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Corticosterone and Color Change in Southern Pacific Rattlesnakes (*Crotalus helleri*)

John Stepanek[1,3], Natalie M. Claunch[1,4,6], Julius A. Frazier[1], Ignacio T. Moore[2], Ben J. Vernasco[2], Camilo Escallón[2,5], and Emily N. Taylor[1]

[1] Department of Biological Sciences, California Polytechnic State University, San Luis Obispo, CA 93407, USA
[2] Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

Abstract: Metachrosis, or color change, in reptiles is used for thermoregulation, crypsis, and many other purposes. The mechanism and function of metachrosis remain unknown for many species, however, especially snakes. Anecdotal observations suggest that some snake species, including rattlesnakes, undergo varying degrees of color change when captured and confined. A possible explanation for this color change is the increase in plasma levels of the primary stress hormone, corticosterone (CORT). In this study, we implanted Southern Pacific Rattlesnakes (*Crotalus helleri*) with either CORT or sham silastic implants and photographed them under standardized lighting in a curtained box at the time of implant and 2 and 4 wk postimplant. We quantified light value (brightness or darkness) of the dark and light bands of the subjects' tails and examined the relationships of these variables to baseline CORT levels (CORT level at time of capture) as well as CORT levels after 1 h of acute confinement stress. CORT-treated snakes had higher baseline CORT than control snakes, but treatment had no direct effect on color. Regardless of treatment group, baseline CORT was positively correlated with lighter light bands, but had no relationship with the dark bands. Additionally, the magnitude of the CORT increase during acute stress was related to greater increase in contrast between light and dark bands. Defensive behavior was negatively correlated with contrast. We discuss potential reasons for the relationship between stress, defensive behavior, and color change.

Key words: Metachrosis; Reptile; Serpentes; Stress

Color is a highly diversified character among animal species and is used for a variety of purposes, including camouflage, mating displays, and thermoregulation (Moreno 2005; Breuer et al. 2007; Hanlon 2007; Geen and Johnston 2014). In many species, color is static; however, some species possess the ability to change color in response to certain stimuli. Metachrosis, the rapid change of color, has been observed in many ectotherms, particularly herpetofauna (Rahn 1942; King et al. 1994; Tanaka 2005; Stuart-Fox and Moussali 2008). Metachrosis is used in lizards for thermoregulation (Velasco and Tattersall 2008; Clusella-Trullas et al. 2009; Krohn and Rosenblum 2016); in anoles, frogs, and boas in response to changing photoperiod regimes (Rahn and Rosendale 1941; McAlpine 1983; Camargo et al. 1999; Wente and Phillips 2003; Stegen et al. 2004; Boback and Siefferman 2010); in anoles and frogs for camouflage (Kleinholz 1936, 1938; King and King 1991; Stegen et al. 2004); and in chameleons and anoles for social signaling (Greenberg 2002, 2003; Yang and Wilczynski 2003; Stuart-Fox and Moussalli 2008). The ability to change color in response to environmental stimuli could have profound consequences for an individual's fitness.

For decades, rattlesnakes have been reported to change color (e.g., Neill and Allen 1955), but there is a limited understanding of the mechanisms and adaptive nature of metachrosis in snakes. Rahn (1942) and Neill and Allen (1955) asserted that rattlesnakes darken in cold temperatures and lighten in warm temperatures, but time of day may have been a confounding factor in these studies and color was subjectively measured with color squares. In some species (*Crotalus adamanteus*, *C. atrox, C. cerastes*, and *C. viridis* [sensu lato]), an individual snake can change color so drastically that it is unrecognizable by the original capturer (Neill and Allen 1955). Herpetologists continue to report drastic and rapid color changes in various rattlesnake species anecdotally, but these claims have not been quantified to date. Observations vary, but the most common pattern reported includes an overall lighter appearance and increased contrast in response to capture (informal social media survey, personal communications, personal observations). As ambush predators and common prey of raptors, rattlesnakes rely on cryptic coloration for hunting and predator avoidance. Understanding the mechanisms and magnitude of color change in rattlesnakes could, therefore, uncover insights into the potential fitness advantages of metachrosis.

Although chameleons mediate color change through neural control (Stuart-Fox and Moussalli 2008), it is evident that color change can also be mediated at least in part by endocrine systems (Kleinholz 1938; Medica et al. 1973; Stuart-Fox and Moussali 2008), including stress hormones (Greenberg and Crews 1990; Greenberg 2002, 2003; Yang and Wilczynski 2003; Calisi and Hews 2007; Korzan et al. 2008; Fitze et al. 2009; San-Jose and Fitze 2013; San-Jose et al. 2013; Kindermann et al. 2013, 2014; Lewis et al. 2017). The role of hormones in metachrosis has been verified by the fact that hypophysectomy eliminates the ability of anoles and rattlesnakes to change color (Rahn 1941; Rahn and Rosendale 1941). The pars intermedia, a part of the pituitary that mediates many diel hormonal pathways, is responsible for regulation of melanophore function, and its removal inhibits metachrosis and leads to permanent pallor in rattlesnakes (Rahn 1941).

Metachrosis in ectotherm species has been associated with elevated stress hormones in several contexts, including toe clipping (Kindermann et al. 2013), topical treatment with epinephrine (Kindermann et al. 2014), mate selection

[3] Present Address: Department of Integrative Biology, Oregon State University, Corvallis OR 97331, USA
[4] Present Address: School of Natural Resources and Environment, University of Florida, Gainesville, FL 32611, USA
[5] Present Address: Departamento de Ciencias Básicas, Universidad de la Salle, Cra 2 No. 10-70, Bogotá, Colombia
[6] Correspondence: e-mail, nmclaunch@ufl.edu

(Greenberg and Crews 1990; Calisi and Hews 2007), and social behaviors for territorial mating dominance (Greenberg 2002, 2003; Korzan et al. 2008). Experimental manipulations of corticosterone (CORT, the primary glucocorticoid stress hormone in reptiles; Moore and Jessop 2003) in lizards have demonstrated a relationship between metachrosis and CORT (Yang and Wilczynski 2003; Fitze et al. 2009; San-Jose and Fitze 2013; San-Jose et al. 2013; Lewis et al. 2017). A link between CORT and metachrosis seems likely in snakes, especially because acute confinement is known to elevate CORT (Moore and Jessop 2003; Schuett et al. 2004; Lutterschmidt et al. 2009; Holding et al. 2014a,b). Such a link is further supported by three lines of evidence: (1) secretion of CORT is controlled by the hypothalamic–pituitary–adrenal (HPA) axis, and hypophysectomy results in a cessation of metachrosis (Rahn 1941); (2) CORT shows negative feedback suppression of the secretion of pro-opiomelanocortin (POMC), the precursor molecule to melanocyte-stimulating hormone (MSH) that controls melanophore activity (Proulx-Ferland et al. 1982; Schimicho-witsch et al. 1994); and (3) metachrosis shows a diel pattern that parallels that of CORT (Rahn and Rosendale 1941; Hedges et al. 1989; Breuner et al. 1999; Jones and Bell 2004; Quillfeldt et al. 2007; Malisch et al. 2008; Boback and Siefferman 2010). Therefore, we can investigate the potential roles of CORT and stress in snake metachrosis by manipulating the HPA system.

Our study tested the hypothesis that CORT mediates metachrosis in rattlesnakes. We implanted free-living rattlesnakes with either CORT-filled or blank ("control") implants and measured the color of the light and dark bands of their dorsal tail patterns before and after implantation. After effects of treatment had worn off, we conducted a second experiment in which we subjected the same individuals to a standardized stressor (i.e., acute confinement) to quantify metachrosis. We predicted that higher baseline CORT (chronically induced) would be associated with greater contrast in the dorsal pattern attributable to darker dark bands, lighter light bands, or both. Similarly, we predicted that snakes would display greater color contrast after acute confinement. Because this experiment was conducted in a field setting—providing the opportunity to examine these interactions in a natural environment—we also quantified the defensive behavior of snakes and examined its relationship with CORT and color.

Materials and Methods

Study Animals and Site

Thirty adult male Southern Pacific Rattlesnakes (*Crotalus helleri*) were captured at the University of California Santa Barbara Sedgwick Reserve, Santa Barbara County, California (34.6928°N, 120.0406°W; datum = WGS 84; elevation = 290 m above sea level) from 13 April to 2 May 2015. Each snake was measured for snout–vent length (SVL), implanted with an intramuscular passive integrated transponder (PIT) tag (MUSICC Chip, AVID Identification Systems, Inc.), and visually marked via nontoxic acrylic paint in the three basal rattle segments with a predetermined color code for future identification. Subjects >55 cm SVL were considered adults. The snakes at the site were found mainly in cattle-disturbed valley oak savannah habitat, with areas of chaparral and coastal sagebrush scattered throughout the landscape.

Snakes were transported to California Polytechnic State University for radio-transmitter and thermal data-logger implantation. Snakes were housed individually in 76.2 × 30.5 × 30.5 cm Visionarium cages (Vision Products) with a heat pad, hide box, and water ad libitum. Snakes were anesthetized via isoflurane inhalation (VetOne, MWI Veterinary Supply Co.) and received intracoelomic implants of radio transmitters weighing 5.3, 11, or 13.5 g (Holohil Systems Ltd.). Radio transmitters and thermal data loggers totaled <5% of snake body mass. Snakes were allowed to recover for 1–2 d before release at the site of capture.

Experimental Administration of CORT

After radio-transmitter implantation, snakes recovered in the field for at least 2 wk before further experimental manipulation in late May 2015. Our project was conducted using the same individuals reported in Claunch et al. (2017), wherein the development and administration of CORT implants, sampling regimes, and evaluation of hormonal and behavioral effects of the implants are detailed. Briefly, snakes were divided into two size classes on the basis of mean and median masses; snakes heavier than 800 g were assigned 15-mm (large) implants, and snakes lighter than 800 g received 7.5-mm (small) implants. Within each size class, snakes were randomly assigned to either a treatment group or control group. Treatment implants consisted of silastic tubing filled with crystalline CORT, plugged with silicone on each end. Control snakes received empty implants. Each snake received two implants: a fast-release implant to induce a dose of CORT shortly after implantation, sustained by CORT from a slow-release implant to ensure relatively even chronic elevation of CORT. The fast-release differed from slow-release by one small hole in the tubing made with an insulin syringe. Effective total dose of treatment implants was estimated at 6.1 mg for large implants and 3.6 mg for small implants. Implants were soaked in 0.9% saline solution for 12 h before administration in the field via intracoelomic injection on each side of the lower third of the body with a sterilized 12-g PIT tag injector, with bevel facing posteriorly. Despite an initially balanced allotment of subjects, variable recapture success yielded data from 26 snakes (7 small and 8 large treatment group snakes, and 7 small and 4 large control group snakes).

Sampling Period and Capture Data

Each snake was sampled (blood sample, mass, body temperature, and photographic imagery) just before implant injection, approximately 2 wk postimplant, and a third time approximately 4 wk postimplant. During each sampling event, we assessed snake defensive behavior before and during capture and restraint using tongs (Whitco Manufacturing Inc.) and a clear snake tube (Midwest Tongs). Cloacal temperature was measured immediately after tubing with a digital thermometer (Mannix HDT303K). To account for the stress response caused by capture, time elapsed from time of disturbance of habitat (if flipped or extracted from a burrow) or tong touch (if encountered in the open) until blood was drawn was recorded (time to bleed). A 0.5-mL sample of blood was collected via caudal venipuncture with a 25-gauge 1.9-cm heparinized syringe within 10 min of capture. Blood

was kept on ice while in the field, temporarily stored at 3°C, and centrifuged within 48 h for 3 min at 10,000 revolutions per min to separate plasma from red blood cells. Thereafter, plasma was stored at or below −18°C until analysis. Ambient temperature was measured at 1 m above the ground in the shade of the observer's body with a Kestrel 3000 pocket weather meter during each sampling event. Typically, only snakes above ground or visible in a burrow were captured to avoid disturbing habitat and inducing unequal stress responses across individuals. A few snakes were deep underground in burrow complexes and were excavated only if the process was estimated to take less than 10 min from start of digging to blood sample collection (two snakes at 2 wk postimplant, five snakes at 4 wk postimplant). If excavation was estimated to exceed 10 min, the snake was sampled at the next sighting. In one instance, 10 min elapsed during the digging process and the snake was not excavated; digging ceased and the snake was not targeted for sampling until the following day.

Field Photography

After blood collection, the tail of each snake was placed into a photo blind for the purpose of digital imagery. The blind consisted of a small box constructed of polyvinyl chloride pipes draped in blackout curtains to block ambient light. The blackout curtains had a small opening at the top through which the camera lens fit snugly. A white cloth was placed on the ground underneath the photo blind to prevent variation in reflectance based on ground cover and vegetation. The interior of the blind was lit with two 45.7-cm fluorescent ultraviolet B (UVB) bulbs (Reptisun 5.0 UVB, Zoo Med) run on a battery-powered portable generator (Rally Manufacturing 7471). A gray, nonreflective ruler was included in the frame of each photograph as a color standard. The ruler served as a gray point for light equalization in Photoshop (Adobe Systems Inc.), and it was nonreflective to minimize glare and reflected light. Photos were taken in RAW format with a Nikon D90 DSLR camera with a fixed 35-mm lens using autofocus and standardized settings (ISO = 800, f/1.8, shutter speed = 1/50, white balance = 5550 K) at a fixed height of 30 cm. Multiple photographs were taken of the dorsal region of the snake's tail with rattle visible, and the sharpest image was selected from each sample for further analysis.

Acute Stress via Confinement

At the 4-wk postimplant sampling event, metachrosis attributable to confinement stress was evaluated after baseline sampling. Snakes were collected, their defensive behaviors assessed, bled, and photographed immediately as described above, and then placed into white pillowcases in individual, white, opaque plastic buckets for 1 h and carried around while the search for other snakes continued. Confinement in buckets is a standardized handling stressor and has been shown to elevate CORT levels in snakes (Lutterschmidt et al. 2009; Holding et al. 2014a,b; Claunch et al. 2017). After 1 h in a bucket, each snake was removed from its bag and the cloacal temperature, blood, and photographs were taken as described above. There was some variability in time in bucket when capture and baseline sampling of additional snakes overlapped with the 1-h time point. We recorded and included time in bucket in our analyses.

Defensive Behavior Assay

Quantifying defensive behaviors is detailed in Claunch et al. (2017). Briefly, behavior was assessed via a scoring method ranking alertness behaviors lowest (tongue flicking = 1, retreating = 2) and active defense highest (rattling = 3, striking = 4, head hiding = 5). This scoring method was applied at sighting, at 5 s of tong touch, while lifting the snake, and when tubing. The method was applied for the baseline sample of the acute stress series, but not after acute confinement stress, as interpretation of this might not be biologically relevant. Scores for each snake were averaged across all time points. Head hiding was rarely observed. A higher mean score indicated that a snake was more likely to exhibit active defense, reveal its position, and remain in contact with a predator, whereas a lower score indicated that a snake was more likely to hold still in its position or retreat from the threat. Defensive behavior was not related to CORT levels, body temperature, or time of day in these snakes (Claunch et al. 2017), but was included as a covariate in color analyses to explore a link between behavior and metachrosis.

CORT Analysis

Plasma CORT levels were determined via radioimmunoassay of plasma as described in Lind et al. (2010). Samples were extracted in dichloromethane, then dried in a 40°C water bath under nitrogen gas. Samples were incubated overnight in 100 μL of antiserum (Esoterix Endocrinology) and 100 μL of tritiated steroid. Unbound steroid was separated from bound steroid using dextran-coated charcoal. A liquid scintillation counter was used to count bound steroid in samples, and final concentrations were corrected for extraction efficiency. Mean recovery for CORT was 64%. Serial dilutions for the standard curves were performed in triplicate (curve range = 2000–4 pg). The limit of detection was 2.5 ng/mL, and the intra-assay coefficient of variation was 7.3%.

Color Analysis

The researcher evaluating snake color was blind to the treatment group and CORT level of rattlesnakes in each photograph. Color ratios in the photographs were equalized using the white balance tool in the camera RAW editing window (Photoshop v5.0). In each image, the 3.3-cm mark on the gray nonreflective ruler was chosen as the gray point for color equalization. The average red, green, and blue (RGB) values of the gray point across all photographs were determined (184.62 ± 17.95, 183.82 ± 20.32, 184.83 ± 17.83, respectively) using the color sampler tool. Subsequently, each image's exposure was equalized by creating a new exposure layer and manually adjusting the exposure (with gamma correction of 1) such that the previously mentioned gray point in each image was equal to the average (184, 184, 184, respectively) on a RGB scale.

Each photo was sampled in RGB (chroma) and lab color schemes using the color sampler tool in Photoshop. The "L" in the lab metric reports value (lightness, *L*), whereas "a" represents the green–red color component and "b" represents the blue–yellow component. High *L* values are

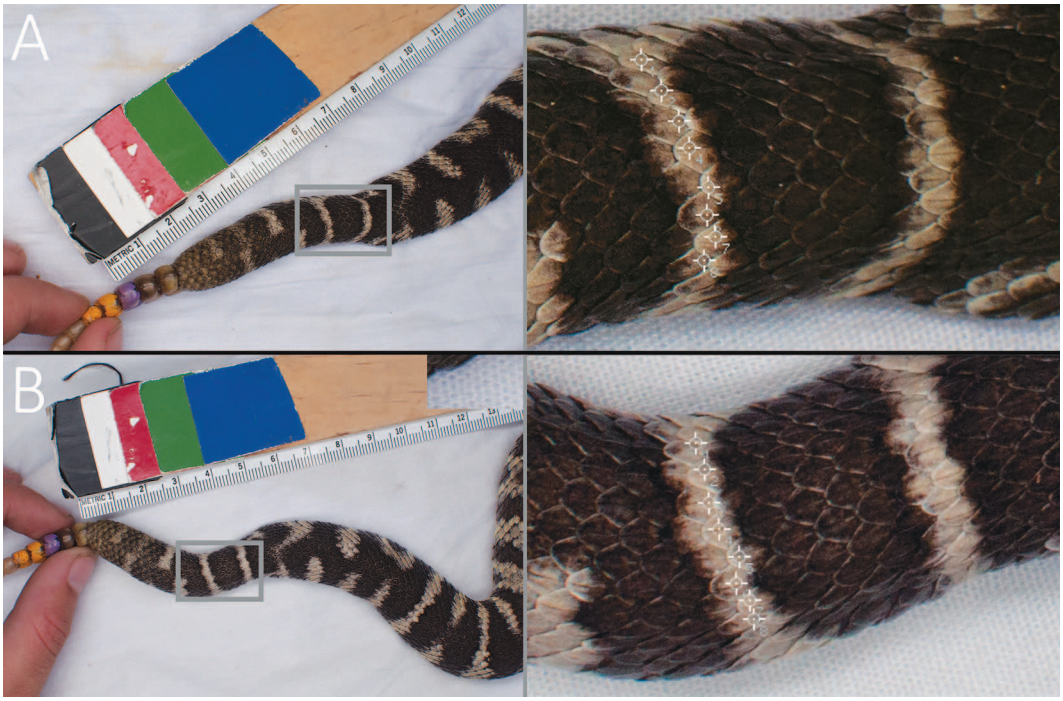

FIG. 1.—Color quantification in an adult male *Crotalus helleri* from digital images using Adobe Photoshop (A) before and (B) after 1 h of acute confinement stress. Note individual snake rattle identification by unique color code.

associated with lighter colors, whereas low *L* values are associated with darker ones. We did not include a and b in our analyses because their interpretation in LAB space requires three-dimensional models on account of their opposing nature (Endler 1990). Because the RGB values generally changed with the same direction and magnitude, which is equal to a change in *L* (Endler 1990), we report *L*. We did not use grayscale metrics because they have lower retention of Euclidian distance information (Indow 1980). The most posterior full light band on each snake was sampled by placing the color sampler tool along the scale row with the band at 31 × 31-pixel average. As anesthesia of snakes was not possible because of the confounding effects on stress hormone levels, precise remeasurement on the same scales was not possible. To account for this, eight evenly spaced areas along the center line of the band were sampled to cover the entire dorsal length of the band (Fig. 1). The second-most posterior light band was sampled similarly. The mean *L* value from all 16 samples of the two light bands on the same snake represent the light-band *L* value for the snake for the time point. A similar procedure was used for sampling the dark band color on each snake, starting with the dark band posterior to the most-posterior light band, and the dark band between the two sampled light bands. The distance from the basal rattle to the posterior-most light band on each snake was recorded, and this location was sampled in subsequent photos of that individual to maintain consistency. To avoid pseudoreplication, only the mean values for light bands and dark bands were used in analyses.

Data Analyses

We used repeated-measures analyses of covariance (ANCOVA) to examine the relationships between baseline stress data and the following responses: *L* value of light band, *L* value of dark band, and contrast (difference between *L* values of light and dark bands). Treatment and plasma CORT levels were included in separate models to avoid potential collinearity between these variables. Otherwise, the parameters for each model were identical. Factors included sample number (time from implant: 0, 2, or 4 wk postimplant) and size class, whereas covariates included time of day, body temperature, temperature differential (between ambient and body temperatures), SVL at capture squared (SVL^2), defensive behavior score, and the time to obtain blood. To account for repeated sampling, snake identification (ID) was included as a random factor. Data were transformed to meet the assumptions of parametric tests: SVL was squared, time to obtain blood was log transformed, and defensive behavior score was square-root transformed. One 4-wk postimplant sample was identified via Dixon's *Q* test (Dean and Dixon 1951) as an outlier with very high CORT levels (122.35 ng/mL, 5.51 SD from the mean) and was excluded from the analyses. All statistical analyses were conducted in JMP (Pro v12.1, SAS Institute Inc.).

We determined the degree of contrast explained by individuals using a repeatability analysis. High repeatability (*R* approaching 1) indicates that color differences are attributed to diversity of color patterns across our study snakes (i.e., color is consistent within individuals), whereas low repeatability (*R* approaching 0) indicates that variation in color is attributed to color changes within individuals. We calculated repeatability using mean-square values from a one-way analysis of variance using snake ID as the predictor on the basis of a previously established method assessing between-snake variance versus within-snake variance (Lessells and Boag 1987; Narayan and Hero 2013).

TABLE 1.—Repeated-measures analyses of covariance of several factors against various color metrics in wild *Crotalus helleri* at approximately 0, 2, and 4 wk after receiving implants of corticosterone (CORT; $n = 15$, 14, and 12, respectively) or blank ($n = 11$, 11, and 10, respectively) capsules. Baseline analyses refer to these time points, whereas the acute CORT change analysis refers to magnitude of CORT change after 1 h of confinement ($n = 22$). Light band represents an average lightness (L) value of 16 different points over the two most posterior light bands on the snakes' dorsal surface, and dark band represents the same of the two most posterior dark bands on the snakes' dorsal surface. Contrast is the difference in L values between the light bands and dark bands. SVL = snout–vent length. Values in bold are statistically significant.

Analysis	Factor/covariate	Response: light band	Response: dark band	Response: contrast
Baseline: plasma CORT	Sample number	$F_{2,35.47} = 1.11$, $P = 0.34$	$F_{2,38.88} = 0.58$, $P = 0.56$	$F_{2,34.48} = 1.83$, $P = 0.18$
	Size class	$F_{1,23.7} = 0.21$, $P = 0.65$	$F_{1,24.63} = 0.02$, $P = 0.90$	$F_{1,22.87} = 0.24$, $P = 0.63$
	CORT	$\mathbf{F_{1,40.56} = 10.14, P = 0.003}$ (+)	$F_{1,47.81} = 0.30$, $P = 0.58$ (+)	$\mathbf{F_{1,39.34} = 13.90, P < 0.001}$ (+)
	Log time to bleed	$F_{1,39.09} = 1.30$, $P = 0.26$	$F_{1,44.9} = 0.120$, $P = 0.73$	$F_{1,37.96} = 1.78$, $P = 0.19$
	Time of day	$\mathbf{F_{1,39.17} = 5.99, P = 0.02}$ (+)	$F_{1,45.91} = 0.27$, $P = 0.61$ (−)	$\mathbf{F_{1,37.99} = 12.54, P = 0.001}$ (+)
	SVL^2	$F_{1,22.62} = 0.10$, $P = 0.76$	$F_{1,22.93} = 0.16$, $P = 0.69$	$F_{1,21.85} = 0.44$, $P = 0.52$
	Temp. differential	$F_{1,41.35} = 2.43$, $P = 0.13$	$F_{1,48.63} = 0$, $P = 0.998$	$F_{1,40.13} = 3.63$, $P = 0.06$
	Body temp.	$F_{1,40.06} = 0.05$, $P = 0.82$	$F_{1,46.35} = 0.76$, $P = 0.39$	$F_{1,38.9} = 0.20$, $P = 0.66$
	Square-root avg. behavior score	$\mathbf{F_{1,39.88} = 9.37, P = 0.004}$ (−)	$F_{1,46.38} = 0.15$, $P = 0.70$ (−)	$\mathbf{F_{1,38.7} = 12.86, P < 0.001}$ (−)
Baseline: treatment group	Sample number	$F_{2,35.88} = 0.41$, $P = 0.67$	$F_{2,37.33} = 0.73$, $P = 0.49$	$F_{2,36.15} = 0.99$, $P = 0.38$
	Size class	$F_{1,22.17} = 0.999$, $P = 0.33$	$F_{1,22.26} = 0.41$, $P = 0.53$	$F_{1,22.28} = 0.73$, $P = 0.40$
	Treatment	$F_{1,22.92} = 1.68$, $P = 0.21$	$F_{1,23.24} = 2.901$, $P = 0.097$	$F_{1,23.06} = 0.26$, $P = 0.61$
	Sample number × treatment	$F_{2,34.47} = 0.47$, $P = 0.63$	$F_{2,35.74} = 0.58$, $P = 0.57$	$F_{2,34.72} = 1.85$, $P = 0.17$
	Log time to bleed	$F_{1,39.9} = 2.01$, $P = 0.16$	$F_{1,42.63} = 0.08$, $P = 0.78$	$F_{1,40.32} = 3.21$, $P = 0.08$
	Time of day	$\mathbf{F_{1,38.91} = 4.68, P = 0.04}$ (+)	$F_{1,41.81} = 0.62$, $P = 0.44$ (−)	$\mathbf{F_{1,39.35} = 10.28, P = 0.003}$ (+)
	Body temp.	$F_{1,43.01} = 0.25$, $P = 0.62$	$F_{1,46.06} = 0.51$, $P = 0.48$	$F_{1,43.48} = 0.018$, $P = 0.90$
	SVL^2	$F_{1,21.47} = 0.29$, $P = 0.59$	$F_{1,21.45} = 0.011$, $P = 0.92$	$F_{1,21.56} = 0.43$, $P = 0.52$
	Temp. differential	$F_{1,44.59} = 0.72$, $P = 0.40$	$F_{1,48} = 0.12$, $P = 0.73$	$F_{1,45.11} = 1.64$, $P = 0.21$
	Sqare-root avg. behavior score	$\mathbf{F_{1,42.66} = 8.14, P = 0.007}$ (−)	$F_{1,45.79} = 0.45$, $P = 0.50$ (−)	$\mathbf{F_{1,43.14} = 9.37, P = 0.004}$ (−)
Acute: CORT change	Size class	$F_{1,8} = 0.002$, $P = 0.97$	$F_{1,8} = 0.09$, $P = 0.78$	$F_{1,9} = 0.20$, $P = 0.66$
	Treatment	$F_{1,8} = 0.15$, $P = 0.70$	$F_{1,8} = 0.37$, $P = 0.56$	$F_{1,9} = 0.52$, $P = 0.49$
	Baseline CORT	$F_{1,8} = 0.0002$, $P = 0.99$	$F_{1,8} = 0.34$, $P = 0.58$	$F_{1,9} = 0.18$, $P = 0.68$
	Body temp.	$F_{1,8} = 2.09$, $P = 0.19$	$F_{1,8} = 0.31$, $P = 0.59$	$F_{1,9} = 3.40$, $P = 0.10$
	Time of day	$F_{1,8} = 1.01$, $P = 0.34$	$F_{1,8} = 0.02$, $P = 0.90$	$F_{1,9} = 2.10$, $P = 0.18$
	Log time to bleed	$F_{1,8} = 1.87$, $P = 0.21$	$F_{1,8} = 1.33$, $P = 0.28$	$F_{1,9} = 3.06$, $P = 0.11$
	Time in bucket	$F_{1,8} = 1.044$, $P = 0.33$	$F_{1,8} = 3.16$, $P = 0.11$	$F_{1,9} = 0.012$, $P = 0.91$
	CORT change stressed − initial	$F_{1,8} = 3.61$, $P = 0.09$ (+)	$F_{1,8} = 0.59$, $P = 0.46$ (−)	$\mathbf{F_{1,9} = 6.05, P = 0.04}$ (+)
	Temp. change stressed − initial	$F_{1,8} = 0.29$, $P = 0.61$	$F_{1,8} = 0.14$, $P = 0.72$	$F_{1,9} = 0.24$, $P = 0.64$
	SVL^2	$F_{1,8} = 0.14$, $P = 0.72$	$F_{1,8} = 2.99$, $P = 0.12$	$F_{1,9} = 0.0001$, $P = 0.99$

To assess the influence of acute CORT increase on metachrosis, the magnitude of change in L value after 1 h of confinement was assessed for light bands, dark bands, and contrast. Factors in this analysis included size class and treatment, whereas covariates included initial CORT, CORT change (stressed − initial), initial temperature, temperature change (stressed − initial), time of day at capture, time to bleed at initial capture, time in bucket (min), and SVL^2.

Variable recapture success led to differing sample sizes for each capture period (preimplant = 15 treatment, 11 control; 2 wk postimplant = 14 treatment, 11 control; 4 wk postimplant = 13 treatment, 9 control). Repeated-measures ANCOVAs are robust to missing values, and snakes with a maximum of one missing sample were included in analyses. We report the sample size by treatment group for each analysis.

RESULTS

Claunch et al. (2017) reported that CORT levels in CORT-implanted snakes increased at 2 wk postimplant and returned to preimplant levels at 4 wk postimplant. A repeated-measures ANCOVA including all three sampling periods of the implant experiment (preimplant, 2 wk postimplant, and 4 wk postimplant) revealed that implant treatment did not influence metachrosis, as there was no significant interaction between treatment type and sample number (Table 1). This might be explained by high variation of light band L values between groups (Fig. 2), which could have masked any effects of treatment thereafter. The observed natural variation in CORT presented an opportunity to conduct a second, fine-scale ANCOVA testing a potential relationship between two continuous variables, color and CORT, independent of treatment. Plasma CORT was positively associated with light band L (effect size = 2.56) but not dark band L, such that light bands were lighter in snakes with higher CORT, leading to greater contrast between the bands (effect size = 3.22; $n = 15$ treatment, 11 control; Table 1; Fig. 3). In both ANCOVAs, time of day was positively related to light band L and contrast but did not influence dark band L (Table 1). In addition, average defensive behavior score was negatively associated with light band L and contrast in both treatment and plasma CORT models such that snakes exhibiting greater contrast were less likely to show active defensive behaviors such as rattling and striking (Table 1; Fig. 4). Variation in color was not significantly related to sample number, size class, body

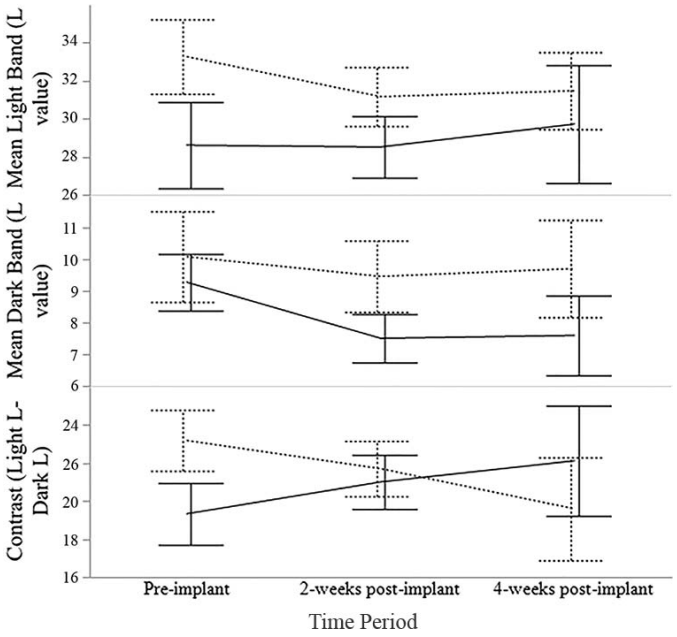

FIG. 2.—Mean values for color lightness (*L*) of light and dark bands in adult male *Crotalus helleri* treated with corticosterone implants (solid) and blank implants (dashed) at three time periods. Bars indicate ± 1 SE.

temperature, SVL^2, temperature differential, or time to bleed (Table 1). Contrast within individuals was not highly repeatable ($R = 0.38$, $P = 0.001$), indicating that 38% of variation in color across all samples can be explained by diversity in color among individual snakes, whereas the majority of color variation (62%) was attributable to within-snake color change.

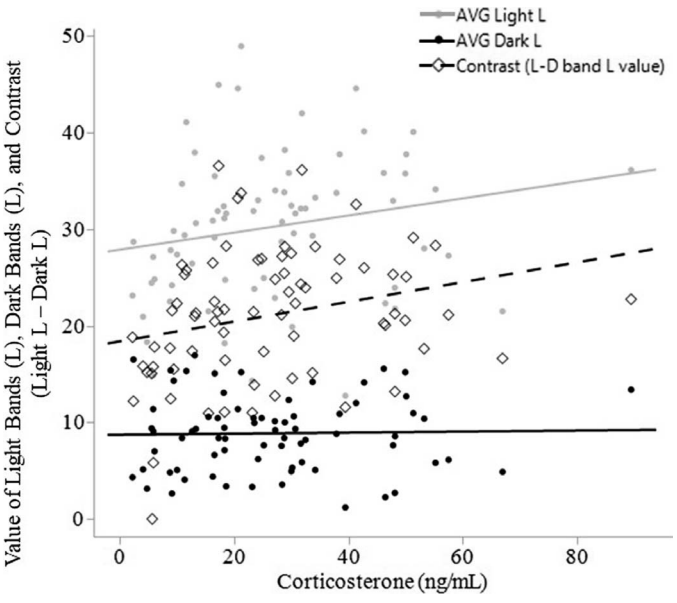

FIG. 3.—Relationships between mean values of color lightness (*L*) and corticosterone levels in adult male *Crotalus helleri* treated with corticosterone or blank implants, shown for light (gray circles) and dark (black circles) bands, as well as the contrast between the two band types (dashed diamonds). Regardless of treatment group, plasma corticosterone was associated with lighter light bands ($P = 0.003$, $R^2 = 0.04$) and more contrast ($P < 0.001$, $R^2 = 0.07$) between light and dark bands.

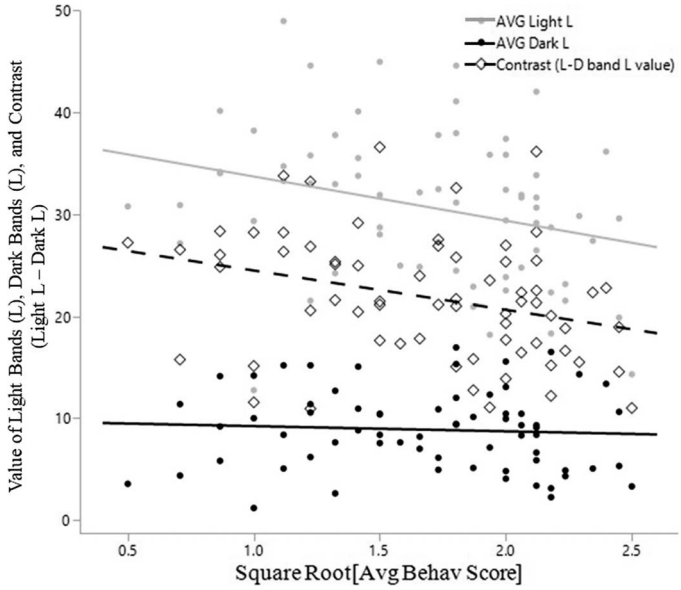

FIG. 4.—Relationships between mean values of color lightness (*L*) and defensive behavior score in adult male *Crotalus helleri* shown for light (gray circles) and dark (black circles) bands, as well as the contrast between the two band types (dashed diamonds). Greater intensity of defensive behaviors (e.g., rattling, striking, etc.) was related to darker light bands ($P = 0.004$, $R^2 = 0.08$) and lower contrast ($P < 0.001$, $R^2 = 0.10$) between light and dark bands.

For the acute stress test, neither treatment nor initial (baseline) CORT level affected the magnitude of color change (difference between baseline value and that after 1 h of confinement) in light bands, dark bands, or contrast ($n = 13$ treatment, 9 control; Table 1). However, a greater magnitude of CORT response (difference between stressed CORT and baseline CORT levels) was significantly related to a greater increase in color contrast (Table 1). Whereas the slopes of the relationship between CORT response and light *L* (positive) and dark *L* (negative) were similar, the directional trends support the relationship between CORT response and contrast (Fig. 5). All other variables regarding color response or change in contrast were not statistically distinguishable.

DISCUSSION

Our data indicate that experimental CORT implants had no direct effect on color or metachrosis in *C. helleri*. However, CORT implants increased baseline plasma CORT in the treatment group (Claunch et al. 2017) and baseline CORT was positively correlated with lighter light bands and higher contrast between bands across both treatment groups. Effect of treatment might have been masked by inherent differences in dorsal patterns between individuals within each group (i.e., inherently lighter bands on most control individuals at all time points; Fig. 4), individual differences in endogenous clearance rates or CORT receptor densities (Cockrem 2013), and small sample size, despite random assignment of subjects. Individuals of the same species can vary in endogenous production and clearance rates of exogenous CORT (Cockrem 2013). Because we created a wide range of endogenous plasma CORT levels, whether naturally occurring or attributable to implant, our results are

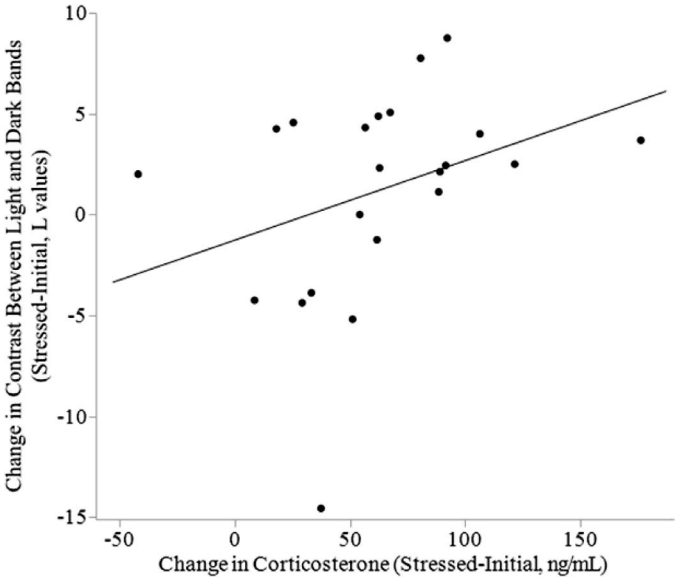

Fig. 5.—Contrast between light and dark bands of adult male *Crotalus helleri* as a function of the change in plasma corticosterone levels after 1 h of confinement ($P = 0.04$, $R^2 = 0.18$).

still interpretable on the basis of analysis of the continuous variable CORT for all individuals. Plasma CORT was positively related to the lightness of light bands and to contrast, with little to no relation to dark bands. Whereas we originally hypothesized that CORT is related to color change, our implant experiment supports the hypothesis that CORT is related simply to color. In the acute stress experiment, however, magnitude of CORT change was positively related to increased contrast between bands, which supports our metachrosis hypothesis.

It is possible that higher levels of CORT are required to induce color change, or that bioregulators other than CORT contribute to metachrosis. We might have observed a more direct effect of CORT on color in our implant treatment if we increased the dosage of CORT. Even at 2 wk post-implantation, CORT-treated snakes had CORT levels completely within naturally occurring baseline values for rattlesnakes (2.18–89.47 ng/mL; cf. Schuett et al. 2004; Lind et al. 2010; Capehart et al. 2016; Heiken et al. 2016) and much lower than CORT levels induced by acute confinement. CORT implantation in Tawny Dragons (Agamidae: *Ctenophorus*) produced a broader range of plasma CORT as compared with our study, leading to decrease in achromatic contrast of throat coloration (Lewis et al. 2017). Thus, even though treatment snakes had higher CORT levels than control snakes, an even greater chronic elevation of CORT might be necessary to induce measurable color changes over longer time periods. Metachrosis might also be affected by bioregulators aside from CORT. During an acute stressor such as confinement, CORT is synthesized de novo, which could delay its action on various processes such that the extent of downstream effects of increased plasma CORT were not observed within the 1-h time frame of this study (Turner and Bagnara 1971). The context of the acute stressor might also influence CORT secretion and metachrosis. Sweet (1985) presented anecdotal evidence that a rattlesnake could transition from dark black to light gray within minutes of sighting if it exhibited continuous defensive behavior during that time. It is possible that active harassment might induce a rapid CORT response and more rapid metachrosis. It is also likely, however, that neural mechanisms or more rapidly acting hormones might affect metachrosis in these cases. In contrast to Sweet's (1985) observations, we observed slower metachrosis in rattlesnakes, including that measured after our 1-h acute confinement protocol, where the change in CORT was related to the change in color.

Although we found some support for the CORT-mediated metachrosis hypothesis, a mechanism-based approach might further our understanding of this phenomenon. CORT is produced by the adrenal glands in response to adrenocorticotropic hormone (ACTH). Both ACTH and MSH are melanocortins that derive from the same precursor, POMC (Proulx-Ferland et al. 1982). Color fixation (e.g., inability to change color) has been induced by removal of the pituitary gland (Kleinholz 1936, 1938; Rahn 1941; Rahn and Rosendale 1941), likely because ACTH and MSH are no longer produced. ACTH and MSH can both independently induce similar responses in any given chromatophore (Nielsen 1978), such as melanophore dispersion or iridophore (blue pigment cell) contraction (Dikstein and Sulman 1964; Ide 1973; Butman et al. 1979; Carter and Shuster 1982; but see Nielsen 1978). Elevated CORT might lead to a decrease in levels of POMC through negative feedback inhibition, reducing ACTH and MSH (Proulx-Ferland et al. 1982; Schimichowitsch et al. 1994). Because ACTH and MSH generally cause darkening on account of melanophore dispersion (Ide 1973; Butman et al. 1979; Carter and Shuster 1982), reduced levels of these hormones caused by higher CORT could have triggered the lightening of the light bands and increased contrast observed in this study. Indeed, higher CORT and iridophore contraction were associated with paler throat coloration in Tawny Dragons (Lewis et al. 2017). This hypothesis could be further evaluated by measuring circulating levels of ACTH and MSH or by treatment with melanocortin receptor antagonist.

Time of day was positively related to light band lightness and contrast in both the treatment and baseline CORT models. Because of the diel rhythm of MSH (Turner and Bagnara 1971; Sherbrooke 1988), we also expected to see a relationship between time of day and color. Our results showed that contrast was higher, with light bands lighter, later in the day. Indeed, several snake species show diel cycles in coloration that corresponded with lighter colors during the most active hours (McAlpine 1983; Hedges et al. 1989; Boback and Siefferman 2010). Whereas diel cycling of MSH is a likely causative agent (Boback and Siefferman 2010), the influence of CORT on this system cannot be discounted because CORT also shows diel cycling and is typically highest during active periods (Breuner et al. 1999; Jones and Bell 2004; Quillfeldt et al. 2007; Malisch et al. 2008). It might be adaptive for snakes to have increased contrast and therefore improved camouflage at certain times of the day, for example when moving through tall vegetation or resting in scattered shadows (Merilaita 2003; Cuthill et al. 2005; Merilaita and Lind 2005; Kang et al. 2016). To achieve a more comprehensive understanding of diel cycles, CORT and color could be quantified at different times over several 24-h periods.

In contrast with other studies (e.g., Rahn 1942; Neill and Allen 1955; Muri et al. 2015), we did not detect any

relationship between body temperature and color. As such, it is unlikely that color changes recorded for our subjects were attributable to thermoregulatory needs. It is possible that metachrosis in the dorsal, distal portion of the tail is not influenced by temperature, but that other areas on the body might be affected by temperature, such as reported for Bearded Dragons (*Pogona vitticeps*; Velasco and Tattersall 2008). Weak evidence for differential metachrosis across the body was found in a study on Sidewinders (*C. cerastes*), but the colors were measured using a color chart that was subject to interpretation (Neill and Allen 1955). To further investigate this phenomenon, our color quantification methods could be replicated over a greater range of the snakes' bodies to evaluate patterns of metachrosis throughout the body, and at different temperatures.

Rattlesnakes with high contrast were less likely to give up their position (i.e., not as likely to rattle, strike, or direct attention to themselves) when disturbed during our defensive behavior assays. The observed relationship between higher color contrast and decreased defensive behavior corroborates the idea that increased contrast is adaptive in certain contexts (Merilaita 2003; Cuthill et al. 2005; Merilaita and Lind 2005; Kang et al. 2016). Rattlesnakes with high contrast might be more successful at evading detection by prey and predators given that tall grasses and savannah duff provide scattered shadows in which snakes with high contrast seem to disappear; this would be especially true if paired with the snakes' reluctance to abandon their crypsis by rattling. The adaptive nature of metachrosis in these rattlesnakes can only be assumed, however, until predatory success or depredation avoidance of rattlesnakes with different levels of contrast are assessed.

We have demonstrated a relationship between circulating CORT and color, and additionally, a relationship between acute stress and color change in Southern Pacific Rattlesnakes. The mechanisms by which CORT interacts to influence color remain to be tested. Studied in an ecological context, snake coloration and metachrosis and the underlying mechanisms might provide insight into the adaptive nature and evolution of metachrosis among reptiles.

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