Trait Covariances in Eastern Box Turtles Do Not Support Pleiotropic Effects of the Melanocortin System on Color, Behavior, and Stress Physiology

Authors: Carlson, Bradley E., and Robinson, William L.

Source: Journal of Herpetology, 56(4) : 478-488

Published By: Society for the Study of Amphibians and Reptiles

URL: https://doi.org/10.1670/22-010

Journal of Herpetology, Vol. 56, No. 4, 478–488, 2022

Trait Covariances in Eastern Box Turtles Do Not Support Pleiotropic Effects of the Melanocortin System on Color, Behavior, and Stress Physiology

Bradley E. Carlson[1,3] and William L. Robinson[1,2]

[1]*Wabash College, Crawfordsville, Indiana, 47933, USA*

Abstract.—**Endocrine systems and individual behavioral differences (temperament) are often linked in animals. In particular, glucocorticoids (corticosterone [CORT]) have been implicated in animal coping styles, or syndromes of integrated temperamental and neuroendocrine variation. Typically, organisms with lower stress-induced elevations of CORT tend to exhibit more proactive behavior. Melanin-based coloration has been further linked to CORT physiology and temperament, with more melanistic individuals typically exhibiting more proactive coping styles. The melanocortin hypothesis proposes that variation in the melanocortin system could drive the repeated covariation in coloration, coping style, and CORT levels. We evaluated the relationships among the CORT stress response, boldness (i.e., responsiveness to risk), and melanization of the shell in Eastern Box Turtles (*Terrapene carolina carolina*), predicting that turtles with bolder temperaments would exhibit lower stress-induced CORT levels and possess darker shells. We also expected stress-induced CORT levels to be lower at cooler body temperatures. Our results generally failed to support the melanocortin hypothesis. We found no significant correlations among behavior, CORT, and melanization, and correlations that approached significance were weak. Moreover, the near significant relationship between CORT levels and boldness is in the opposite direction predicted. We also found that temperature had a strong positive effect on CORT levels, and there were population differences in plastron melanization and boldness.**

Animal temperament is present when there is significant among-individual variation in behavior (Réale et al., 2007; also known as personality, behavioral syndromes, and other terms, MacKay and Haskell, 2015) and is ubiquitous across taxa (Bell et al., 2009). In addition to behavior, endocrine profiles often vary substantially within populations (Kempenaers et al., 2008; Williams, 2008; Cockrem, 2013). Given the integrative role of the endocrine system in diverse physiological and behavioral activities, hormones could function as mediators of variation in temperament (Koolhaas et al., 1999), though the lower individual consistency of hormone levels relative to behavior suggests they are unlikely to drive behavioral variation (Holtmann et al., 2017).

Alternatively, endocrine systems and temperament could be coevolved, with correlational selection favoring particular temperaments in individuals with certain states (i.e., physiological characteristics; Wolf and Weissing, 2010). In particular, the glucocorticoid corticosterone (CORT) has been implicated in coping styles, or syndromes of integrated temperamental and neuroendocrine variation (Koolhaas et al., 1999). Corticosterone is produced predominantly in the adrenal or interrenal glands of vertebrates as part of the hypothalamic–pituitary–adrenal/interrenal (HPA/I) axis, and levels of CORT exhibit individual repeatability (Taff et al., 2018). At the onset of a potential stressor (i.e., a threat to survival or homeostasis), CORT levels are generally increased from baseline levels, altering physiological activities to cope with current or future stressors, and CORT is therefore often generally referred to as a stress hormone (Sapolsky et al., 2000). One function of CORT is to mobilize energy reserves (i.e., glucose), along with a wide variety of other activities (Sapolsky et al., 2000; Vera et al., 2017). Corticosterone rises over a period of minutes to hours and has slow-acting effects via changes in gene transcription (Sapolsky et al., 2000) but can stimulate certain responses quickly by binding to membrane-bound receptors (Moore and Orchinik, 1994; Evanson et al., 2010). Typically, individuals that exhibit proactive coping styles are characterized by readily attacking or escaping threats and having lower stress-induced CORT than conspecifics who are reactive (e.g., exhibiting an immobility response; Koolhaas et al., 1999).

Additionally, melanin-based coloration has been linked to both CORT physiology and temperament, with more melanistic individuals typically exhibiting proactive coping styles (Ducrest et al., 2008; Santostefano et al., 2019). The melanin-based coloration syndrome may be the result of pleiotropic effects of the melanocortin system, and in particular the peptide hormones derived from the proopiomelanocortin gene (*POMC*) and their associated receptors (Ducrest et al., 2008). The POMC protein, encoded by the *POMC* gene, is cleaved to produce adrenocorticotropic hormone (ACTH), an upstream hormone in the HPA/I axis that stimulates the release of CORT. However, ACTH can be further spliced into α-melanocyte stimulating hormone (α-MSH), while other splicing products of POMC include β-MSH and γ-MSH. As melanizing hormones, MSH darkens melanin-based coloration (Ducrest et al., 2008). As shared cleavage products of the POMC protein, ACTH and MSH are likely to covary in their levels depending on the expression of *POMC* (Roulin et al., 2011). Moreover, these hormones are all capable of binding with variable affinity to the several melanocortin receptors (MC1–5R) that regulate several biological activities related to coloration, stress physiology, and behavior (e.g., ACTH can stimulate melanocyte activity by binding to MC1R in the skin in addition to stimulating the HPA/I axis in the adrenal glands; Ducrest et al., 2008). Consequently, the melanocortin hypothesis proposes that the melanocortin system could drive the repeated covariation of these diverse traits related to hormones, coping, and temperament.

The melanocortin hypothesis has not, however, received consistent support, with some studies finding that melanin-based coloration was independent of behavior and/or stress physiology (e.g., Jenkins et al., 2013; San-Jose and Roulin, 2018; Santostefano et al., 2019). Moreover, though the link between CORT and temperament has generally become well-established

[2]Present address: Indiana Department of Environmental Management, Indianapolis, Indiana 46204, USA
[3]Corresponding author. E-mail: carlsonb@wabash.edu
DOI: 10.1670/22-010

in mammals and birds (Cockrem, 2007; Øverli et al., 2007; Carere et al., 2010; Hau and Goymann, 2015), evidence for this pattern is mixed (Westrick et al., 2019). For instance, the relationship between CORT and temperament can vary among age classes (Seltmann et al., 2012) and may be undetectable in wild populations under strong stabilizing selection (Archard et al., 2012 and citations therein). Furthermore, sympathetic nervous system reactivity may also be a key physiological correlate of temperament differences (Qu et al., 2018), and the combination of sympathetic and HPA/I axis reactivity could yield a more complex array of behavioral phenotypes (i.e., in addition to bold and shy temperaments, individuals may be docile or panicky, for example; Koolhaas et al., 2010).

A promising avenue for better evaluating support for the melanocortin hypothesis is to expand the taxonomic breadth of study. Nonavian reptiles (e.g., snakes, lizards, turtles, and crocodilians, which together are twice as speciose as mammals; Pincheira-Donoso et al., 2013) have been neglected in studies examining the relationships among stress physiology, temperament, and melanization as per the melanocortin hypothesis (but see Seddon and Hews, 2016), though some studies have evaluated correlations between pairs of these variables (e.g., Thaker et al., 2009; Mafli et al., 2011; Ibáñez et al., 2016; Mell et al., 2016; Currylow et al., 2017; Herr et al., 2017; Brashears et al., 2020). Furthermore, reptiles are ectotherms and therefore generally exhibit lower metabolic rates than endotherms (White et al., 2006) and lifestyles characterized by energy conservation (Sandmeier and Tracy, 2014). Consequently, energy mobilization in the stress response may carry different strategic value compared with the endotherms that have been the focus of stress physiology research (Herr et al., 2017). Indeed, reptiles with higher CORT levels typical of a reactive coping style have been found to exhibit more aggression toward humans (Mell et al., 2016; Herr et al., 2017; Brashears et al., 2020) and to flee sooner from predators (Thaker et al., 2009), behaviors that are more consistent with a proactive coping style (Koolhaas et al., 1999). A link between a reactive coping style and high CORT levels may occur because proactive behavioral strategies often require additional energy expenditure, and higher CORT levels may permit such behavior in otherwise energy-conserving animals. Additionally, as poikilotherms, reptile body temperatures are determined by a thermally variable environment. Reptile temperament (e.g., Citadini and Navas, 2013) and CORT physiology (e.g., Telemeco and Addis, 2014; Jessop et al., 2016; Moleón et al., 2018; Racic et al., 2020) can be temperature-dependent, and melanization can affect thermoregulation (Clusella Trullas et al., 2007). As a result, temperature may be independently associated with coping styles and melanization in reptiles and therefore should be accounted for when evaluating correlations between traits.

We evaluated the relationships among the CORT stress response, boldness (i.e., responsiveness to risk), and melanization of the shell in Eastern Box Turtles (*Terrapene carolina*, specifically the Woodland Box Turtle subspecies, *T. c. carolina*). We hypothesized that turtles with bolder temperaments would exhibit lower stress-induced CORT levels, based on coping style theory (Koolhaas et al., 1999), and that these bolder turtles would also have darker, more melanized shells (Mafli et al., 2011; Ibáñez et al., 2016), reflecting pleotropic effects of the melanocortin system. We also expected fluctuations in temperature (attributable to ambient conditions or performance of thermoregulatory behavior) to play a critical role: stress-induced CORT levels should be lower in cooler individuals because of limitations on physiological activity (Gienger and Urdiales, 2017), boldness should remain consistent or moderately increase with higher temperatures as observed previously (Kashon and Carlson, 2018; Pich et al., 2019; Carlson and Tetzlaff, 2020), and darker turtles should be warmer because of faster heating during basking behavior (Clusella Trullas et al., 2007).

Materials and Methods

Study Species.—Eastern Box Turtles (*T. carolina*) are predominantly terrestrial turtles found in much of the eastern United States, primarily occupying forested habitats (Dodd, 2001). Box turtles (*Terrapene* spp.) are characterized by a domed carapace (upper shell) and hinged plastron (lower shell), with the latter allowing the shell to seal completely around the head and limbs (Dodd, 2001). It is difficult for most predators to successfully attack an adult turtle enclosed in the shell, yet individuals seem to vary in the extent to which they emerge from the shell and employ more active defenses (e.g., fleeing, biting, urinating, etc.; Dodd, 2001; Pich et al., 2019). Individual Eastern Box Turtles exhibit very consistent differences in response to human handling and confinement stress, with some individuals' heads emerging consistently from their shells immediately or quickly after confinement (i.e., bold) and others remaining within their shell for a prolonged period (i.e., shy; Kashon and Carlson, 2018; Pich et al., 2019; Carlson and Tetzlaff, 2020). The head emergence time was found to be highly repeatable across several years, both in captive juveniles and in wild adults (Carlson and Tetzlaff, 2020). Moreover, this variation in boldness was not correlated with other phenotypic characteristics (e.g., sex or body size) or most testing conditions (e.g., the number of trials to which they have been subjected; Kashon and Carlson, 2018), though in some analyses there were modest reductions in head emergence time in warmer individuals (Pich et al., 2019; Carlson and Tetzlaff, 2020).

Eastern Box Turtles are also highly variable in shell color patterns (Dodd, 2001), most notably in the extent of black versus brown-yellow coloration on the carapace and plastron (Fig. 1). Ontogenetic change in coloration could be one source of color pattern variability in *T. c. carolina*. Ontogenetic color change occurs in some other turtle species (Tucker et al., 1995; Ennen et al., 2015), including a different subspecies of Eastern Box Turtle (*T. c. triunguis*; Leuck and Carpenter, 1981), the coloration of which is distinct from *T. c. carolina*. We know of no studies documenting the development of shell coloration across the lifespan in *T. c. carolina*, but a general trend is apparent: hatchlings possess almost uniformly dark brown carapaces that become lighter brown with little patterning as subadults, with mature animals being distinctly patterned with contrasting dark and light areas (Fig. 1). We therefore restricted our analysis to adult turtles (>115 mm plastron length; Williams and Parker, 1987). Dramatic differences in shell color still occur among mature adults (Dodd, 2001), and we note that in our study population, several individuals are known to have been marked 58 yr prior to this study and were already adults (≥10 yr old) at the time (Williams and Parker, 1987), and these now >68-yr-old individuals display the full range of variation in shell melanization.

Corticosterone levels in Eastern Box Turtles have been previously reported to vary across years and seasons but not to differ between sexes (West and Klukowski, 2018). A previous study evaluated correlations between CORT and behavioral responses of Eastern Box Turtles to simulated attacks and found no relationship; however, individual animals received different

FIG. 1. Example carapace (upper row) and plastron (lower row) images of turtles demonstrating a range of melanization from low (left) to high (right). Upper row and lower row images are not paired and represent different individual turtles.

types of simulated attack treatments with small sample sizes within each treatment, temperature and melanization were not incorporated into the study, and stressor duration may have been insufficient to elicit elevated CORT levels (Preston et al., 2020).

Study Sites.—We conducted our study at Wabash College's Allee Memorial Woods (AMW), a 72-ha forested preserve in Parke County, Indiana, USA, and at Weiler–Leopold and Black Rock Barrens Nature Preserves (WLBRB), two adjacent, predominately forested preserves comprising 113 ha in Warren County, Indiana, USA. Allee Memorial Woods is located approximately 56 km south of WLBRB and is the site of an Eastern Box Turtle population that was studied from 1958 to 1983, largely by capturing and marking turtles (Williams and Parker, 1987). Beginning in September 2014, we began collecting and marking turtles again at AMW. Subsets of these turtles at AMW were used for disease surveillance (having blood samples and mouth and cloacal swabs collected), tracked by radiotelemetry, and repeatedly subjected to behavioral assays over a period of 4 wk. Some of the turtles included in the present study were also used in one or both of these previous studies (1958–1983 and/or beginning in 2014), though there is little evidence of effects of repeated handling on head emergence time (Kashon and Carlson, 2018) and head emergence is consistent over years (Carlson and Tetzlaff, 2020). The turtles at WLBRB have not been studied but may have had occasional experience with human visitors to the nature preserve.

Field Data Collection.—Turtles were collected from 16 May to 15 June 2016 and between 14 May and 28 June 2018 by visual search. Once located, we immediately used an infrared thermometer to measure plastron temperature of an encountered turtle, and then we collected approximately 200 μL of blood from the subcarapacial sinus using a 22-gauge needle (the baseline sample). We recorded the time of initial encounter and the time blood draws were complete, which varied from 1 to 5 min (mean = 2.7 min). Blood samples were immediately stored in heparinized tubes on ice. We then placed the turtle in a zippered, opaque, black nylon bag (18 × 32 cm) in which a small amount of movement was possible. After 30 min, we removed the turtle and immediately drew another blood sample (~100 μL; the stress-induced sample). This process was completed in 0.5–7 min, resulting in a total time of 32–41 min from first contact with the turtle to completion of the final blood draw (the stressor exposure time; mean = 34.7 min). One person then placed the turtle on the ground at or close to its original position and then walked away while another person quietly observed the turtle from approximately 10 m away. The observer recorded the time elapsed until the head of the turtle emerged (emergence latency), defined by the eyes surpassing the anterior margin of the carapace (modified after Ibáñez et al., 2014) and waiting up to a maximum of 10 min.

We recorded plastron length and sex of the turtle as covariates for analysis, though neither has been previously found to correlate with CORT levels or boldness (Kashon and Carlson, 2018; West and Klukowski, 2018). We used calipers to measure the length of the plastron as a metric of overall body size. We sexed the turtles primarily by the presence of a pronounced indentation on the plastron in males, as well as the position of the cloaca and length of the tail (Dodd, 2001).

We photographed the carapace and plastron of each turtle. For all photographs, we used a D7100 DSLR Camera with an AF-S DX Micro-NIKKOR 40mm f/2.8G lens (Nikon) with a macro ring flash (Sigma). We used identical settings for all photographs to minimize instrumental sources of variation (aperture = f/8, ISO = 200, RAW+JPEG file encoding, and 3 levels of exposure to ensure there was at least one well-exposed photograph per individual). Next to the turtle in each photo was an 18% gray reflectance standard to account for environmental sources of variation in color.

Within 7 h of collection, blood samples were centrifuged at 4,000 revolutions per minute for 2 min to separate plasma, which was then stored separately at −80°C until processing. We excluded from later analysis all turtles for which plasma samples were contaminated with lymph during collection or that experienced observable hemolysis during centrifugation, resulting in a final sample size of $n = 68$ turtles (29 females and 39 males; 59 from AMW and 9 from WLBRB).

Hormone Measurement.—We measured CORT levels in plasma using a high sensitivity enzyme immunoassay (EIA) kit for corticosterone (Immunodiagnostic Systems Limited, product number AC-15F1). The EIA kit that we used has been successfully used with lizards (Trompeter and Langkilde, 2011), snakes (Herr et al., 2017; Tylan et al., 2020), amphibians (Graham et al., 2012), and birds (Pike and Petrie, 2006). We determined recovery and linearity of dilution to validate this kit for Eastern Box Turtles. Serial dilutions of pooled plasma samples were linear and parallel to serial dilutions of kit standards. We spiked pooled plasma samples with known concentrations of CORT to evaluate recovery, which averaged 110.2%, indicating high accuracy with a small positively biased error.

Assays were performed using five kits. For the first two kits, plasma had been stored for 115–151 d prior to assays; for the third kit, samples had been stored for 361–397 d; for the fourth and fifth kits, samples had been stored for 39–84 d. CORT is generally stable for long-term storage while frozen (Reimers et al., 1982; Garde and Hansen, 2005; Herring and Gawlik, 2009), and there was no variation among CORT kits in estimated CORT levels despite the large differences in storage time (likelihood ratio test of censored regression models [see below]: $\chi^2_4 = 5.63$, $P = 0.23$). We diluted 15 μL of plasma in 35 μL of assay buffer and followed kit instructions. Given the specified lowest sensitivity of the kit (0.17 ng/mL) and the dilution used, we were unable to detect CORT levels in plasma below 0.57 ng/mL. Intraassay coefficients of variation ranged from 2.7 to 6.6%, and the mean interassay coefficient of variation was 15.6%.

Color Pattern Analysis.—Our approach to characterizing the extent of melanization was to determine the proportion of shell area covered by dark (black to dark brown) versus light (yellow to light brown) pigmentation. We adopted this pattern-based, binary approach (dark versus light areas) rather than quantifying the value or lightness of the shell because of uncontrollable sources of variation in value: the curvature of the shell produces uneven reflectance of light, making certain areas appear brighter, and soil encrusted between annuli on the scutes altered perceived coloration. We used the program ImageJ (Schneider et al., 2012) for all analyses. For carapace photographs, we cropped images to only include the second vertebral scute, because it most reliably was oriented approximately parallel to the camera (whereas patterning on much of the dome-shaped carapace would be distorted as a result of perspective), and because relative differences in melanization among turtles appeared to be consistent across all carapacial scutes. We used the Smooth tool to blur out small bright spots (specular highlights) associated with surface irregularities. We measured the area of the entire scute (in pixels) and then used the Color Threshold tool to select only the dark regions of the scute. Threshold values were adjusted until there was a good match between the selected area and the actual, visually assessed patterning of the shell. The area of the selected region was measured and divided by the total area to determine the percent of the scute that was melanized (dark).

Color patterns are relatively indistinct on the plastron, with darker regions usually grading into lighter regions. To describe the proportion of the plastron that exhibited dark coloration, we visually scored the percent of the plastron that appeared dark in the photographs, treating moderately darkened areas as intermediate in melanization. Only the region of the plastron posterior to the hinge was considered because the anterior portion was frequently bent away from the plane of the camera when turtles were enclosed in the shell. To validate this subjective assessment of melanization, we used the Multispectral Imaging plugin (Troscianko and Stevens, 2015) to normalize images to the 18% gray standard included in the photographs and then measured the luminance of the posterior plastron. Our image analysis approach was objective but was limited in reliability by the presence of brighter areas associated with the curvature of the plastron. Luminance values correlated well with our visual assessments ($r = 0.84$), and we therefore used the latter because they describe melanization as percent of area, similar to how we analyzed the carapace. Importantly, all aspects of measuring melanization were performed blind to associated morphometric, behavioral, or hormonal data and should therefore be free of unintentional bias.

Statistical Analysis.—We first tested whether there was evidence that confinement stress increased CORT levels, demonstrating that postconfinement CORT levels reflected a stressed state. Because 92.6% and 42.6% of pre- and postconfinement CORT levels, respectively, were below the detection limit (left-censored), we used censored regression to analyze these data. We used all data without imputing a value (such as zero, or the detection limit) in place of censored values, and the analysis incorporates information on whether or not data points were above or below the detection limit as well as known values for data points that were not censored (Helsel, 2005; Chambers et al., 2011). We used the censReg package in R (Henningsen, 2017) to test for the effect of blood sampling period (pre- versus postconfinement) on log-transformed CORT values, including individual identity as a factor to allow a paired comparison.

For the remainder of the analysis, our objective was to evaluate correlations among behavior, stress physiology, and melanization, both with and without accounting for the effects of covariates. We used ln(x+1)-transformed head-emergence latency as the measure of boldness, ln(x)-transformed postconfinement CORT levels as the measure of stress physiology, and the percent of darkly colored area on both the carapace and plastron as measures of melanization. We chose to restrict the analysis to postconfinement CORT levels because only 7.4% of preconfinement CORT levels were above the detection limit,

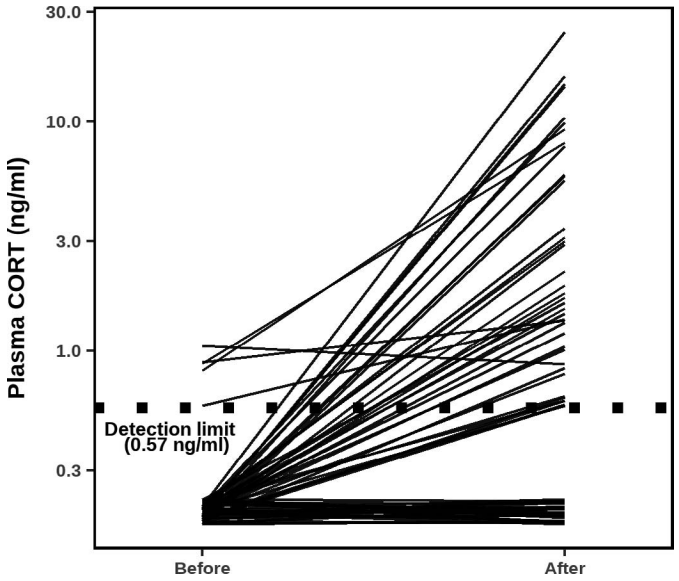

FIG. 2. Plasma corticosterone (CORT) levels from before and after confinement stress. Values from the same individual are linked by the lines. Values below 0.57 ng/mL (indicated by the horizontal dotted line) were below the detection limited and treated as censored in the analysis. The y-axis is presented on a logarithmic scale.

and thus there was little information available about baseline levels of CORT. We used both carapace and plastron melanization in the analysis because they separately correlate with different behaviors in tortoises (Mafli et al., 2011). To draw inferences about correlations among these four traits (boldness, CORT, and carapace and plastron melanization), we used the traits as response variables in a multivariate linear model fit with Bayesian Monte Carlo Markov Chain (MCMC) in the MCMCglmm package (Hadfield, 2010) in Program R. Our approach and this package allowed us to draw inferences about the correlations among traits (using the posterior distribution of the variance–covariance matrix of the traits), to examine the residual correlations among traits after correcting for the influence of covariates, and to use the censored Gaussian distribution for boldness (which was right-censored at a maximum observation period of 600 sec) and CORT level (which was left-censored at the detection limit; see above).

We first fit intercept-only MCMCglmm models with the four traits as responses, an unstructured residual variance–covariance matrix (permitting all covariances to be estimated), and Gaussian distributions (for carapace and plastron color) or censored Gaussian distributions (for boldness and CORT). We then fit models that also included covariates that could influence or associate with trait values: plastron length as a proxy for body size and age; sex; source population (AMW or WLBRB); plastron temperature; Julian date to account for potential seasonal effects on boldness and CORT levels; year to estimate interannual differences in boldness and CORT levels; time of day to account for diel variation in boldness and CORT levels; and total duration of stressor exposure to account for how differences in handling, confinement, and blood draw times could influence boldness and CORT levels. The MCMCglmm package uses Bayesian MCMC methods and thus requires the use of prior probabilities. For covariate effects, we used the default priors. For the variance–covariance matrices, we evaluated models with four priors to limit how much our results depended on the selected priors: 1) very low variances and belief parameter $v = 5$ ("V = diag(4)*1e-6, nu = 5"), the flat improper prior for correlations (J. Hadfield, pers. comm.); 2) very low variances and $v = 1$, the flat prior for variances and covariances (J. Hadfield, pers. comm.); 3) variance = 1 and $v = 3.002$, a weakly informative prior (Dingemanse and Dochtermann, 2013); and 4) the calculated raw variance–covariances among the four traits as the prior variance, with $v = 3.002$ (Brommer et al., 2014). All priors yielded qualitatively similar conclusions, and thus we restrict the presentation of our results to prior (1) above.

After 5×10^6 iterations, we excluded the first 1×10^6 as burn-in and generated the posterior distributions from 1,000 values (thinning to every 4,000th) from the MCMC chain. This yielded good convergence as assessed from plots of posterior distributions, traces, and Geweke's diagnostic, with low levels of autocorrelation ($r < 0.10$). We report results as 95% Bayesian credibility intervals (CIs, estimated using the highest posterior density interval) and MCMC *P*-values (pMCMC, using the package MCMC.OTU; Green et al., 2014), and we use $\alpha = 0.05$ to determine statistical significance. The data sets and code used during this study are available in the Figshare repository (https://doi.org/10.6084/m9.figshare.19103282).

RESULTS

Postconfinement CORT levels were significantly higher than baseline CORT levels in Eastern Box Turtles (censored regression: $t_{70} = 13.88$, $P < 0.0001$). Therefore, our confinement and handling techniques successfully induced a stress response in the subjects (Fig. 2).

There was little evidence for correlations among boldness, stress-induced CORT levels, and shell melanization that are predicted by the melanocortin hypothesis, regardless of whether trait correlations were adjusted for covariates (Table 1; Fig. 3). Carapace and plastron melanization were significantly though not strongly correlated (Fig. 3), indicating a tendency for the melanin content of both parts of the shell to vary in similar directions but with substantial independence. However, neither boldness nor stress-induced CORT levels were associated with the melanization of either part of the shell (Fig. 3), indicating that darker colored turtles did not predictably differ in behavior or stress response. When not adjusting for covariates, there was a trend toward turtles with darker carapaces having low stress-induced CORT levels ($P_{MCMC} = 0.11$; Table 1; Fig. 3). Additionally, there was a potential relationship between boldness and stress-induced CORT levels such that bolder (fast-emerging) turtles tended to have higher levels of CORT (Fig. 3), but again with 95% CIs that marginally included 0 (raw trait correlation: $P_{MCMC} = 0.08$, covariate-corrected trait correlation: $P_{MCMC} = 0.12$), indicating only moderate support for this relationship (Table 1).

In the model that included covariates, neither plastron length nor sex had an effect on any of the four traits (Table 2). Effects of date, year, and time of day were only assessed for boldness and stress-induced CORT levels (for which they could have biologically meaningful effects), and they did not influence either trait (Table 2). Populations differed in boldness and plastron melanization, with WLBRB turtles taking significantly longer to emerge from the shell and having significantly lighter colored plastrons (Fig. 4). Plastron temperature and total stressor exposure time were both only associated with stress-induced CORT levels: turtles that were warmer or had been handled longer had higher stress-induced CORT levels (Fig. 5).

TABLE 1. Correlations among behavioral, corticosterone (CORT), and shell melanization traits as estimated from posterior distribution of the variance–covariance matrix in multivariate Generalized Linear Mixed Models. Values shown are correlations (95% credibility interval), and Bayesian Monte Carlo Markov Chain *P*-values, with significant relationships in bold text. Values above the diagonal are uncorrected for covariates, while values below the diagonal account for covariates (extrinsic factors associated with sampling conditions or intrinsic differences between individuals; see Table 2).

	Head emergence latency	Stress-induced CORT levels	Carapace melanization	Plastron melanization
Head emergence latency		−0.16 (−0.48, 0.02) $P = 0.08$	−0.02 (−0.24, 0.22) $P = 0.97$	0.16 (−0.11, 0.34) $P = 0.32$
CORT levels	−0.26 (−0.48, 0.05) $P = 0.12$		−0.22 (−0.43, 0.04) $P = 0.11$	0.04 (−0.21, 0.27) $P = 0.80$
Carapace melanization	0.02 (−0.25, 0.30) $P = 0.88$	−0.04 (−0.39, 0.12) $P = 0.31$		**0.35 (0.12, 0.52)** $\mathbf{P = 0.008}$
Plastron melanization	0.16 (−0.06, 0.39) $P = 0.20$	−0.03 (−0.24, 0.26) $P = 0.90$	**0.29 (0.10, 0.53)** $\mathbf{P = 0.008}$	

DISCUSSION

Our results generally failed to support the melanocortin hypothesis in Eastern Box Turtles. We found no significant correlations among behavioral, CORT, and shell melanization traits, and correlations that approached significance were weak. Moreover, the near significant relationship between stress-induced CORT levels and boldness ($P = 0.08$ uncorrected for covariates, $P = 0.12$ corrected for covariates) is in the opposite direction from what we would have expected, with higher stress-induced CORT levels tending to occur in the bolder, more proactive turtles who emerged faster from the shell; in previous studies, higher CORT levels were generally associated with more reactive and shy individuals (Koolhaas et al., 1999; Atwell et al., 2012; Baugh et al., 2012; Mazza et al., 2019). We found a significant difference between populations in plastron melanization and boldness and a clear effect of plastron temperature on stress-induced CORT levels. Curiously, we also found that plastron melanization and carapace melanization were significantly correlated but apparently the correlation was too weak to demonstrate differences in the degree of carapace melanization between populations.

When uncorrected for covariates, we found no significant effect of melanization on stress-induced CORT levels although the value of our *P*-value was small ($P = 0.11$). Therefore, melanized turtles did not tend to have lower CORT levels. The *POMC* gene is expressed as a peptide chain (POMC) that is spliced into 5 melanocortin hormones, one of which (ACTH) stimulates the release of CORT from the adrenal–interrenal glands, affecting numerous aspects of behavior and physiology (Sapolsky et al., 2000; Ducrest et al., 2008). The ACTH peptide chain can be further modified to produce α-MSH, which darkens coloration by increasing melanin in the integument (Ducrest et al., 2008). Therefore, CORT and melanin are linked because of their reliance on a single hormone (ACTH; Ducrest et al., 2008). Splicing ACTH to produce α-MSH disables its stimulatory effect on CORT release and, as such, higher melanization should yield lower CORT release. For instance, lizard populations with higher circulating α-MSH levels had lower baseline CORT levels (Seddon and Hews, 2020).

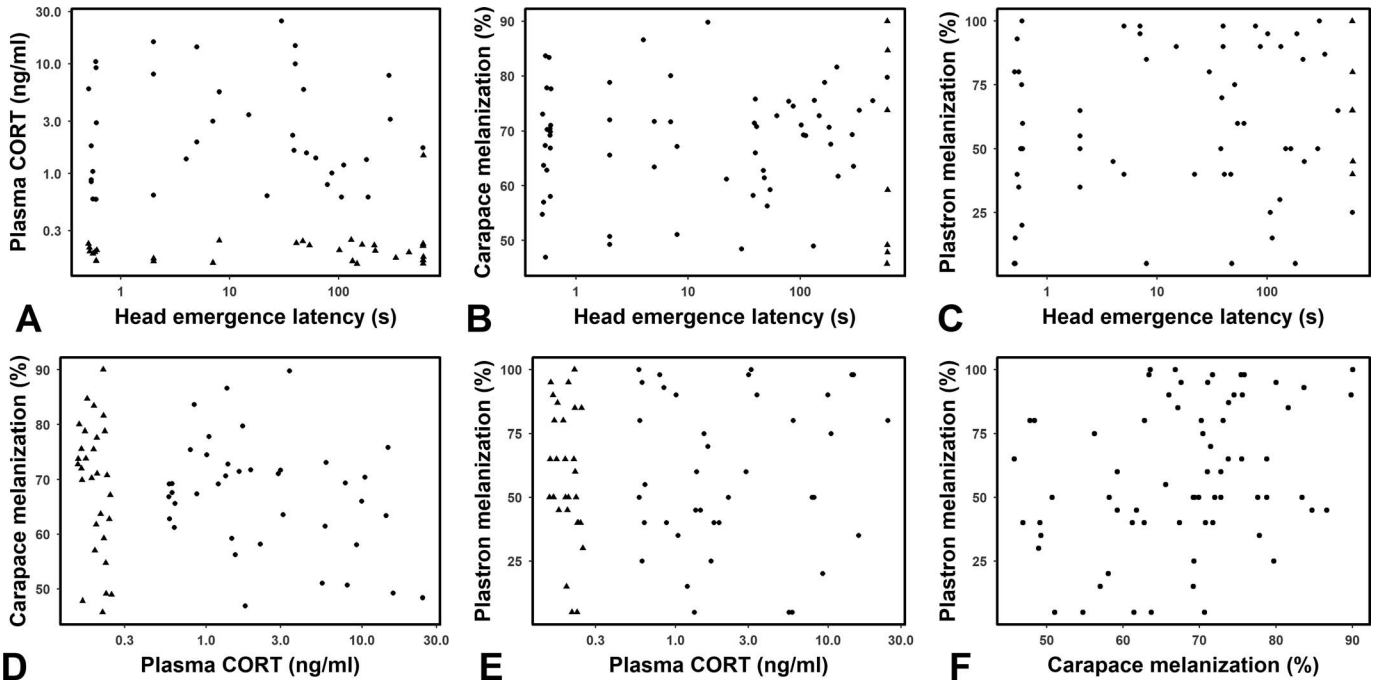

FIG. 3. Relationships among behavioral, corticosterone (CORT), and shell melanization traits. Stress-induced plasma CORT and boldness (head emergence latency) are shown on a logarithmic scale. Censored values of CORT levels (<0.57 ng/mL) and boldness (head emergence latencies >600 sec) are indicated by triangular symbols.

TABLE 2. Covariate effects on behavioral, corticosterone (CORT), and shell melanization traits estimated from multivariate Generalized Linear Mixed Models. Results are shown as the estimated effect β (95% credibility interval) and Bayesian Monte Carlo Markov Chain *P*-values, with significant effects in bold text. Unestimated effects are blank.

	Trait			
Covariate	Head emergence latency	Stress-induced CORT levels	Carapace melanization	Plastron melanization
Plastron length	−0.06 (−0.79, 0.55) $P = 0.74$	0.03 (−0.50, 0.43) $P = 0.95$	0.65 (−2.46, 2.89) $P = 0.67$	2.73 (−5.19, 8.14) $P = 0.64$
Sex (M)	0.18 (−0.85, 1.77) $P = 0.44$	−0.30 (−1.44, 0.33) $P = 0.27$	−0.59 (−5.26, 5.15) $P = 0.85$	−9.76 (−23.4, 4.75) $P = 0.20$
Population (WLBRB)[a]	**3.23 (0.35, 5.01)** $\boldsymbol{P = 0.01}$	−0.55 (−2.26, 1.13) $P = 0.48$	1.96 (−7.87, 7.88) $P = 0.94$	**−23.6 (−42.0, −1.47)** $\boldsymbol{P = 0.04}$
Plastron temperature, °C	−0.52 (−1.31, 0.32) $P = 0.25$	**0.79 (0.19, 1.33)** $\boldsymbol{P = 0.006}$	−1.59 (−4.86, 0.59) $P = 0.14$	−1.02 (−7.48, 6.09) $P = 0.94$
Date	−0.67 (−1.24, 0.31) $P = 0.23$	−0.40 (−0.91, 0.20) $P = 0.15$	-	-
Year (2018)	−1.17 (−2.83, 0.39) $P = 0.10$	0.11 (−0.70, 1.29) $P = 0.64$	-	-
Time of day	−0.04 (−0.74, 0.86) $P = 0.89$	−0.29 (−0.82, 0.27) $P = 0.26$	-	-
Total stressor exposure time	0.02 (−0.32, 0.47) $P = 0.62$	**0.34 (0.03, 0.55)** $\boldsymbol{P = 0.02}$	-	-

[a] Weiler–Leopold/Black Rock Barrens.

However, greater expression of the *POMC* gene could produce both higher ACTH and α-MSH levels, resulting in a positive relationship between CORT and melanin levels (Roulin et al., 2011). In addition, ACTH is also capable of directly increasing melanization by binding to the MC1R receptor, which would mean elevated ACTH could both increase melanization and CORT release. As such, higher CORT could be correlated positively or negatively with melanization depending on the mechanism behind melanization in a specific organism (Ducrest et al., 2008). In Eastern Box Turtles, this complexity may have obscured the relationship between CORT and melanization.

Moreover, the mechanisms underlying differences in shell coloration among individual turtles are unknown and may be unrelated to *POMC* expression. For example, melanization in one species of lizard was uncorrelated with α-MSH levels (Seddon and Hews, 2020). The *MC1R* gene encodes several melanocortin receptors to which *POMC* products bind, and thus *MC1R* may play a role in melanization in some species (Hosoda et al., 2005; Mundy, 2005), and agouti-signaling protein acts as an antagonist to α-MSH and other melanocortin hormones and promotes production of phaeomelanin (responsible for yellow and brown coloration) over eumelanin (responsible for blacker color; Ollmann et al., 1998). Production of predominately phaeomelanin when compared with eumelanin could explain the lack of relationship we see between coloration and behavior, warranting further research on the mechanisms underlying

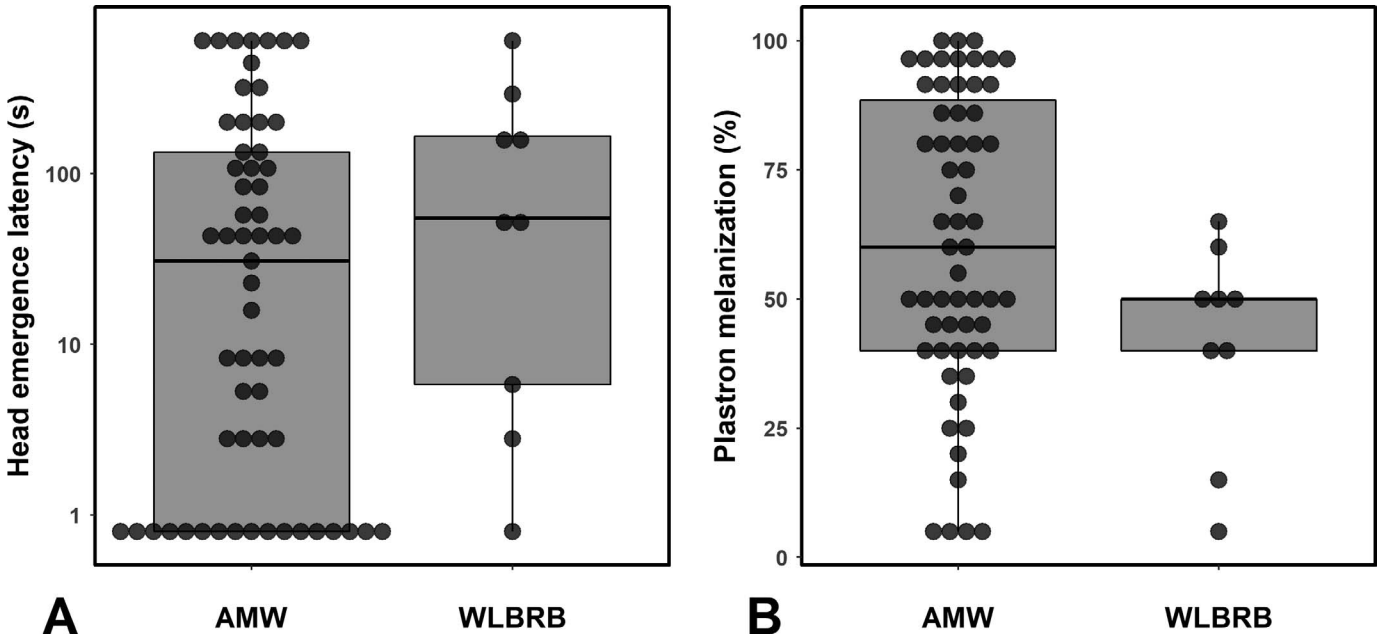

FIG. 4. Differences in boldness (A) and plastron melanization (B) between the two populations, Allee Memorial Woods (AMW) and Weiler–Leopold/Black Rock Barrens (WLBRB) nature preserves. Boldness (head emergence latency) is depicted on a logarithmic scale, and each circle represents a value for an individual turtle. Boxes represent the 25th to 75th percentiles, thick lines within the boxes represent the median, and whiskers (vertical lines) extend to all observed values within a range of 1.5 times the interquartile range from the boxes; points beyond 1.5 times the interquartile range are shown unconnected by whiskers in (B).

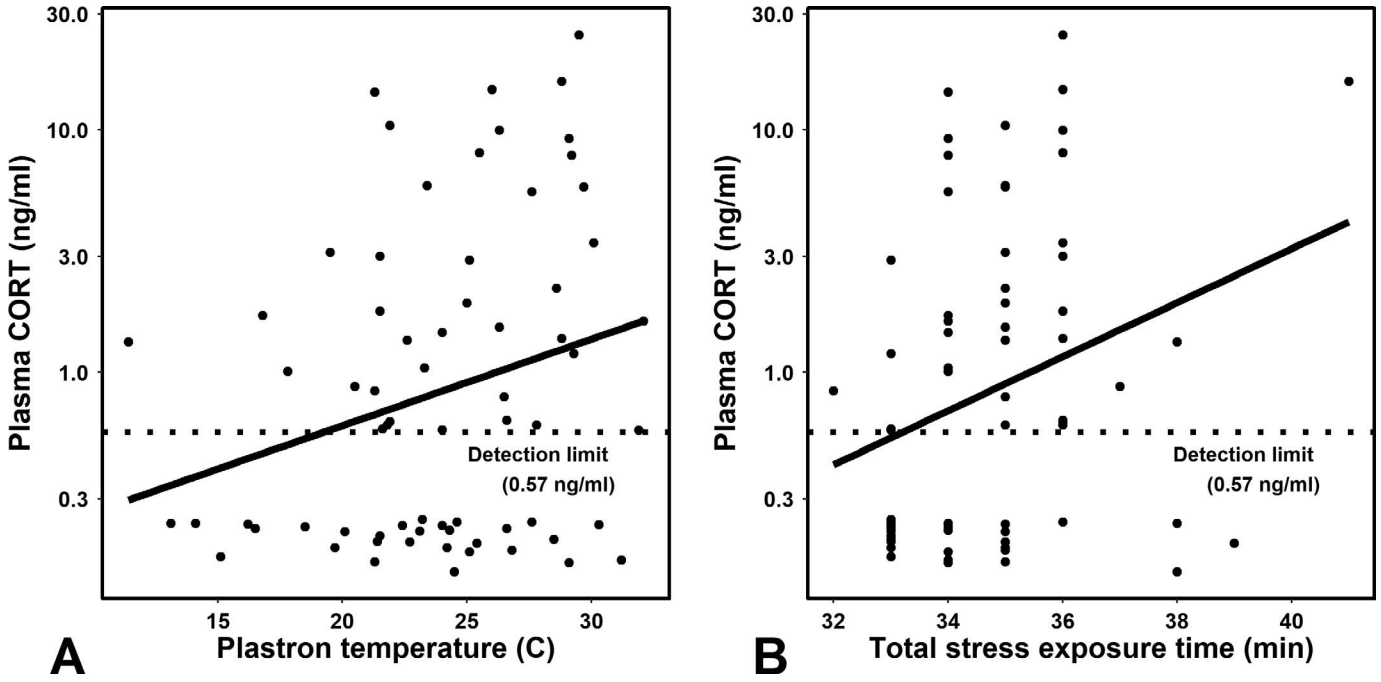

FIG. 5. Effects of plastron temperature (A) and total stress exposure time (B) on stress-induced plasma corticosterone (CORT) levels. Total stress exposure time is the elapsed time from initial contact to completion of the final (stress-induced) blood sampling, including confinement, handling, and bleed times. CORT levels are shown on a logarithmic scale. Censored values of CORT levels (<0.57 ng/mL) appear below the horizontal line representing the detection limit.

turtle color patterns. Further research on the development of coloration within Eastern Box Turtles would also be valuable for understanding drivers of variation in carapace coloration. There is a well-known but poorly documented pattern of ontogenetic shell color change, and the development of carapace coloration is subject to some environmental influence: a complete absence of sunlight on carapacial scutes prevents darkening of the scutes in growing *T. c. carolina* (Belzer and Seibert, 2010), and juveniles of many turtle species develop darker carapaces when reared on dark substrates, though this tendency was nonsignificant in *T. c. carolina* specifically (Rowe et al., 2014). Our observations point toward substantial coloration differences even among mature adults from the same habitat, but in-depth experimental and longitudinal studies of carapace coloration and comparisons of related individuals could yield important insights into ontogenetic, extrinsic, and heritable drivers of shell coloration in Eastern Box Turtles.

Our nearly significant findings in which bolder turtles tended to have higher stress-induced CORT is the reverse of what has been suggested as a general pattern (Koolhaas et al., 1999) in which bolder individuals are expected to have lower levels of CORT, whereas in these turtles, bolder individuals tended toward higher levels of CORT. Similar relationships have been observed in other reptiles, where bolder or more aggressive animals have higher, rather than lower, levels of CORT (Mell et al., 2016; Herr et al., 2017; Brashears et al., 2020; but see Thaker et al., 2009; Claunch et al., 2017), and in other animals the links between boldness and CORT may be variable among species (Tablado et al., 2021). In Eastern Box Turtles, the nature of the defensive strategies used by the putatively bold individuals may be important to explaining their tendency toward higher stress-induced CORT levels. Specifically, fleeing is likely more energetically demanding than remaining enclosed within the shell, and therefore high glucose mobilization following CORT elevation may be advantageous in bolder individuals. Moreover, neuroendocrine activity in the sympathetic nervous system may play a complicating or predominant role in Eastern Box Turtle behavior under stress. In particular, norepinephrine and epinephrine levels represent another physiological variable that interacts with CORT, and this physiological system may be most important in fight–flight–freeze responses to immediate threats (Wingfield, 2005). Indeed, in rats these hormone levels can positively correlate with at least some types of proactive behaviors (which typically would correlate with boldness; Ducrest et al., 2008). Direct evaluation of sympathetic nervous activity may better illuminate the physiological correlates of differences in boldness in Eastern Box Turtles.

The general lack of correlations predicted by the melanocortin hypothesis may also reflect a statistical limitation for our data set. Though boldness in Eastern Box Turtles is highly repeatable ($r = 0.69–0.77$; Kashon and Carlson, 2018; Pich et al., 2019; Carlson and Tetzlaff, 2020), repeatability of CORT levels is not known but is often moderate in other species (approximately $r = 0.20–0.40$, depending on conditions; Schoenemann and Bonier, 2018; Taff et al., 2018). Even with substantial repeatability, any given measurement of behavior or hormone levels will deviate from an individual animal's mean value. Such deviations mean that the use of single values for each animal (rather than repeated measurements) could statistically obscure the underlying correlation between traits; moreover, such variance in the data can conflate correlations that occur between individuals' mean values (e.g., between an animal's long-term boldness and its average CORT level) with correlations that occur between observation-level deviations within individuals (e.g., between individual measurements of boldness and CORT levels; Garamszegi and Herczeg, 2012; Fürtbauer et al., 2015; Niemelä and Dingemanse, 2018). Further exploration of the melanocortin hypothesis in turtles would be strengthened by repeated measurements of behavior and CORT levels within individuals.

We found that temperature had a strong positive effect on CORT levels. Many studies in reptiles, amphibians, and birds have found similar effects (Narayan et al., 2012; Telemeco and Addis, 2014; Lynn and Kern, 2017; Brischoux et al., 2018; Racic et al., 2020), though low temperature stress can also be associated with higher CORT (Dupoué et al., 2013; Telemeco and Addis, 2014). Furthermore, the scale can matter when examining stress responses because temperature may play a role in stress physiology in birds but at a climate-scale, in which long-term average conditions shape stress physiology (Jessop et al., 2016). Increased CORT in warmer animals may reflect stress responses to thermal challenge or, especially in ectotherms, enhanced physiological activity and increased metabolic rates. Notably, higher temperatures can slightly increase boldness in Eastern Box Turtles (Pich et al., 2019) and bolder turtles tend to maintain higher body temperatures (Kashon and Carlson, 2018), and therefore higher temperatures could cause a relationship between boldness and stress-induced CORT levels. Alternatively, in an observational study such as this, existing differences in CORT can affect body temperature by influencing an organism's thermoregulation. Manipulation of CORT levels can alter thermoregulatory behavior in reptiles: in one lizard species, thermoregulatory behavior increases body temperatures in CORT-elevated individuals (Preest and Cree, 2008), whereas in another lizard species the elevation of CORT either increased or decreased time spent basking, depending on whether CORT was applied to prenatal or postnatal lizards (Belliure and Clobert, 2004; Belliure et al., 2004). We know of no research on the effects of CORT levels on thermoregulation in turtles.

Our populations differed in plastron coloration and in boldness, with turtles from the AMW population being bolder and their plastrons less melanized than turtles from the WLBRB population (Table 2). The AMW turtles presumably had more contact history with humans (Williams and Parker, 1987) but this is unlikely to explain their increased boldness because previous research has found that repeated behavioral trials did not affect behavior (Kashon and Carlson, 2018). The two populations may differ in predator abundance or assemblage because exposure to predators has been found to influence boldness via plasticity (Hellström and Magnhagen, 2011) and differential mortality (Lapiedra et al., 2018). Alternatively, other behaviors or phenotypic traits that are correlated with boldness (Koolhaas et al., 1999; Sih et al., 2004) may be targets of selection. With respect to plastron melanization, substrate and habitat differences can influence carapace coloration of some turtles (Rowe et al., 2006, 2014; McGaugh, 2008) and could be driving differences in the two populations in this study, but such influences on plastron coloration have not been documented in other turtles (Reinke et al., 2018).

In conclusion, we find little support for the melanocortin hypothesis in Eastern Box Turtles, a species with highly consistent individual differences in boldness and with often dramatic variation in melanization. However, we found a clear effect of temperature on stress-induced CORT levels and population differences in melanization and boldness, pointing to the added complexity that comes with expanding this research question to more diverse taxa. Further work in this and similar systems promises to enhance our understanding of the melanocortin system and its generality.

Acknowledgments.—We thank R. Borland, N. Brown, F. Kashon, S. Khoo, K. Klein, and C. Rhodes for assistance in data collection; M. Allender for guidance on performing blood draws; and G. McCormick and T. Langkilde for initial assistance with CORT analysis. The manuscript was much improved by comments from J. Van Dyke and an anonymous reviewer. This work was supported by the Byron K. Trippet fund at Wabash College. The research activities were approved and permitted by Indiana Scientific Purposes License #18-147 and #16-154, Indiana DNR Division of Nature Preserves Research and Collecting Permit #NP19-25, the NICHES Land Trust, and the Wabash College Institutional Animal Care and Use Committee.

Literature Cited

Archard, G. A., R. L. Earley, A. F. Hanninen, and V. A. Braithwaite. 2012. Correlated behaviour and stress physiology in fish exposed to different levels of predation pressure. Functional Ecology 26:637–645.

Atwell, J. W., G. C. Cardoso, D. J. Whittaker, S. Campbell-Nelson, K. W. Robertson, and E. D. Ketterson. 2012. Boldness behavior and stress physiology in a novel urban environment suggest rapid correlated evolutionary adaptation. Behavioral Ecology 23:960–969.

Baugh, A. T., S. V. Schaper, M. Hau, J. F. Cockrem, P. de Goede, and K. van Oers. 2012. Corticosterone responses differ between lines of great tits (*Parus major*) selected for divergent personalities. General and Comparative Endocrinology 175:488–494.

Bell, A. M., S. J. Hankison, and K. L. Laskowski. 2009. The repeatability of behaviour: a meta-analysis. Animal Behaviour 77:771–783.

Belliure, J., and J. Clobert. 2004. Behavioral sensitivity to corticosterone in juveniles of the wall lizard, *Podarcis muralis*. Physiology & Behavior 81:121–127.

Belliure, J., S. Meylan, and J. Clobert. 2004. Prenatal and postnatal effects of corticosterone on behavior in juveniles of the common lizard, *Lacerta vivipara*. Journal of Experimental Zoology Part A: Comparative Experimental Biology 301A:401–410.

Belzer, W., and S. Seibert. 2010. Photo-dependent localized color development in the eastern box turtle carapace. Philadelphia Herpetological Society Special Publication 2009(02). Available from: http://herpetology.com/belzer2/colorintro.htm

Brashears, J. A., H. B. Fokidis, and D. F. DeNardo. 2020. Fear-based aggression and its relationship to corticosterone responsiveness in three species of python. General and Comparative Endocrinology 289:113374.

Brischoux, F., O. Lourdais, A. Boissinot, and F. Angelier. 2018. Influence of temperature, size and confinement on testosterone and corticosterone levels in breeding male spined toads (*Bufo spinosus*). General and Comparative Endocrinology 269:75–80.

Brommer, J. E., P. Karell, K. Ahola, and T. Karstinen. 2014. Residual correlations, and not individual properties, determine a nest defense boldness syndrome. Behavioral Ecology 25:802–812.

Carere, C., D. Caramaschi, and T. W. Fawcett. 2010. Covariation between personalities and individual differences in coping with stress: converging evidence and hypotheses. Current Zoology 56: 728–740.

Carlson, B. E., and S. J. Tetzlaff. 2020. Long-term behavioral repeatability in wild adult and captive juvenile turtles (*Terrapene carolina*): implications for personality development. Ethology 126: 668–678.

Chambers, D. L., J. M. Wojdak, P. Du, and L. K. Belden. 2011. Corticosterone level changes throughout larval development in the amphibians *Rana sylvatica* and *Ambystoma jeffersonianum* reared under laboratory, mesocosm, or free-living conditions. Copeia 2011: 530–538.

Citadini, J. M., and C. A. Navas. 2013. Inter-individual variation and temperature-dependent antipredator behavior in the snake *Tomodon dorsatus* (Dipsadidae). Behavioural Processes 97:11–17.

Claunch, N. M., J. A. Frazier, C. Escallón, B. J. Vernasco, I. T. Moore, and E. N. Taylor. 2017. Physiological and behavioral effects of exogenous corticosterone in a free-ranging ectotherm. General and Comparative Endocrinology 248:87–96.

Clusella Trullas, S., J. H. van Wyk, and J. R. Spotila. 2007. Thermal melanism in ectotherms. Journal of Thermal Biology 32:235–245.

Cockrem, J. F. 2007. Stress, corticosterone responses and avian personalities. Journal of Ornithology 148:169–178.

———. 2013. Individual variation in glucocorticoid stress responses in animals. General and Comparative Endocrinology 181:45–58.

Currylow, A. F. T., E. E. Louis, and D. E. Crocker. 2017. Stress response to handling is short lived but may reflect personalities in a wild, Critically Endangered tortoise species. Conservation Physiology 5: 793–817.

Dingemanse, N. J., and N. A. Dochtermann. 2013. Quantifying individual variation in behaviour: mixed-effect modelling approaches. Journal of Animal Ecology 82:39–54.

Dodd, C. K. J. 2001. North American Box Turtles: A Natural History. University of Oklahoma Press, USA.

Ducrest, A.-L., L. Keller, and A. Roulin. 2008. Pleiotropy in the melanocortin system, coloration and behavioural syndromes. Trends in Ecology & Evolution 23:502–510.

Dupoué, A., F. Brischoux, O. Lourdais, and F. Angelier. 2013. Influence of temperature on the corticosterone stress-response: an experiment in the Children's python (*Antaresia childreni*). General and Comparative Endocrinology 193:178–184.

Ennen, J. R., P. V. Lindeman, and J. E. Lovich. 2015. Intersexual allometry differences and ontogenetic shifts of coloration patterns in two aquatic turtles, *Graptemys oculifera* and *Graptemys flavimaculata*. Ecology and Evolution 5:2296–2305.

Evanson, N. K., J. P. Herman, R. R. Sakai, and E. G. Krause. 2010. Nongenomic actions of adrenal steroids in the central nervous system. Journal of Neuroendocrinology 22:846–861.

Fürtbauer, I., A. Pond, M. Heistermann, and A. J. King. 2015. Personality, plasticity and predation: linking endocrine and behavioural reaction norms in stickleback fish. Functional Ecology 29:931–940.

Garamszegi, L. Z., and G. Herczeg. 2012. Behavioural syndromes, syndrome deviation and the within- and between-individual components of phenotypic correlations: when reality does not meet statistics. Behavioral Ecology and Sociobiology 66:1651–1658.

Garde, A. H., and Å. M. Hansen. 2005. Long-term stability of salivary cortisol. Scandinavian Journal of Clinical and Laboratory Investigation 65:433–436.

Gienger, C. M., and E. M. Urdiales. 2017. Influences on standard metabolism in Eastern Box Turtles (*Terrapene carolina*). Chelonian Conservation and Biology 16:159–163.

Graham, S. P., C. Kelehear, G. P. Brown, and R. Shine. 2012. Corticosterone–immune interactions during captive stress in invading Australian cane toads (*Rhinella marina*). Hormones and Behavior 62:146–153.

Green, E. A., S. W. Davies, M. V. Matz, and M. Medina. 2014. Quantifying cryptic *Symbiodinium* diversity within *Orbicella faveolata* and *Orbicella franksi* at the Flower Garden Banks, Gulf of Mexico. PeerJ 2:e386.

Hadfield, J. D. 2010. MCMC methods for multi-response generalized linear mixed models: the MCMCglmm R package. Journal of Statistical Software 33:1–22.

Hau, M., and W. Goymann. 2015. Endocrine mechanisms, behavioral phenotypes and plasticity: known relationships and open questions. Frontiers in Zoology 12:S7.

Hellström, G., and C. Magnhagen. 2011. The influence of experience on risk taking: results from a common-garden experiment on populations of Eurasian perch. Behavioral Ecology and Sociobiology 65: 1917–1926.

Helsel, D. R. 2005. More than obvious: better methods for interpreting nondetect data. Environmental Science & Technology 39:419A–423A.

Henningsen, A. 2017. censReg: censored regression (tobit) models. R package version 0.5. Available from: https://CRAN.R-project.org/package=censReg

Herr, M. W., S. P. Graham, and T. Langkilde. 2017. Stressed snakes strike first: hormone levels and defensive behavior in free ranging cottonmouths (*Agkistrodon piscivorus*). General and Comparative Endocrinology 243:89–95.

Herring, G., and D. E. Gawlik. 2009. Stability of avian fecal corticosterone metabolite levels in frozen avian feces. Journal of Wildlife Management 73:1010–1013.

Holtmann, B., M. Lagisz, and S. Nakagawa. 2017. Metabolic rates, and not hormone levels, are a likely mediator of between-individual differences in behaviour: a meta-analysis. Functional Ecology 31: 685–696.

Hosoda, T., J. J. Sato, T. Shimada, K. L. Campbell, and H. Suzuki. 2005. Independent nonframeshift deletions in the MC1R gene are not associated with melanistic coat coloration in three mustelid lineages. Journal of Heredity 96:607–613.

Ibáñez, A., P. López, and J. Martín. 2014. Inter-individual variation in antipredator hiding behavior of Spanish Terrapins depends on sex, size, and coloration. Ethology 120:742–752.

Ibáñez, A., D. Pellitteri-Rosa, R. Sacchi, P. López, and J. Martín. 2016. Melanin-based coloration covaries with hiding and exploratory behavior in male Spanish Terrapins. Ethology 122:30–36.

Jenkins, B. R., M. N. Vitousek, and R. J. Safran. 2013. Signaling stress? an analysis of phaeomelanin-based plumage color and individual corticosterone levels at two temporal scales in North American barn swallows, *Hirundo rustica erythrogaster*. Hormones and Behavior 64: 665–672.

Jessop, T. S., M. L. Lane, L. Teasdale, D. Stuart-Fox, R. S. Wilson, V. Careau, and I. T. Moore. 2016. Multiscale evaluation of thermal dependence in the glucocorticoid response of vertebrates. American Naturalist 188:342–356.

Kashon, E. A. F., and B. E. Carlson. 2018. Consistently bolder turtles maintain higher body temperatures in the field but may experience greater predation risk. Behavioral Ecology and Sociobiology 72:9.

Kempenaers, B., A. Peters, and K. Foerster. 2008. Sources of individual variation in plasma testosterone levels. Philosophical Transactions of the Royal Society of London B: Biological Sciences 363:1711–1723.

Koolhaas, J. M., S. M. Korte, S. F. De Boer, B. J. Van Der Vegt, C. G. Van Reenen, H. Hopster, I. C. De Jong, M. A. Ruis, and H. J. Blokhuis. 1999. Coping styles in animals: current status in behavior and stress-physiology. Neuroscience & Biobehavioral Reviews 23:925–935.

Koolhaas, J. M., S. F. de Boer, C. M. Coppens, and B. Buwalda. 2010. Neuroendocrinology of coping styles: towards understanding the biology of individual variation. Frontiers in Neuroendocrinology 31: 307–321.

Lapiedra, O., T. W. Schoener, M. Leal, J. B. Losos, and J. J. Kolbe. 2018. Predator-driven natural selection on risk-taking behavior in anole lizards. Science 360:1017–1020.

Leuck, B. E., and C. C. Carpenter. 1981. Shell variation in a population of three-toed box turtles (*Terrapene carolina triunguis*). Journal of Herpetology 15:53–58.

Lynn, S. E., and M. D. Kern. 2017. Ecologically relevant cooling early in life alters prefledging adrenocortical response in free-living songbirds. Physiological and Biochemical Zoology 90:118–123.

MacKay, J., and M. Haskell. 2015. Consistent individual behavioral variation: the difference between temperament, personality and behavioral syndromes. Animals 5:455–478.

Mafli, A., K. Wakamatsu, and A. Roulin. 2011. Melanin-based coloration predicts aggressiveness and boldness in captive eastern Hermann's tortoises. Animal Behaviour 81:859–863.

Mazza, V., M. Dammhahn, J. A. Eccard, R. Palme, M. Zaccaroni, and J. Jacob. 2019. Coping with style: individual differences in responses to environmental variation. Behavioral Ecology and Sociobiology 73: 142.

McGaugh, S. E. 2008. Color variation among habitat types in the Spiny Softshell Turtles (Trionychidae: *Apalone*) of Cuatrociénegas, Coahuila, Mexico. Journal of Herpetology 42:347–353.

Mell, H., R. Josserand, B. Decencière, P. Artacho, S. Meylan, and J. F. Le Galliard. 2016. Do personalities co-vary with metabolic expenditure and glucocorticoid stress response in adult lizards? Behavioral Ecology and Sociobiology 70:951–961.

Moleón, M. S., M. V. Parachú Marcó, E. O. Pietrobon, G. A. Jahn, P. M. Beldomenico, and P. A. Siroski. 2018. Corticosterone levels and immunological indices in stressed juvenile broad-snouted caimans. Journal of Zoology 304:151–158.

Moore, F. L., and M. Orchinik. 1994. Membrane receptors for corticosterone: a mechanism for rapid behavioral responses in an amphibian. Hormones and Behavior 28:512–9.

Mundy, N. I. 2005. A window on the genetics of evolution: MC1R and plumage colouration in birds. Proceedings of the Royal Society B: Biological Sciences 272:1633–1640.

Narayan, E. J., J. F. Cockrem, and J. M. Hero. 2012. Effects of temperature on urinary corticosterone metabolite responses to short-term capture and handling stress in the cane toad (*Rhinella marina*). General and Comparative Endocrinology 178:301–305.

Niemelä, P. T., and N. J. Dingemanse. 2018. On the usage of single measurements in behavioural ecology research on individual differences. Animal Behaviour 145:99–105.

Ollmann, M., M. Lamoreux, B. Wilson, and G. Barsh. 1998. Interaction of Agouti protein with the melanocortin 1 receptor in vitro and in vivo. Genes & Development 12:316–330.

Øverli, Ø., C. Sørensen, K. G. T. Pulman, T. G. Pottinger, W. Korzan, C. H. Summers, and G. E. Nilsson. 2007. Evolutionary background for

stress-coping styles: relationships between physiological, behavioral, and cognitive traits in non-mammalian vertebrates. Neuroscience & Biobehavioral Reviews 31:396–412.

Pich, J. M., A. J. Belden, and B. E. Carlson. 2019. Individual variation in boldness in turtles is consistent across assay conditions and behavioural measures. Behaviour 156:1039–1056.

Pike, T. W., and M. Petrie. 2006. Experimental evidence that corticosterone affects offspring sex ratios in quail. Proceedings of the Royal Society B: Biological Sciences 273:1093–1098.

Pincheira-Donoso, D., A. M. Bauer, S. Meiri, P. Uetz, and C. Kuczynski. 2013. Global taxonomic diversity of living reptiles. PLoS One 8: e59741.

Preest, M. R., and A. Cree. 2008. Corticosterone treatment has subtle effects on thermoregulatory behavior and raises metabolic rate in the New Zealand Common Gecko, *Hoplodactylus maculatus*. Physiological and Biochemical Zoology 81:641–650.

Preston, V. L., J. M. Vannatta, and M. Klukowski. 2020. Behavioural and physiological responses to simulated predator-induced stress in the eastern box turtle, *Terrapene carolina carolina*. Amphibia-Reptilia 41: 387–398.

Qu, J., Q. E. Fletcher, D. Réale, W. Li, and Y. Zhang. 2018. Independence between coping style and stress reactivity in plateau pika. Physiology & Behavior 197:1–8.

Racic, A., C. Tylan, and T. Langkilde. 2020. Effects of temperature on plasma corticosterone in a native lizard. Scientific Reports 10:1–8.

Réale, D., S. M. Reader, D. Sol, P. T. McDougall, and N. J. Dingemanse. 2007. Integrating animal temperament within ecology and evolution. Biological Reviews 82:291–318.

Reimers, T. J., J. P. McCann, R. G. Cowan, and P. W. Concannon. 1982. Effects of storage, hemolysis, and freezing and thawing on concentrations of thyroxine, cortisol, and insulin in blood samples. Proceedings of the Society for Experimental Biology and Medicine 170:509–516.

Reinke, B., S. Pearson, and W. Selman. 2018. Plastron pigmentation variation in a coastal turtle species of conservation concern (*Malaclemys terrapin*). Herpetologica 74:141–145.

Roulin, A., G. Emaresi, P. Bize, J. Gasparini, R. Piault, and A.-L. Ducrest. 2011. Pale and dark reddish melanic tawny owls differentially regulate the level of blood circulating POMC prohormone in relation to environmental conditions. Oecologia 166:913–921.

Rowe, J. W., D. L. Clark, C. Ryan, and J. K. Tucker. 2006. Effect of substrate color on pigmentation in Midland Painted Turtles (*Chrysemys picta marginata*) and Red-Eared Slider Turtles (*Trachemys scripta elegans*). Journal of Herpetology 40:358–364.

Rowe, J. W., B. J. Miller, M. A. Stuart, C. Snyder, J. K. Tucker, D. L. Clark, L. W. Wittle, and J. T. Lamer. 2014. Substrate color-induced melanization in eight turtle species from four chelonian groups. Zoology 117:245–252.

Sandmeier, F. C., and R. C. Tracy. 2014. The metabolic pace-of-life model: incorporating ectothermic organisms into the theory of vertebrate ecoimmunology. Integrative and Comparative Biology 54:387–395.

San-Jose, L. M., and A. Roulin. 2018. Toward understanding the repeated occurrence of associations between melanin-based coloration and multiple phenotypes. American Naturalist 192:111–130.

Santostefano, F., K. V. Fanson, J. A. Endler, and P. A. Biro. 2019. Behavioral, energetic, and color trait integration in male guppies: testing the melanocortin hypothesis. Behavioral Ecology 30:1539–1547.

Sapolsky, R. M., L. M. Romero, and A. U. Munck. 2000. How do glucocorticoids influence stress responses? integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21:55–89.

Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9:671–675.

Schoenemann, K. L., and F. Bonier. 2018. Repeatability of glucocorticoid hormones in vertebrates: a meta-analysis. PeerJ 2018:e4398.

Seddon, R. J., and D. K. Hews. 2016. Phenotypic correlates of melanization in two *Sceloporus occidentalis* (Phrynosomatidae) populations: behavior, androgens, stress reactivity, and ectoparasites. Physiology & Behavior 163:70–80.

———, and ———. 2020. Melanization, α-melanocyte stimulating hormone and steroid hormones in male western fence lizards from nine populations. General and Comparative Endocrinology 285: 113287.

Seltmann, M. W., M. Öst, K. Jaatinen, S. Atkinson, K. Mashburn, and T. Hollmén. 2012. Stress responsiveness, age and body condition interactively affect flight initiation distance in breeding female eiders. Animal Behaviour 84:889–896.

Sih, A., A. Bell, and J. C. Johnson. 2004. Behavioral syndromes: an ecological and evolutionary overview. Trends in Ecology & Evolution 19:372–378.

Tablado, Z., Y. Bötsch, V. Bókony, F. Angelier, Á. Z. Lendvai, S. Jenni-Eiermann, and L. Jenni. 2021. Factors modulating the behavioral and physiological stress responses: do they modify the relationship between flight initiation distance and corticosterone reactivity? Hormones and Behavior 132:104979.

Taff, C. C., L. A. Schoenle, and M. N. Vitousek. 2018. The repeatability of glucocorticoids: a review and meta-analysis. General and Comparative Endocrinology 260:136–145.

Telemeco, R. S., and E. A. Addis. 2014. Temperature has species-specific effects on corticosterone in alligator lizards. General and Comparative Endocrinology 206:184–192.

Thaker, M., S. L. Lima, and D. K. Hews. 2009. Alternative antipredator tactics in tree lizard morphs: hormonal and behavioural responses to a predator encounter. Animal Behaviour 77:395–401.

Trompeter, W. P., and T. Langkilde. 2011. Invader danger: lizards faced with novel predators exhibit an altered behavioral response to stress. Hormones and Behavior 60:152–158.

Troscianko, J., and M. Stevens. 2015. Image calibration and analysis toolbox—a free software suite for objectively measuring reflectance, colour and pattern. Methods in Ecology and Evolution 6:1320–1331.

Tucker, J. K., R. J. Maher, and C. H. Theiling. 1995. Melanism in the red-eared slider (*Trachemys scripta elegans*). Journal of Herpetology 29: 291–296.

Tylan, C., K. Camacho, S. French, S. P. Graham, M. W. Herr, J. Jones, G. L. McCormick, M. A. O'Brien, J. B. Tennessen, C. J. Thawley, A. Webb, and T. Langkilde. 2020. Obtaining plasma to measure baseline corticosterone concentrations in reptiles: how quick is quick enough? General and Comparative Endocrinology 287:113324.

Vera, F., R. Zenuto, and C. D. Antenucci. 2017. Expanding the actions of cortisol and corticosterone in wild vertebrates: a necessary step to overcome the emerging challenges. General and Comparative Endocrinology 246:337–353.

West, J. M., and M. Klukowski. 2018. Seasonal changes in baseline corticosterone, association with innate immunity, and effects of confinement in free-ranging Eastern Box Turtles, *Terrapene carolina carolina*. General and Comparative Endocrinology 262:71–80.

Westrick, S. E., F. van Kesteren, R. Palme, R. Boonstra, J. E. Lane, S. Boutin, A. G. McAdam, and B. Dantzer. 2019. Stress activity is not predictive of coping style in North American red squirrels. Behavioral Ecology and Sociobiology 73:113.

White, C. R., N. F. Phillips, and R. S. Seymour. 2006. The scaling and temperature dependence of vertebrate metabolism. Biology Letters 2: 125–127.

Williams, E. C. J., and W. S. Parker. 1987. A long-term study of a box turtle (*Terrapene carolina*) population at Allee Memorial Woods, Indiana, with emphasis on survivorship. Herpetologica 43:328–335.

Williams, T. D. 2008. Individual variation in endocrine systems: moving beyond the 'tyranny of the Golden Mean.' Philosophical Transactions of the Royal Society of London B: Biological Sciences 363:1687–1698.

Wingfield, J. C. 2005. The concept of allostasis: coping with a capricious environment. Journal of Mammalogy 86:248–254.

Wolf, M., and F. J. Weissing. 2010. An explanatory framework for adaptive personality differences. Philosophical Transactions of the Royal Society of London B: Biological Sciences 365:3959–68.

Accepted: 10 July 2022.
Published online: 19 December 2022.