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Handling Stress and Plasma Corticosterone Levels in Captive Male Western Diamond-backed Rattlesnakes (*Crotalus atrox*)

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Reptiles are similar to other vertebrates in that they show both behavioral and physiological stress responses to a wide variety of stimuli (reviewed by Greenberg and Wingfield 1987; Lance 1990; Guillette et al. 1995; Wingfield and Romero 2001; Greenberg 2002; Greenberg et al. 2002; Moore and Jessop 2003). Frequently reported stressors include extreme temperature (Jessop et al. 2000), crowding (Hayes 1997), social dominance (Alberts et al. 1994; Matter et al. 1998; Schuett et al. 1996; Greenberg 2002, 2003), starvation (Romero and Wikelski 2001), as well as a range of anthropogenic factors (Hofer and East 1998) including capture and handling (Moore et al. 1991; Kreger and Mench 1993; Cash et al. 1997; Lance and Elsey 1999; Cree et al. 2000; Moore et al. 2000; Gregory and Schmid 2001; Mathies et al. 2001; Franklin et al. 2003; Jessop et al. 2003).

The hypothalamo-pituitary-adrenal (HPA) axis is unquestionably the best-studied physiological system concerning the stress response (Selye 1973), and there is a relatively long history on adrenal hormones (glucocorticoids) in reptiles (Greenberg and Wingfield 1987; Lance 1990). Cortisol and corticosterone (CORT) are adrenal glucocorticoids released in response to stressors, and when elevated have important metabolic functions in converting stored energy (e.g., lipids and proteins) to available glucose for escape or defense (Dallman et al. 1995; Guillette et al. 1995). Chronically high levels, however, can have profoundly negative

downstream effects on immune function (Guillette et al. 1995; Moynihan 2003). The primary glucocorticoid in reptiles is CORT (Greenberg and Wingfield 1987)

Researchers interested in measuring levels of circulating CORT for studies on metabolism or reproduction face the problem of obtaining blood samples quickly to avoid adversely influencing "true state" levels through handling stress. It is thus important to understand the influence of handling stress on CORT levels. A quantitative sense of the timing of the CORT response during handling permits evaluation of handling techniques as potential confounding variables in the analysis of CORT itself, other steroids such as testosterone (Lance et al. 2004), and a wide range of physiological parameters immediately removed from but affected by elevated levels of CORT (e.g., immune responses) (Guillette et al. 1995; Moynihan 2003)

The goal of this study was to determine whether certain handling procedures cause a significant CORT response in a laboratory colony of male Western Diamond-backed Rattlesnake (*Crotalus atrox*) derived from the wild. The measurements of CORT we report are not intended to stand as surrogate values for subjects living in the wild. Rather, they denote important baseline data for laboratory analyses and prospective field studies.

Materials and Methods

Subjects.—Seventeen adult male Western Diamond-backed Rattlesnakes (*Crotalus atrox*) were used. All subjects were long-term (3–4 years), healthy captives (housed in the Life Sciences Department, Arizona State University West, Animal Care Facility), and were collected as adults from several areas in central Maricopa County, Arizona, near the vicinity of Phoenix. Mean (± 1 SE) snout–vent length (SVL) was 89.1 ± 2.39 cm, range 74.0 to 98.0 cm, and mean (± 1 SE) body mass was 480.45 ± 32.81 g, range 278.80 to 639.3 g. Subjects were housed individually in glass enclosures (91 L x 30 W x 25 H cm) fitted with screen covers, with the front end heated by commercial heat tape (8 cm wide; 32°C) during photophase. Newsprint was used as a floor covering. Artificial lighting (eight 40 W fluorescent tubes) positioned 3 m above the cage was electronic timer-controlled to simulate natural (Arizona time) photoperiod year round. Laboratory rodents were offered weekly during the active season (March through October), and water was available in glass bowls *ad libitum*.

Testing procedures.—Experimental procedures for testing handling stress were performed in the laboratory. Handling procedures involved the following. The experimental subjects were randomly selected and placed in three groups: group 1 (N = 5), the control, and two treatment groups, group 2 (N = 5) and group 3 (N = 7). Testing occurred in a large observation arena (2.5 L x 1.5 W x 1 H m). The arena floor had three commercial strips of heat tape (16 cm wide; 32°C) running its length, and on top of them newsprint covered the entire arena floor. Each trial (N = 12) involved placing a single subject in the middle of the test arena, and each was gently and consistently (every 5 sec) prodded and grabbed with a commercial snake grabber (snake tongs; 1 m in length) for a total time of 5-min. Following this procedure they were immediately returned to their individual permanent enclosures, and bled at 15-min (group 2) and 30-min (group 3) post-handling, respectively, and returned to their permanent enclosures. The control (group 1) involved placing subjects (N = 5) into the arena for 5-min but they were not prodded nor grabbed. They were bled immediately after removal from the arena, and returned to their individual permanent enclosures. All tests occurred during the late morning of 24 March 2000.

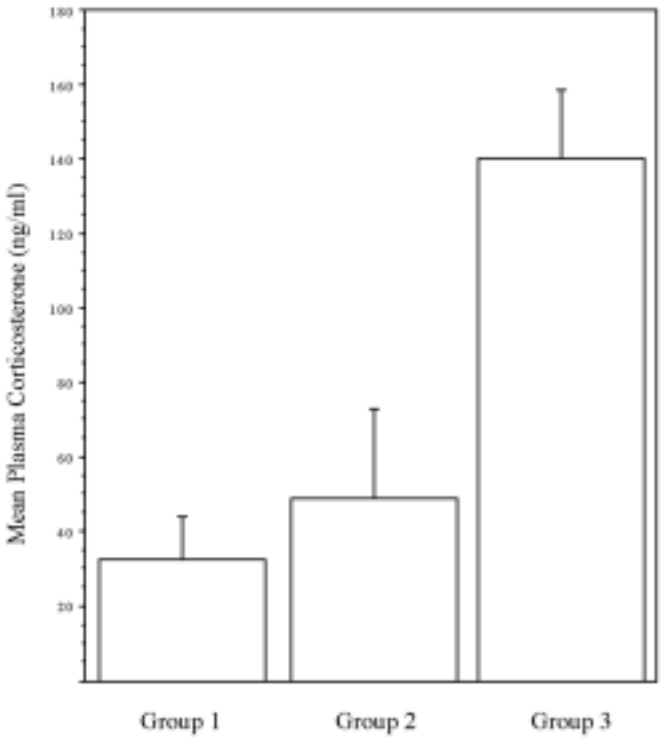

FIG. 1. Mean (± 1 SE) concentration of plasma corticosterone (ng/ml) for 17 adult male *Crotalus atrox* in three independent experimental groups. Control Group: Group 1 = no handling stress and blood sampling at 5-min post arena exposure. Treatment Groups: Group 2 = 5-min handling stress and blood sampling at 15-min post-handling. Group 3 = 5-min handling stress and blood sampling at 30-min post-handling.

Collection of blood and plasma.—Subjects were gently removed from their individual permanent enclosures, quickly restrained in a standard squeeze-box, bled, and returned to their enclosures. The entire process required 1–3 min. Blood was obtained from tail vessels using sterile 1.0 ml tuberculin syringes (25-G5/8") treated with porcine-derived heparin sodium (1,000 units/ml). Blood was immediately placed on ice (Taylor and Schuett 2004), followed by centrifugation for 4-min at 6000 rpm. Plasma was individually collected in 1.5 ml centrifuge tubes and stored in an ultra-low freezer (- 80ºC) until radioimmunoassays (RIAs) could be performed (< 1 year).

Radioimmunoassay of plasma.—The general procedures for conducting RIAs for measurement of CORT are published elsewhere (Schuett et al. 1996; Schuett and Grober 2000), but will be briefly described below. RIA kits were used (ImmuChem double antibody corticosterone, RIA I; ICN Biomedicals, Inc.). Validation for the RIA was by quantitative recovery and parallelism. Quantitative recovery of CORT added to snake plasma was 100%, and parallelism was demonstrated between the inhibition curve for the standards and dilutions. In this analysis CORT did not require an extraction procedure. The minimum detectable concentration was ≥ 50 pg. Samples (N = 34) were analyzed in duplicate in one RIA. The intra-assay coefficient of variation was 2.4%. All CORT values are pre-

sented as arithmetic means ± 1 SE (ng/ml).

Statistical analyses.—Prior to performing statistical tests (StatView 5.01, SAS Institute, Inc.), data were inspected for outliers, normality (skewness and kurtosis), and equality of variance. Outliers were not detected, and conditions for normality and equality of variance were met. Because of the tendency of snakes with larger body mass to have relatively higher levels of circulating gonadal steroids (Schuett et al. 2001a, b; 2002; Taylor and Schuett 2004), we inspected body mass as a potential covariate for ANCOVA. Although body mass of individuals ranged widely, mean body mass of the three treatment groups was not significantly different (ANOVA, $F_{2, 14} = 0.854$, $p = 0.447$), and linear regression showed no significant relationship between body mass and levels of plasma CORT (Group 1: $r^2 = 0.411$; ANOVA, $F_{1, 3} = 2.097$, $p = 0.243$. Group 2: $r^2 = 0.515$; ANOVA, $F_{1, 5} = 5.305$, $p = 0.070$. Group 3: $r^2 = 0.236$; ANOVA, $F_{1, 3} = 0.926$, $p = 0.410$.). Accordingly, ANOVA was used instead of ANCOVA. All tests were two-tailed; the α-level of significance was set at $p < 0.05$.

Results

Mean (± 1 SE) concentrations of plasma CORT in the three experimental groups of *C. atrox* are presented in Fig. 1. ANOVA demonstrated a significant treatment effect ($F_{2, 14} = 9.829$, $p = 0.0022$), and post hoc (Fisher's PLSD) analyses showed that the mean concentration of CORT in group 3 (blood obtained at 30-min post-handling) was significantly greater than in group 2 (blood obtained at 15-min post-handling) or in group 1 (control). There was no significant difference ($p = 0.578$) in the mean CORT levels between group 1 and group 2. Therefore, in adult male *C. atrox* subjected to mild handling stress, the response time for detection of significantly elevated levels of CORT is at least 30-min post-handling.

Discussion

The present data show that wild-collected adult male *C. atrox* held long-term (3–4 years) in a laboratory situation show a relatively rapid and significant CORT response to mild handling stress at 30-min post-handling. Our results should not be used as surrogate values for free-ranging subjects, but they emphasize the importance of obtaining blood for CORT measurements relatively quickly to obtain "real state" values. The duration of capture and the time required to obtain blood are potential confounding variables in analyzing CORT and other steroid hormones (Moore et al. 1991; Lance et al. 2004). If captivity has the influence of reducing the time course of the stress response due to habituation, we suspect that free-ranging *C. atrox* might be even more sensitive to handling stress (i.e., show a greater and more rapid CORT response). We do not, however, have data to support this view in snakes. Interestingly, the subjects in this study showed no signs of behavioral habituation with respect to defensive behaviors (e.g., striking, rattling) despite being removed from the wild as adults for 3–4 years. In a non-reptilian example, a battery of stress tests using wild and domestic cavies (Rodentia, *Cavia aperea*) showed that long-term breeding (30 generations) in captivity did not result in significant changes in behavioral and CORT responses (Künzl et al. 2003). A similar study on snakes and other reptiles would be valuable for comparison.

Other researchers investigating handling stress and CORT levels in snakes obtained results similar to ours. Kreger and Mench (1993) used three common handling techniques to investigate stress responses in captive Ball Pythons (*Python regius*). Although their study had potential problems because of repeated-sampling of a small number of subjects (N = 4), none of the short-term (10-min) handling procedures evoked a significant elevation of plasma CORT. A study on Brown Treesnakes (*Boiga irregularis*) in Guam showed that protracted (1 night) confinement stress in traps showed significantly elevated plasma CORT, and there was limited evidence that other sex steroids were affected negatively (Mathies et al. 2001). Moore et al. (2000) showed that capture stress in male Red-sided Garter Snakes (*Thamnophis sirtalis parietalis*) did not result in suppression of courtship. There was, however, a significant elevation in plasma CORT, as well as a significant negative correlation between levels of plasma CORT and testosterone (Moore et al. 2000). Nonetheless, Moore et al. (2000) suggested that the behavioral stress response is uncoupled from the hormonal stress response during mating to maximize reproductive success. All of the above examples clearly show that capture and handling stress can elicit a CORT response, and that there are complex issues concerning species differences, as well as seasonal and sex differences.

We would like to specify several limitations of this study and point to further research. First, we have few concerns regarding diel effects in this study based on the fact that all sampling occurred in a narrow time frame in a single day (Cree et al. 2000; Summers and Norman 1988; Tokarz et al. 1998; Tyrell and Cree 1998). Although we have not determined in either captive or wild adult male *C. atrox* whether or not plasma CORT concentrations show diel patterns, we do know that there is no significant variation in plasma CORT during their active season (Mar.–Oct.) (Taylor et al. 2004). Second, we have not tested adult female *C. atrox* for CORT responses to handling. Several studies have demonstrated that there are sex differences in the CORT response (Lance et al. 2001; Mathies et al. 2001; Moore and Jessop 2003), and in females differences can be seasonal and influenced by reproductive states (Cree et al. 2000; Lance et al. 2001). During parturition, for example, mean plasma CORT levels in *C. atrox* are exceedingly high (> 350 ng/ml) yet their behavioral state appears to be stress-free. In fact, females appear to be tranquil and unremarkable (Schuett et al., *in press*). Third, there are few data on stress in juvenile reptiles. Behavioral (e.g., social) stressors can affect growth and immunocompetency (Alberts et al. 1994; Guillette et al. 1995). Finally, because our data set is limited to captive animals, tests of handling stress in free-ranging *C. atrox* across their active season and spanning different contexts (e.g., reproductive vs. non reproductive) would provide a broader range of knowledge.

In conclusion, our results on handling stress have direct utility for investigations of CORT in *C. atrox* in a wide range of laboratory-based studies of physiology and behavior (Guillette et al. 1995), as well as in free-ranging populations. Moreover, there are implications for studies of stress and CORT levels in the collection of free-ranging individuals for commercial uses, such as in rattlesnake roundups (Fitzgerald and Painter 2000).

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