1987; Lance, 1990; Mathies et al., 2001). Increased GC secretion in response to environmental perturbations potentially enhances organismal fitness by allowing greater access to energy stores and increasing the ability to escape predation or other stressful situations (Wingfield and Romero, 2001; Langkilde and Shine, 2006). However, chronically elevated GCs may impair reproduction, suppress immunity and cause neuronal cell death (Wingfield and Romero, 2001).

There is increasing concern that human encounters associated with the rising popularity of outdoor recreation and ecotourism into natural habitats may be stressful to and have negative impacts on wildlife (Parent and Weatherhead, 2000). Investigations have concentrated on behavioral effects in birds and mammals (Boyle and Sampson, 1985) and indicate adverse impacts related to activities such as migration, reproduction, foraging, and roosting (Burger and Gochfeld, 1994; Parent and Weatherhead, 2000; Burger, 2001). Comparatively fewer studies have examined hormonal (GC) responses to human disturbance (e.g., Romero and Wikelski, 2002; Müllner et al., 2004; Walker et al., 2006). Disturbance effect studies are uncommon in reptiles, perhaps

because of their secretive and solitary nature (Burger, 2001). However, reptiles may be vulnerable to human disturbance because of limited ability to move to less disturbed areas and the physiological constraints of ectothermy that may tie them to specific periods of activity or specific habitats (Parent and Weatherhead, 2000).

In snakes, behavioral but not hormonal responses to human presence have been investigated. Prior and Weatherhead (1994) found that *Sistrurus catenatus* in disturbed areas continued to respond to people as if they were predators. Examining the influence of human disturbance on basking snakes (*Nerodia sipedon* and *Thamnophis sirtalis*), Burger (2001) indicated that *N. sipedon* continued to respond to humans even in disturbed areas and that walking past snakes without stopping results in less disturbance than stopping to watch them. Further, Parent and Weatherhead (2000) showed that, for *S. catenatus*, daily movement decreased, and gravid females responded to human disturbance by becoming more cryptic.

Corticosterone (CORT) is the principal GC in reptiles (Greenberg and Wingfield, 1987; Guillette et al., 1995) and is secreted in response to various types of stressors including capture and holding. Indeed, snakes including *Agkistrodon piscivorus*, *Boiga irregularis*, *T. sirtalis*, and *N. sipedon* perceive capture and confinement as stressful as indicated by an increase in plasma CORT in response to capture (Moore et al., 2000,

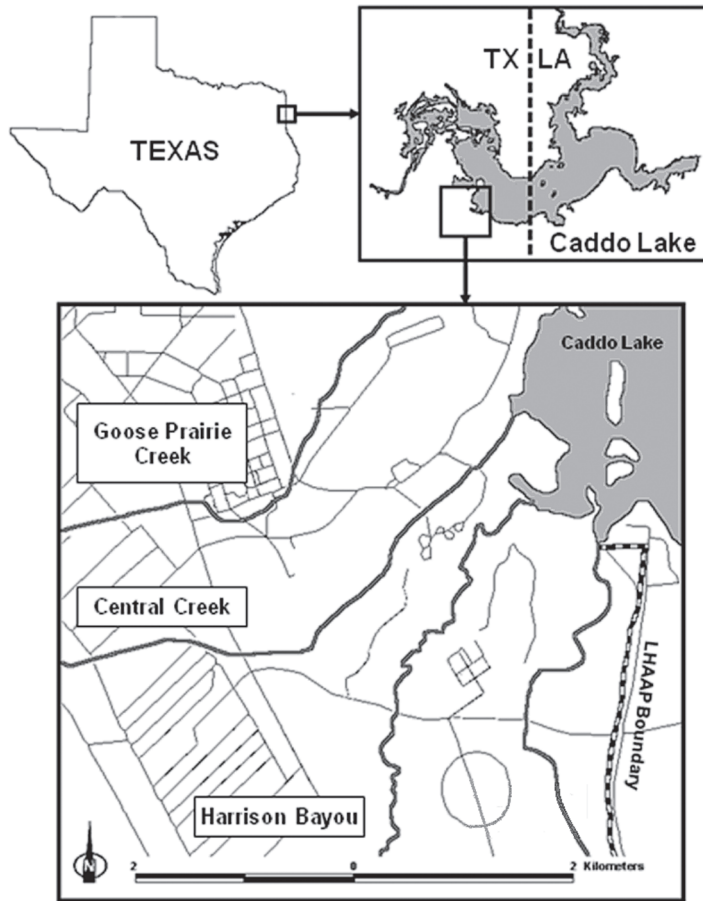[2] Corresponding Author. E-mail: fcbailey@mtsu.edu

Fig. 1. Map of the Longhorn Army Ammunitions Plant (LHAAP), Texas. Cottonmouths were collected from Goose Prairie Creek, Central Creek, and Harrison Bayou (modified from Rainwater et al., 2005).

2001; Mathies et al., 2001; Graham, 2006). The major objective of our study was to determine whether mere human presence elicits a strong CORT response in snakes as does capture and confinement. We chose to use Cottonmouths, *A. piscivorus*, as our test species because their behavioral responses to humans have recently received considerable attention (Gibbons and Dorcas, 2002; Roth and Johnson, 2004; Glaudas and Winne, 2007). We hypothesized that short-term close encounters with humans would not elicit a CORT response in Cottonmouths different from control snakes (background levels) because it would seemingly be disadvantageous for a snake to have a stress response to the simple presence of every potential predator.

MATERIALS AND METHODS

Study Site.—Caddo Lake National Wildlife Refuge, formerly Longhorn Army Ammunitions Plant (LHAAP) consists of 3,437 ha of mostly bottomland hardwood forest and scattered wetlands containing four creek drainages into the southwestern region of Caddo Lake in northeastern Texas (Fig. 1). Human access to the refuge has been restricted or limited since 1942. We sampled snakes along three creeks (Goose Prairie Creek, Central Creek, and Harrison Bayou). All creeks were forested with Goose Prairie and Central Creeks having 2–3 m embankments while Harrison Bayou had little embankment.

Snake Treatments.—During May 2007, on four consecutive clear sunny days between 1000 and 1800 h, four of us slowly walked in pairs along the banks of the creeks in an effort to observe cottonmouths before being detected by them. We assigned one of three predetermined behavioral treatments to snakes before encounters. Behavioral treatments were as follows. (1) Immediate (control)—consisted of immediately capturing the snake on observation and collecting a blood sample as quickly as possible. (2)

Observe—consisted of slowly approaching a stationary snake to 3 m, sitting and watching the snake for 30 min, then capturing the snake and collecting a blood sample. During this period, only one person remained with the snake. The observer would exhibit movement to make sure the snake was initially aware of the intruder (as evidenced by increased tongue flicks or slight movement of the snake's head in the direction of the observer), then sit and face the snake. If a snake was initially assigned to the Observe treatment but attempted to crawl or swim away upon initial observation, we would default it to an Immediate or Confine treatment. Snakes that began to crawl away during the observation period were excluded from any experimental group. (3) Confine—consisted of immediately capturing the snake and placing it in a nylon snake bag for 30 min, then removing the snake from the bag for blood sampling. We handled all snakes by capturing them with snake tongs and placing them into clear acrylic tubes for blood sampling. For each snake, sex, snout–vent length (SVL), body mass, time of capture, time to bleed, and treatment type were recorded. Repeated sampling of individuals was prevented by sampling different sections of the streams each day and by injecting tested snakes intraabdominally with radiofrequency identification microchips (Avid Identification Systems, Inc., Folsom, LA) after blood sampling.

Blood Sampling and ELISA.—We attempted to capture, tube, and bleed snakes within <5 min of a treatment. Timing began at capture for the Immediate and Observe groups and upon removal from the bag for the Confine group. We used 3-ml syringes with 21-gauge needles to obtain whole blood from the caudal vein. Depending on the size of the snake, 70–420 μl of whole blood was collected in heparinized capillary tubes for storage until processing later the same day. Not more than 5% of the blood volume was removed from any snake, assuming 70 ml of blood per kg of animal (Dessauer, 1970). We centrifuged blood samples for 15 min at 1,000 g, removed the blood plasma using a 25-μl Hamilton gas chromatography syringe (cleaned with deionized water prior to each use), and stored the plasma in 1.5-ml microcentrifuge tubes at −20°C for 1–4 days then at −80°C until analysis in July 2007.

We analyzed circulating levels of corticosterone using an enzyme-linked immunosorbant assay (ELISA; Correlate-EIA™ Corticosterone kit 900-097, Assay Designs, Inc., Ann Arbor, MI). We diluted 20-μl plasma aliquots with 20 μl of steroid displacement reagent, let samples sit for 10 min, and further diluted samples 34-fold with assay buffer. Competitive binding

was accomplished by adding alkaline phosphatase labeled corticosterone. After a 2-h incubation period, we washed the plate, added p-nitrophenyl phosphate substrate, incubated samples for 60 min, and read by spectrophotometry (Optimax microplate reader, Molecular Devices, Sunnyvale, CA) at 405 nm. All samples were run in duplicate in a single assay. A pooled plasma sample from both male and female snakes known to have high corticosterone exhibited parallelism to the standard curve when serially diluted. The intraassay coefficient of variation was 7%, and the limit of detection for the assay was 0.027 ng/ml.

Statistical Analysis.—We examined snake body size (body mass and SVL) for sex differences by treatment using two-way ANOVAs. Also a two-way ANOVA was used to investigate the effects of sex and treatment on plasma CORT. If differences were seen in main effects of this two-way ANOVA, a Dunnett's post hoc test was run to determine differences between means of treatment groups compared to the control (Immediate). Snake body condition was calculated as the residuals of mass regressed against SVL and then was used to examine potential relationships with CORT within treatments using linear regressions. We compared the time it took to sample (capture and bleed or remove from a bag and bleed) snakes in each treatment using a one-way ANOVA while potential relationships of CORT levels against blood sampling times, snake body mass, SVL, and body condition were examined using simple linear regressions. We report all data as means ± 1 SE with the level of significance being α = 0.05. All statistical tests were performed using SigmaStat 3.1 (Systat Software, Inc., 2004, San Jose, CA).

RESULTS

Of 40 encountered Cottonmouths, we obtained blood samples from 34 individuals that corresponded to one of the three treatment groups: Immediate, Observe, Confine (for body size measurements, see Table 1). Snakes did not differ in body mass by treatment ($F_{2,28} = 2.54$, $P = 0.097$), sex ($F_{1,28} = 0.94$, $P = 0.341$), or interaction ($F_{2,28} = 2.89$, $P = 0.072$). Nor did snakes differ in SVL by treatment ($F_{2,28} = 2.58$, $P = 0.094$), sex ($F_{1,28} = 0.05$, $P = 0.816$) or interaction ($F_{2,28} = 2.02$, $P = 0.152$). We found circulating CORT levels to be significantly different by treatment ($F_{2,28} = 8.86$, $P = 0.001$) but not by sex ($F_{1,28} = 0.012$, $P = 0.914$) nor the interaction of sex and treatment ($F_{2,28} = 0.684$, $P = 0.513$). Snakes in the Confine treatment exhibited a distinct stress response (Fig. 2) with a mean CORT level (42.5 ± 11.4 ng/ml) that

TABLE 1. Mean (SE) body size measurements for Cottonmouths (*Agkistrodon piscivorus*) used in each of three behavioral treatments.

	Confine	Observe	Immediate
Female			
SVL (cm)	51.4 (1.26)	49.1 (4.31)	51.9 (3.08)
Mass (g)	156.0 (16.4)	153.3 (27.50)	181.0 (27.83)
N	4	7	8
Male			
SVL (cm)	65.3 (5.55)	40.5 (1.00)	50.0 (7.38)
Mass (g)	393.0 (83.93)	80.5 (6.50)	179.6 (76.42)
N	8	2	5
Sexes combined			
SVL (cm)	60.7 (4.13)	47.2 (3.53)	51.2 (3.24)
Mass (g)	314.0 (64.4)	137.1 (23.60)	180.5 (32.04)
N	12	9	13

FIG. 3. Relationship of plasma corticosterone in the Cottonmouth (*Agkistrodon piscivorus*) against the time it took to capture and collect blood samples for the Immediately captured (control) treatment.

was significantly higher (Dunnett's test, $P = 0.0002$) than the Immediate (8.1 ± 1.3 ng/ml). There was no significant difference between the Observe (16.2 ± 4.4 ng/ml) and Immediate treatment groups (Dunnett's test, $P = 0.073$).

Mean blood sampling time (i.e., time to bleed) among the treatment groups was 3.1 ± 0.3 min (range 1.0–9.5 min) and was not significantly different among treatments ($F_{2,29} = 3.1$, $P = 0.062$). For the Immediate treatment, we observed a positive relationship ($R^2 = 0.51$, $P < 0.009$) between bleed time and CORT level (Fig. 3) but not in the Confine ($R^2 = 0.00$, $P = 0.997$) and Observe ($R^2 = 0.014$, $P = 0.778$) treatments. Also, no relationships were observed between CORT level and body mass (Immediate, $N = 13$, $R^2 = 0.09$, $P = 0.313$; Observe, $N = 9$, $R^2 = 0.13$, $P = 0.335$; Confine, $N = 12$, $R^2 = 0.03$, $P = 0.614$), SVL (Immediate, $N = 13$, $R^2 = 0.09$, $P = 0.309$; Observe, $N = 9$, $R^2 = $
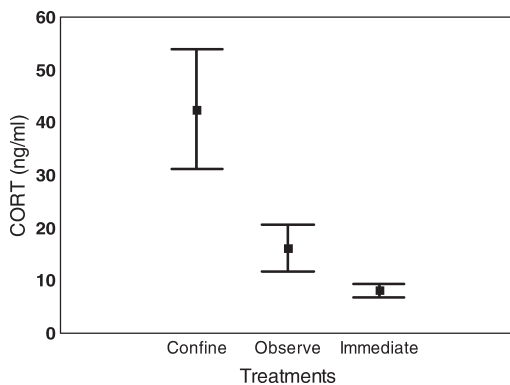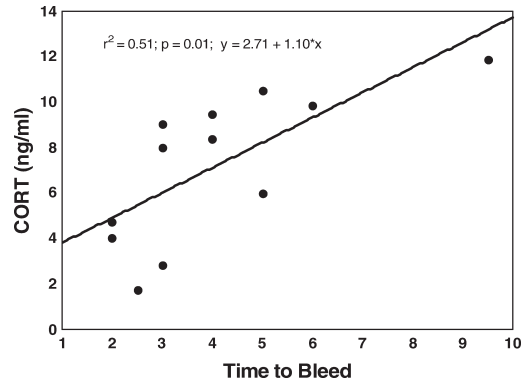

FIG. 2. Plasma corticosterone levels in the Cottonmouth (*Agkistrodon piscivorus*) after exposure to three treatments. Boxes indicate means, and bars indicate $\pm$ 1 SE. CORT levels differed significantly across treatment groups ($F_{2,28} = 8.86$, $P = 0.001$).

0.06, $P = 0.542$; Confine, $N = 12$, $R^2 = 0.01$, $P = 0.816$), or body condition (Immediate, $N = 13$, $R^2 = 0.03$, $P = 0.555$; Observe, $N = 9$, $R^2 = 0.00$, $P = 0.901$; Confine, $N = 12$, $R^2 = 0.06$, $P = 0.428$) within treatments.

DISCUSSION

As we predicted based on prior studies (Moore et al., 2000; Mathies et al., 2001; Graham, 2006), snakes that were captured and confined (Confine treatment) for 30 min had elevated levels of plasma CORT compared to controls (Immediate treatment). However, the amount of variation in the stress response exhibited by Cottonmouths in the Confine treatment was unexpected. Although most Confine group snakes exhibited high plasma CORT levels, two individuals had levels within background range after 30 min of capture indicating individual snakes may perceive predator encounters or at least capture and confinement differently.

Moore et al. (2001) showed that even within a species (*T. sirtalis*), different populations can differ in CORT response to capture and confinement. Herzog et al. (1989) demonstrated interspecific differences in habituation rates of defensive behaviors among closely related species of *Thamnophis* as well as large differences in habituation rates between individuals within a species. Moreover, Glaudas (2004) showed that Cottonmouths habituate to human handling to some degree, with their behavioral responses to handling becoming more passive over a five-day test period. Also, in a study related to human disturbance with birds, Walker et al. (2006) showed that Magellanic Penguins from a tourist-visited area had lower CORT responses to human visitation and

capture and confinement than those from more undisturbed areas. However, because of prior restricted access to LHAAP, it is not likely that any of the Cottonmouths sampled in the present study had been handled by humans before or had many previous human encounters.

Background CORT levels for Cottonmouths in this study are comparable to those previously reported for *B. irregularis* (Mathies et al., 2001) and *Thamnophis sirtalis concinnus* (Moore et al., 2000, 2001) and are much lower than those reported for background levels in *Agkistrodon contortrix* (Schuett et al., 1996; Schuett and Grober, 2000), *A. piscivorus* (Graham, 2006), *Crotalus atrox* (Schuett et al., 2004), and *Thamnophis sirtalis parietalis* (Whittier et al., 1987; Moore et al., 2001). Graham (2006) implied that CORT levels he observed in Cottonmouths may not be indicative of background levels because of the relatively long time it took to collect blood samples (on average, ~13 or 22 min vs. ~3 min this study; see Romero and Reed, 2005). CORT levels in captive Cottonmouths (Graham, 2006) were over twofold higher than those in the Confine treatment group in our study. The high CORT levels in Graham's (2006) captive snakes may also have been influenced by a relatively long interval between capture and blood collection (2–24 h) compared to the present study (30-min holding time). Regardless, these studies suggest the influence of captivity on Cottonmouth stress.

The effects of human disturbance or presence on snakes are somewhat variable in the literature. The work of Gibbons and Dorcas (2002) and Prior and Weatherhead (1994) have shown that Cottonmouths and *S. catenatus*, respectively, are not as aggressive to humans upon close encounter as generally perceived. Because human encounters do not typically elicit aggressive responses from Cottonmouths, it may be true that they are not stressful to the snakes. However, the absence of outwardly aggressive behaviors does not necessarily indicate the absence of a physiological stress response. In the case of *S. catenatus* (Prior and Weatherhead, 1994), >60% of the snakes did not show any outward response to human disturbance. This may be because the snakes were not stressed by the encounters, or as was suggested by Prior and Weatherhead (1994), it may be that, for a slow-moving species like the *S. catenatus* (and presumably Cottonmouths), remaining cryptic may simply offer more protection than moving and drawing attention to an individual's presence.

In contrast, the work of Burger (2001) showed that humans stopping near and watching caused Northern Watersnakes (*N. sipedon*) and Common Gartersnakes (*T. sirtalis*) to rapidly flee in a high percentage of encounters, possibly indicating stress. Burger's data indicate that walking past snakes without stopping results in less disturbance than stopping to watch them and that there is a clear species difference in response to human disturbance with water snakes being more responsive than gartersnakes.

The hypothesis that there would be no significant difference in plasma CORT between the Immediate (control) and Observe treatment snakes was confirmed, indicating that Cottonmouths did not find the presence of humans at close range to be stressful. However, it is interesting to note that there was a significant positive relationship between time to blood sample and plasma CORT in the Immediate treatment group (Fig. 3) but not in the Observe group. Presumably, if there is no difference between the Observe and Immediate groups, the two groups would show the same time to blood sample versus plasma CORT relationship. It is possible that there is a small CORT response because of the presence of humans that has elevated the CORT levels enough to mask the time to sample versus plasma CORT relationship in the Observe group, although no statistically significant elevation was detected in our study.

The positive relationship between CORT and time to blood sample in the Immediate treatment also indicates that some of the samples in this group have slightly elevated CORT levels because of the amount of time it took to obtain the samples and are not truly "background." Ideally, all samples would have been taken in less than 2–3 min to observe true background levels of CORT (see Romero and Reed, 2005). However, because of field conditions and the care necessary in handling venomous snakes, this was not always possible (half of the snakes in this group were sampled in ≤3 min). Nevertheless, we consider the Immediate treatment CORT levels to be a reasonable approximation of background for Cottonmouths.

Different groups of snakes, such as venomous ambush predators and active, less cryptic species, face different fitness challenges and may respond to similar situations differently. Moore and Jessop (2003) state that individuals may respond to the same challenges differently depending on the social and environmental context. Furthermore, the hypothalamic-pituitary-adrenal axis may be modulated such that functions necessary for certain activities (e.g., reproduction) can be accomplished (Moore and Jessop, 2003). In essence, by modulating the stress response, an individual is altering its sensitivity to particular stressors. For an ambush predator like the Cottonmouth, a strong

CORT response to every human (or other potential predator) may cause individuals to move from their locations more frequently than is usual or preferred. The cost to these snakes in loss of feeding opportunities from leaving preferred ambush sites too often could be high and lead to fitness effects. However, it would be expected that at some point the costs of not responding to the presence of a potential predator would outweigh the benefits of a potential meal.

To our knowledge, this is the first study to examine the CORT response of snakes to human presence. Although it is difficult to infer how snakes may perceive noninvasive human interactions, our data suggest the stress response may be minimal compared to the response to capture and confinement, at least in a typical venomous ambush species. However, examination of CORT response in other less naïve cottonmouth populations and other snake species is clearly needed before any inference is made concerning stress in snakes caused by human presence in general. Perhaps there is considerable relevance in Glaudas's (2004) statement that ''Snakes are not simple automatons in their defensive behavior, but make sophisticated judgments about their enemies and are capable of learning.''

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