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Temperature has species-specific effects on corticosterone in alligator lizards

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ABSTRACT

In response to conditions that threaten homeostasis and/or life, vertebrates generally increase production of glucocorticoid hormones, such as corticosterone (CORT), which induces an emergency physiological state referred to as the stress response. Given that extreme temperatures pose a threat to performance and survival, glucocorticoid upregulation might be an important component of a vertebrate ectotherm's response to extreme thermal conditions. To address this hypothesis, we experimentally examined the effects of body temperature (10, 20, 28, and 35 °C; 5-h exposure) on CORT in two congeneric species of lizard naturally exposed to different thermal environments, northern and southern alligator lizards (*Elgaria coerulea* and *Elgaria multicarinata*, respectively). In both species, CORT was similarly elevated at medium and high temperatures (28 and 35 °C, respectively), but CORT was only elevated at low temperatures (10 °C) in southern alligator lizards. We also examined CORT before and after adrenocorticotrophic hormone (ACTH) challenge. In both species, ACTH induced higher CORT levels than any temperature, suggesting that these animals could respond to further stressors at all experimental temperatures. Finally, we compared our laboratory results to measurements of CORT in field-active southern alligator lizards. Plasma CORT concentrations from our laboratory experiment had the same mean and less variance than the field lizards, suggesting that our laboratory lizards displayed CORT within natural levels. Our results demonstrate that body temperature directly affects CORT in alligator lizards. Moreover, the CORT response of these lizards appears to be adapted to their respective thermal environments. Species-specific differences in the thermal CORT response might be common in vertebrate ectotherms and have implications for species' biogeography and responses to climate change.

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Introduction

Temperature broadly affects the performance and function of organisms (Angilletta, 2009; Huey and Stevenson, 1979). In general, the relationship between body temperature (T_B) and performance can be described by a left-skewed, hump-shaped curve (thermal response curve): performance increases with T_B until an optimum is reached, then rapidly drops off (Huey and Kingsolver, 1989; Huey and Stevenson, 1979). Within a range around the thermal optimum, organisms can maintain homeostasis and physiological performance is high (Angilletta et al., 2002; Huey and Kingsolver, 1989). At extreme T_B (above and below the optimal range), organisms are unable to maintain homeostasis and performance is compromised (Angilletta, 2009; Bradshaw, 2003; Huey and Kingsolver, 1989). Ectothermic animals have limited capacity to metabolically control their T_B, and primarily thermoregulate behaviorally (Angilletta, 2009; Avery, 1982; Chown and Terblanche, 2007). To avoid extreme T_B, many ectotherms seek thermal refugia and cease activity when temperatures become too extreme for successful thermoregulation (Cowles and Bogert, 1944; Grant and Dunham, 1988; Sinervo et al., 2010).

Exposure to extreme T_B is likely stressful for ectotherms (Bradshaw, 2003; Cowles and Bogert, 1944; Van Berkum et al., 1986). While the term "stress" has many connotations, we follow the convention of considering an organism physiologically "stressed" when an environmental perturbation (i.e., stressor) drives them out of homeostasis (Bradshaw, 2003; Greenberg and Wingfield, 1987; Orchiinik, 1998; Romero et al., 2009; Selye, 1936, 1950). "Stressful" situations/environments challenge homeostasis and must be countered by the individual to maintain homeostatic function and high performance (Bradshaw, 2003; Greenberg and Wingfield, 1987).

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One mechanism whereby vertebrates perceive and respond to stressors is through the upregulation of glucocorticoid hormones (GCs, Bradshaw, 2003; Greenberg and Wingfield, 1987; Norris, 2007). High GC levels induce an emergency physiological state: digestion, immunity, and reproduction are impaired, while energy is mobilized for emergency use (Greenberg and Wingfield, 1987; Norris, 2007; Romero et al., 2009). In addition, behaviors such as territory defense, courtship, and foraging may be suppressed while escape behaviors are promoted (Belliure et al., 2004; Belthoff and Dufty, 1998; Greenberg and Wingfield, 1987; Orchiinik, 1998). These GC mediated changes are frequently adaptive, allowing organisms to escape stressful environments and maintain homeostasis (Bradshaw, 2003; Nelson, 2011). However, extreme elevations of GCs, either in duration or magnitude (i.e., chronic stress/homeostatic overload/allostatic overload), can reduce fitness and even induce pathology (Bradshaw, 2003; Sapolsky, 1992; Selye, 1936). Because GCs are an important component of an organism's response to environmental stressors, GC elevation is often used to indicate whether or not organisms are exposed to stressful environments (Broom and Johnson, 1993; Busch and Hayward, 2009; Romero, 2004).

Because extreme T_B can disrupt homeostasis and even cause death (Bradshaw, 2003; Norris, 2007), GC elevation might be an important component of a vertebrate ectotherm's response to non-optimal temperature (Bradshaw, 2003; Cree et al., 2003; Dupoué et al., 2013). Given that both temperature and GC production have direct effects on metabolism (Bradshaw, 2003; Preest and Cree, 2008; Sykes and Klukowski, 2009), any relationship between GCs and T_B could have important biological implications. To date, little is known about the relationship between GC hormones and T_B in reptiles and other ectothermic vertebrates. In reptiles, corticosterone (CORT) is the primary GC (Idler, 1972). Multiple researchers have found correlations between CORT and T_B in reptiles in the field (e.g., Cree et al., 2003; Dunlap and Wingfeld, 1995; Jessop et al., 2000; Tyrrell and Cree, 1998; Woodley et al., 2003). However, such correlations could arise through three, non-mutually exclusive, pathways (outlined in Fig. 1). First, CORT and T_B might be simultaneously affected by an outside factor. For example, CORT can fluctuate with time of day and season (Dickmeis, 2009; Eikenaar et al., 2012; Romero, 2002), but temperature also varies temporally. Second, CORT might directly induce changes in T_B. Experimental elevation of CORT affects thermoregulatory behavior in some lizards, and not necessarily in the same way (Belliure and Clobert, 2004; Belliure et al., 2004; Preest and Cree, 2008). Finally, T_B might directly affect CORT concentrations. Experimental exposure to non-optimal temperatures in two snake species induced elevated CORT levels (Dupoué et al., 2013; Schwartz and Bronikowski, 2013) but had little effect on CORT in a third species (Sykes and Klukowski, 2009). While these results are limited, they suggest that T_B and CORT might be functionally linked in reptiles.

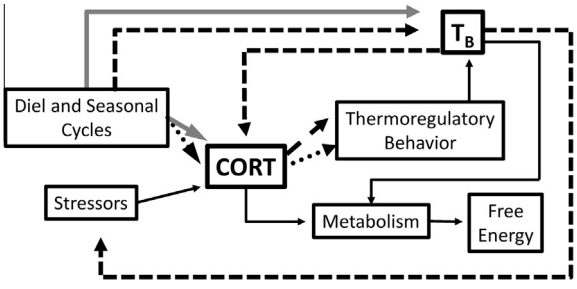

Fig. 1. Concept map of the potential causal relationships explaining correlations between plasma corticosterone concentration (CORT) and body temperature (T_B) in vertebrate ectotherms. The thin solid lines represent well-supported relationships. The grey lines represent hypothesis (1) diel and seasonal cycles affect both CORT and T_B independently. The dotted lines represent hypothesis (2) CORT affects T_B through its effects on thermoregulatory behavior. The dashed lines represent hypothesis (3) T_B directly affects CORT (either baseline or stress levels), and this represents a pathway whereby temperature affects metabolic rate. Importantly, many of the connections proposed under these hypotheses are not mutually exclusive. Free energy refers to energy available for immediate use by the organism.

We expand on this work by experimentally examining the effect of T_B on CORT in two congeneric species of alligator lizard (family Anguidae), northern alligator lizards and southern alligator lizards (*Elgaria coerulea* (Wiegmann, 1828) and *Elgaria multicarinata* (Blainville, 1835), approximately 6.6 million years divergent (Macey et al., 1999)). These lizards occur in different thermal environments: *E. coerulea* occurs at higher elevation and latitude (i.e., colder environments) than *E. multicarinata* (Beck, 2009a,b; Stebbins, 2003). Alligator lizards are best described as facultative thermoregulators (Kingsbury, 1994), and will remain active across a broad range of temperatures. *E. coerulea* and *E. multicarinata* are naturally active at remarkably similar body temperatures (both species display a mean active temperature of ~24–25 °C with a range of ~10–35 °C, Cunningham, 1966; Kingsbury, 1994; Sheen, 2001; Stewart, 1984). This observation seems paradoxical because species' thermal tolerances are predicted to be locally adapted to their thermal environment (Gilchrist, 1995; Huey and Kingsolver, 1993; Kingsolver and Gomulkiewicz, 2003). Because *E. coerulea* persist in colder environments than *E. multicarinata*, their thermal optimum range is predicted to be colder than that of *E. multicarinata*. However, the response of these animals to extreme thermal events might be more important than their average active temperatures (Bradshaw, 2003; McKenchnie and Wolf, 2009; Pörtner and Knust, 2007). We generally consider extreme temperatures to be those that are beyond an organism's optimal performance range. In practice, this could be measured as temperatures outside the 80% performance breadth (Angilletta et al., 2002) or greater than two standard deviations from mean activity temperatures (Telemeco et al., 2013).

First, we tested the hypothesis that T_B affects CORT in *E. coerulea* and *E. multicarinata*. We quantified CORT after exposing lizards to four controlled temperature treatments (constant 10, 20, 28, and 35 °C for 5 h each), as well as laboratory control conditions (at ~23.5 °C) and an adrenocorticotrophic hormone (ACTH) challenge. ACTH challenge induced CORT secretion, and thus allowed estimation of the potential CORT-response capacity of the lizards (Klukowski, 2011; Phillips and Klukowski, 2008; Romero and Wikelski, 2006). Next, we used these data to test the hypothesis that *E. coerulea* and *E. multicarinata* differ in their CORT response to temperature. If variation in the thermal stress response contributes to biogeographic variation in these species, we predict that *E. coerulea* will have elevated CORT levels at warm temperatures that do not affect *E. multicarinata*, and vice versa. Finally, we examined CORT in field sampled *E. multicarinata* for comparison to our experimental results.

2. Materials and methods

2.1. Laboratory experiment

2.1.1. Lizard collection and general laboratory maintenance

We collected adult *E. multicarinata* and *E. coerulea* during the active seasons of 2010 and 2011. Most lizards were collected from the central-coast region of California, which corresponds to the southern-most region where *E. multicarinata* and *E. coerulea* coexist. A few individuals, however, were collected in north-central California. Precise capture locations and body-size data are given in Table A1. Lizards were collected by hand via active searching in appropriate habitat, primarily from under rocks, logs, or anthropo-

genic debris. After collection, we transported the lizards to Iowa State University (ISU) and entered them into captive colonies. During active seasons (March–December), we housed the lizards in plastic containers with mesh tops. In 2010 and 2011, we housed the lizards either individually or in size-matched, male–female pairs whereas, in 2012, all lizards were housed individually. Enclosures housing individual lizards measured 33 cm long × 20 cm wide × 14.5 cm tall, whereas enclosures housing pairs measured 39 cm long × 26 cm wide × 29 cm tall. Each enclosure was outfitted with plastic hides (14 cm diameter × 2 cm tall, one per lizard) and water dishes with standing water provided *ad libitum*. Twice per week, we fed each lizard 3 crickets (*Acheta domesticus*) dusted with reptile vitamins (1:1 mixture Exo-Terra calcium and multivitamin powder supplements). We misted the cages with water daily. Enclosures were stored on a metal shelving system and illuminated with 40 W ReptiSun 5.0 UVB bulbs (ZooMed Inc.) set on a 12 h light cycle suspended above each enclosure. Additional room lights turned on 1 h before the enclosure lights and turned off 1 h after. Flex Watt heat tape (7.6 cm wide) on the shelves, under the rear portion of each enclosure, maintained a surface heat gradient within the enclosures ranging from 26 to 32 °C for 5 h/day (middle of the 12 h light cycle) to allow behavioral thermoregulation.

Outside of the active season (mid-December to mid-February), we hibernated the lizards at 6 °C in a dark room to mimic natural conditions. During hibernation, we housed the lizards individually in small containers with moist peat moss. Prior to the onset of hibernation, room temperature and light exposure was gradually reduced over 1 month. Similarly, temperature and lighting was gradually increased over 1 month at the end of hibernation.

We quantified body temperatures maintained by thermoregulating lizards in our captive colony in September 2011. We affixed iButton thermal data loggers (Maxim Integrated, San Jose, CA; diameter: 15 mm, height: 6 mm, mass: 3.3 g) programmed to record temperature every 10 min to six post-reproductive *E. coerulea* (see Telemeco et al., 2010 for detailed methods) that were each housed individually. We affixed the data loggers to the dorsum of the lizards with superglue, above the pectoral girdle. The data loggers did not affect lizard movement, and body surface temperatures measured in this way correlate highly with internal body temperatures in lizards (Robert and Thompson, 2003). We collected the data loggers after they naturally fell from the lizards as they sloughed. Grand-mean lizard body temperatures ± 1.0 s.e. over 10 days were 21.22 ± 0.13 °C when both the heaters and lights were off, 23.56 ± 0.20 °C when only the lights were on, and 27.70 ± 0.29 °C when both the heaters and lights were on.

2.1.2. Experimental protocol

We examined thirty adult lizards for the present study: 15 *E. coerulea* (6 male, 9 female) and 15 *E. multicarinata* (12 male, 3 female). When the lizards were brought out of hibernation in February 2012, we measured them (snout-vent length [SVL] and mass) and placed them individually in home enclosures for the season. These lizards received minimal human interaction until the onset of experiments in April 2012. During this period and throughout the experiments, lizard care was highly controlled, with lizards misted daily and fed each Tuesday and Saturday. We identified the sex of each lizard after the experiments by examining the gonads of euthanized individuals (data in Table A1). Because they were housed individually following hibernation, no females were pregnant/gravid.

On 12 April 2012, we collected blood samples (for details see Section 2.3, below) before (laboratory control) and after ACTH challenge. Beginning at 09:10 h, we removed individual lizards from their enclosures and bled them at approximately 5 min intervals. When the laboratory control samples were collected, the enclosure lights, but not heaters, were switched on. We therefore estimate that lizard body temperatures were approximately 23.5 °C (see Section 2.1.1, above) at the time of blood collection. Immediately following blood collection, each lizard was injected with ACTH (Sigma A6303, fragments 1–39 porcine) in the anteroventral portion of the right hindlimb using a 30G disposable insulin syringe. Injections consisted of 0.1 IU ACTH/μL/g body mass (based on mass measured in Feb 2012, Table A1). In previous studies, this dosage was effective but not supraphysiological in both squamate reptiles and birds (Klukowski, 2011; Phillips and Klukowski, 2008; Romero and Wikelski, 2006; Romero and Wingfield, 1999). After ACTH injection, we returned the lizards to their home enclosures and allowed them to rest for 60 min (±1.5 min) before collecting a second blood sample. Previous studies have shown that lizards display a CORT response to ACTH 60 min after exposure (e.g., Klukowski, 2011 and citations therein). During the waiting period, the lizards were at room temperature (~22 °C). After the second blood collection, we returned the lizards to their home enclosures on the shelving units.

We allowed the lizards to rest with minimal human contact until thermal experiments began in June 2012. For these experiments, we exposed each lizard, in random order, to four temperature treatments (10, 20, 28, and 35 °C) for 5 h each. These temperatures spanned the range of possible active T_B in *E. coerulea* and *E. multicarinata*. The thermal extremes are approximately the minimum and maximum active T_B recorded in both species (Brattstrom, 1965; Cunningham, 1966; Kingsbury, 1994; Stewart, 1984). Moreover, prior work suggests that T_B of 10 and 35 °C challenge homeostatic function in *E. multicarinata* (Dawson and Templeton, 1966). For example, breathing rate, heart rate, and evaporative water loss are exceptionally high above 33 °C, while breathing rate drops precipitously below 15 °C (for breathing rates, $Q_{10} > 10$ in both of these ranges; Dawson and Templeton, 1966). 28 °C optimizes ATPase and skeletal muscle activity (Licht, 1964, 1967) in *E. multicarinata* and is their preferred body temperature in the field and laboratory (Dawson and Templeton, 1966; Kingsbury, 1994; Licht, 1964). 28 °C thus appears to correspond to the physiological optimum for *E. multicarinata*. Similar published data are not available for *E. coerulea,* but we found that *E. coerulea* in our colony maintained body temperatures near 28 °C when allowed to thermoregulate (see Section 2.1.1, above). Finally, 20 °C approximates the average active temperature for both species (Brattstrom, 1965; Cunningham, 1966; Kingsbury, 1994; Sheen, 2001; Stewart, 1984).

The temperature experiments were separated by one week, beginning 8 June 2012. For each experiment, we placed the lizards in one of four thermal chambers (7 or 8 lizards per chamber) illuminated with small LED lights that did not measurably radiate heat. We confirmed chamber temperatures using iButton thermal data loggers. The time when lizards were in each chamber was staggered to allow post-treatment blood collection: we placed lizards in the 10 °C chamber from 09:30 to 14:30, the 20 °C chamber from 09:45 to 14:45, the 28 °C chamber from 10:00 to 15:00, and the 35 °C chamber from 10:15 to 15:15 (±1.0 min for each experiment). Thus, lizards were exposed to each temperature treatment for 5 h, which is sufficient for alligator lizard body temperatures to equilibrate to treatment temperatures (Dawson and Templeton, 1966). The timing of the temperature treatments approximately corresponded to when under-cage heaters were on in the animal room as well as the warmest part of the day in nature. The chambers provided no opportunity for behavioral thermoregulation and evaporative cooling has little effect on body temperature in these species (Dawson and Templeton, 1966). Thus, lizard body temperatures should have equilibrated with treatment temperatures by the end of each treatment. To reduce external stressors, we left the lizards in their original home enclosures while in the thermal chambers, and only handled the lizards to

collect blood. In addition, at 17:30 the evening prior to each experiment, we moved the lizards (within their home enclosures) from their animal room to the room that housed our thermal chambers. The lizards then stayed overnight in the thermal-chamber room, which was maintained at ~21.5 °C and had the same light cycle as the animal room. Immediately following each 5-h temperature treatment, we collected blood from each lizard (for details see Section 2.3, below) and returned the lizards to their shelves in the animal room. After each experiment, we allowed the lizards to rest one week, after which we repeated the entire process until each lizard was exposed to each thermal treatment. Over the course of the experiment, every lizard was exposed to every temperature treatment, but the order that lizards were exposed to each temperature treatment was randomized. This protocol allowed us to have a complete, randomized, repeated-measures statistical design.

2.2. Assessment of field corticosterone and body temperature

To assess natural alligator lizard CORT levels, we collected plasma samples and T_B measurements from 10 field-active *E. multicarinata*. These lizards were located via active searching in July 2013 in Sonoma County, California (precise capture locations and body-size data in Table A1). After capture, we immediately collected a blood sample and measured cloacal body temperature using a pre-calibrated thermocouple and digital thermometer. All blood samples and body temperatures were collected within 2.5 min of initially observing the lizards. We then measured lizard body mass and SVL, and released the lizards where they were originally observed.

2.3. Blood collection

At each sampling time, we collected ~50 μL of blood from a lizard by piercing its post-orbital sinus with a 75 μL heparinized micro-hematocrit capillary tube (Fisher #22-362-566). After blood was drawn into the tubes by capillary action, we carefully removed the tube and applied pressure to the orbital region using a clean cotton pad until bleeding stopped (usually a few seconds). We then returned the lizards to their home enclosures (laboratory experiment) or released them (field study). Blood was usually collected within 1 min of the onset of handling, and the entire process was usually complete within 2 min. We placed the blood samples on ice immediately following collection. Within 5 h, we centrifuged the blood samples (7000 rpm for 10 min), then pippetted off the plasma and stored it at −80 °C for later corticosterone quantification. Field-collected plasma samples were shipped to the laboratory at ISU on dry ice within 2 days of collection and stored at −80 °C.

Collecting blood from the post-orbital sinus in lizards induces minimal stress (Langkilde and Shine, 2006). We observed resumption of normal behaviors (e.g., foraging, exploring, etc.) within seconds of blood collection. For our laboratory experiments, we collected six blood samples from each lizard. To reduce the potential negative consequences of repeated blood collection, we alternated orbital sinuses at each bleeding (each sinus was bled three times in experimental lizards), leaving at least 2 weeks for recovery between consecutive blood draws from the same orbital sinus.

2.4. Quantification of plasma corticosterone

We quantified plasma CORT concentration using the ImmunChem Double Antibody Corticosterone I-125 RIA kit (Catalogue #07-120103, MP Biomedical, Orangeburg, NY; average% recovery = 100.1%), as modified for squamate reptiles (Robert et al., 2009). To validate the use of this radioimmunoassay with alligator lizards, we tested for parallelism between the kit standards and serial dilutions of a pool derived from our plasma samples (hereafter "plasma pool," derived from 8 *E. multicarinata*, 2 samples from each temperature treatment). The serial dilutions of the standards and our plasma pool were parallel after logit transformation (alligator lizard: slope = −1.963, R^2 = 0.96; CORT standards: slope = −1.939, R^2 = 0.99), confirming the validity of quantifying plasma CORT in alligator lizards with this radioimmunoassay.

Following validation, we assayed two replicates of each sample at a 1:40 dilution. We re-assayed samples whenever the intra-assay coefficient of variation (CV) of replicate samples was >10, or if CORT estimates were outside the bounds of the standard curve. For the former, samples were re-assayed until the intra-assay CV was ⩽10, and for the latter, samples were further diluted until they fell within the standard curve (dilutions were accounted for when calculating final CORT concentration). After re-assaying the samples, the mean (±s.e.) intra-assay CV was 3.31 ± 0.22. During each assay, we also quantified CORT from replicate samples of the plasma pool to calculate inter-assay variation, the CV of which was 25.96. To control for this variation, we transformed all CORT estimates prior to analyses such that the plasma-pool estimates were equal across assays.

2.5. Statistical analyses

2.5.1. Laboratory experiment

All analyses were performed using the program R (version 3.0.1, R Core Team, 2013). Prior to analyses, we assessed normality graphically using boxplots, histograms, and q–q plots (Zuur et al., 2009). CORT concentration estimates were non-normally distributed, so we log-transformed these data to meet the assumptions of parametric statistics. After transformation, we used boxplots to identify outliers (points greater than 1.5 times the interquartile range for each species and treatment). These points (N = 5 out of 180) were removed prior to analyses to meet the assumptions of our statistical tests. No more than one measurement was removed for any individual. We used generalized linear mixed-effects models (GLMM) to test for effects of treatment on CORT concentrations in *E. multicarinata* and *E. coerulea.* We examined two models: the first tested for effects of our experimental temperature treatments (10, 20, 28, and 35 °C, "temperature model" hereafter) on CORT, whereas the second model compared CORT before and after ACTH challenge ("ACTH model" hereafter). Experimental treatment, species, and their interaction were included as fixed-effects in these models, and individual lizard was included as a random intercept. We selected random-intercept-only models because they modeled the data better than models that included random intercepts and slopes (ΔBIC = −20.27 and −7.90, respectively, and analyses of residuals showed better homogeneity of the random-intercept-only model in both cases). Because we found a significant interaction between species and treatment, we also tested for effects of treatment on CORT for each species separately. We used the "lme" function in the *nlme* package for these analyses (Pinheiro et al., 2013). We validated the assumptions of the final models graphically by examining histograms of the residuals, and plots of the residuals vs. fitted values (Zuur et al., 2009). To assess pairwise differences in plasma CORT concentrations between the thermal treatments, the laboratory control (at ~23.5 °C), and in response to ACTH challenge, we used *post hoc* Tukey tests (function "lsmeans" in the lsmeans package, Lenth, 2013).

The *E. multicarinata* that we examined were collected from a broad geographic area and likely represent multiple populations (Table A1). While population boundaries are unclear, evidence from mitochondrial DNA suggest that *E. multicarinata* can be divided into two major clades (Feldman and Spicer, 2006). Based on their geographic location at collection and the predicted distribution of the mitochondrial DNA clades from Feldman and Spicer

(2006), we assigned each *E. multicarinata* to a clade. Early in our analyses, we included clade of origin as a fixed-effect factor in our models. Neither clade of origin nor its interactions significantly affected CORT in any model ($P > 0.10$ for all). We therefore removed clade of origin from the final models. In addition, models including the order that the lizards were exposed to each temperature treatment and/or sex showed that neither of these factors (nor their interactions) affected CORT concentration ($P > 0.10$ for all), so we also removed these factors from the final models. Time of day when blood-samples were collected could not be included in the models because we sampled each treatment at the same time during each experiment, thereby conflating thermal and temporal effects. Even so, all samples were collected within the same hour (although on different days), which should minimize any effect of the diel cycle on our results. Moreover, the lack of an effect of experiment order on our results suggests that slight differences in timing of blood collection did not significantly affect our results.

While examining boxplots of the original, untransformed data, we noticed apparent variation in the dispersion of plasma CORT between the two species. We tested this hypothesis using *F*-tests comparing CORT variation between the two species within each treatment. For this analysis, we used our original, untransformed, CORT estimates and the "var.test" function in the base installation of R.

2.5.2. Field study

As with the laboratory experiment, we log transformed the CORT estimates from our field-sampled *E. multicarinata* so that the data met the assumptions of parametric statistics. We examined concordance between our laboratory and field CORT estimates by comparing their mean and variance using a Welch's two-sample *t*-test and an *F*-test, respectively. We used log-transformed data for the *t*-test but untransformed data for the *F*-test. In addition, we tested for a correlation between body temperature and plasma CORT using linear and quadratic regressions. For these analyses, we created a "laboratory" group by pooling our CORT estimates from *E. multicarinata* exposed to all four experimental temperature treatments.

3. Results

3.1. Laboratory experiment

Both the "temperature" and "ACTH" models found a significant interaction between species and treatment on plasma CORT concentration (temperature model: $F_{3,81} = 4.42$, $P = 0.0063$, ACTH model: $F_{1,26} = 9.30$, $P = 0.0052$, Fig. 2). When analyzed separately, experimental temperature affected CORT in both *E. coerulea* ($F_{3,41} = 8.62$, $P = 0.0001$, Fig. 2A) and *E. multicarinata* ($F_{3,40} = 7.36$, $P = 0.0005$, Fig. 2C). Generally, the relationship between temperature and CORT was similar between species (Fig. 2A and C). In both species, CORT was lowest at 20 °C and increased with warmer temperatures, leveling off at 28 °C (Fig. 2A and C, Table 1). In *E. multicarinata*, CORT also increased as temperature cooled to 10 °C (Fig. 2C), but CORT did not increase with colder temperatures in *E. coerulea* (Fig. 2A, Table 1). In both species, ACTH challenge elevated CORT levels (*E. coerulea*: $F_{1,12} = 60.41$, $P < 0.0001$; *E. multicarinata*: $F_{1,14} = 150.75$, $P < 0.0001$) much higher than any temperature treatment, and this increase was greater for *E. multicarinata* than *E. coerulea* (Fig. 2B and D, Table 1). We estimated, *a priori*, that the laboratory control samples represent samples from ~23.5 °C (see Sections 2.1.1 and 2.1.2 for details). Concordant with this hypothesis, mean CORT levels in these samples fell between those from the 20 and 28 °C treatments in both species (Fig. 2A and C). When ACTH samples were collected, lizard T_B was ~22 °C (Section 2.1.2), thus ACTH greatly elevated CORT beyond that expected from T_B alone. Whenever plasma CORT concentration differed between the species, *E. multicarinata* had higher levels than *E. coerulea* (Fig. 2, Table 2). The dispersion of CORT estimates also differed between the two species (Table 3). For all treatments, CORT

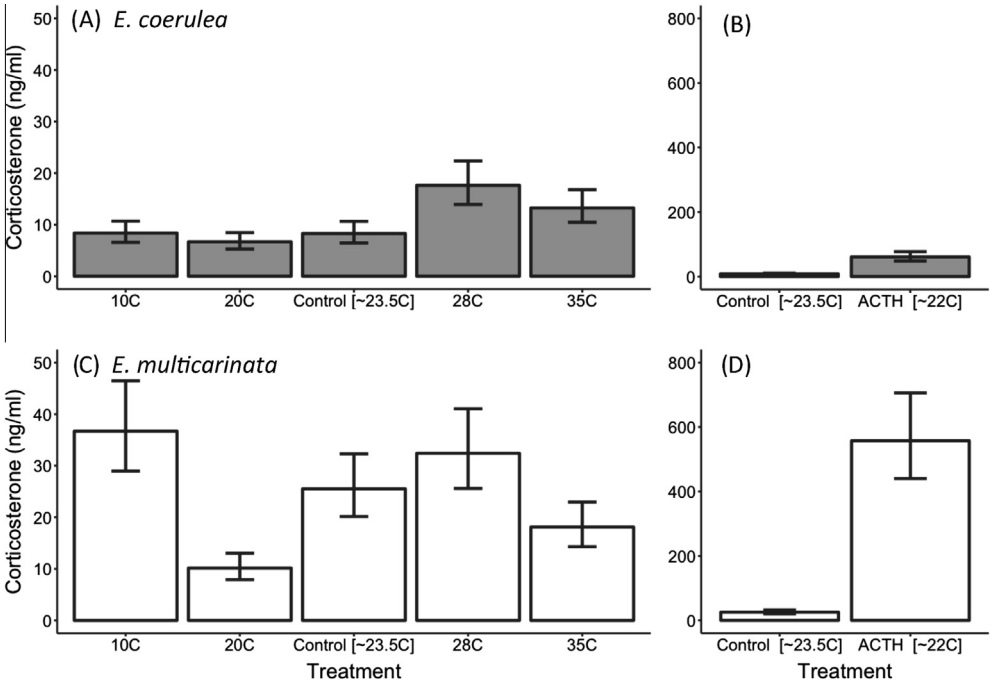

Fig. 2. Treatment effects on plasma corticosterone (CORT) concentrations in northern alligator lizards (*Elgaria coerulea*, A and B) and southern alligator lizards (*E. multicarinata*, C and D). Back-transformed least-squares means ± standard errors are displayed from the experimental temperature treatments (A and C) and the laboratory control and ACTH treatments (lizard T_B's were approximately 23.5 and 22 °C for these treatments, respectively, B and D). Models also included species and the interaction between treatment and species as fixed effects, and individual as a random effect. Note that panels (A) and (C) have the same *y*-axis scale as do panels (B) and (D), but that the scale of (A) and (C) differs from that of (B) and (D). Values for the "Control [~23.5]" treatment are identical in (A) and (B), and (C) and (D), respectively, and can be used to assess differences in scale. Results from pairwise tests are given in Tables 1 and 2.

Table 1
Matrix of results from between-treatment pairwise comparisons of plasma corticosterone levels in northern and southern alligator lizards (*Elgaria coerulea* and *E. multicarinata*). Untransformed mean (±s.e.) plasma corticosterone concentrations (ng/ml) for each treatment are presented on the diagonals (shaded regions). *Z*-scores and *P*-values from pairwise tests are displayed below and above the diagonals, respectively. While exact, uncorrected *P*-values are presented, bold-font indicates significant differences ($P < 0.05$) after a Tukey correction for multiple comparisons. Control indicates laboratory control samples and ACTH indicates samples after adrenocorticotrophic hormone challenge (lizard T_B's were approximately 23.5 and 22 °C, respectively).

Treatment	10C	20C	28C	35C	Control	ACTH
Elgaria coerulea						
10C	9.3 ± 1.3	0.41693	**0.00776**	0.10116	0.97191	**<0.00001**
20C	0.814	9.0 ± 2.0	**0.00039**	**0.01241**	0.44838	**<0.00001**
28C	-2.66	-3.54	21.1 ± 3.0	0.29652	**0.00829**	**0.00001**
35C	-1.641	-2.50	1.04	14.9 ± 1.9	0.10115	**<0.00001**
Control	0.04	-0.76	-2.64	-1.64	8.8 ± 1.0	**<0.00001**
ACTH	-7.10	-8.07	-4.53	-5.57	-6.98	85.3 ± 17.9
Elgaria multicarinata						
10C	89.0 ± 31.5	**0.00001**	0.65025	0.01005	0.18452	**<0.00001**
20C	4.49	14.1 ± 5.5	**0.00005**	0.04303	**0.00129**	**<0.00001**
28C	0.45	-4.06	68.71 ± 23.0	0.03393	0.38234	**<0.00001**
35C	2.57	-2.02	2.12	23.5 ± 3.9	0.21221	**<0.00001**
Control	1.33	-3.22	-0.87	1.25	54.2 ± 22.2	**<0.00001**
ACTH	-9.93	-14.01	-10.38	-12.50	-11.26	765.0 ± 156.7

Table 2
Results from pairwise comparisons of plasma corticosterone between northern and southern alligator lizards (*E. coerulea* and *E. multicarinata*) at each experimental treatment level. *Z*-values and *P*-values are shown. While exact, uncorrected *P*-values are presented, bold-font indicates significant differences ($P < 0.05$) after a Tukey correction for multiple comparisons. Control indicates laboratory control samples and ACTH indicates samples after adrenocorticotrophic hormone challenge (lizard T_B's were approximately 23.5 and 22 °C, respectively). Untransformed means (±s.e.) for each species and treatment level are given in Table 2.

Treatment	*Z*	*P*
10C	−4.36	**0.00001**
20C	−1.22	0.22294
28C	−1.82	0.06913
35C	−0.93	0.35007
Control	−3.26	**0.00110**
ACTH	−6.62	**<0.00001**

Table 3
Dispersion of plasma corticosterone concentrations in southern and northern alligator lizards (*Elgaria multicarinata* [*E.m.*] and *E. coerulea* [*E.c.*]) exposed to each experimental treatment. Coefficients of variation (CV), sample variance (s^2) and results from *F*-tests for equal variances with untransformed data are displayed. Control indicates laboratory control samples and ACTH indicates samples after adrenocorticotrophic hormone challenge (lizard T_B's were approximately 23.5 and 22 °C, respectively). Significant *P*-values (<0.05) are in bold font.

Treatment	CV		s^2		*F*-value	d.f.	*P*-value
	E.m.	*E.c.*	*E.m.*	*E.c.*			
10C	137.0	52.1	14873.9	23.7	627.82	14,13	**<0.00001**
20C	139.3	86.1	386.5	60.5	6.39	12,14	**0.00161**
28C	129.8	54.6	7957.0	132.5	60.07	14,14	**<0.00001**
35C	64.1	48.1	226.8	51.4	4.41	14,14	**0.00886**
Control	158.3	40.1	7368.0	12.5	587.92	14,12	**<0.00001**
ACTH	79.3	81.0	368069.1	4777.6	77.04	14,14	**<0.00001**

estimates for *E. multicarinata* were at least an order of magnitude more variable (s^2) than those for *E. coerulea* (Table 3). Similarly, the coefficient of variation for CORT was an order of magnitude higher for *E. multicarinata* (pooled CV = 163.7) than for *E. coerulea*

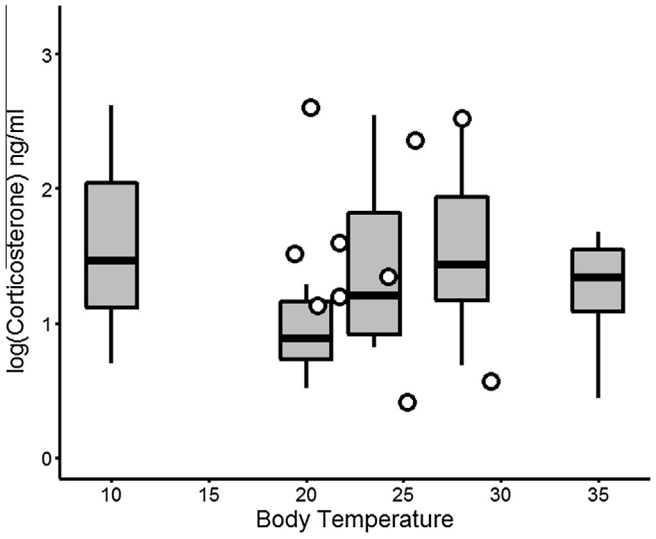

Fig. 3. Effect of body temperature on CORT in southern alligator lizards (*Elgaria multicarinata*) from the laboratory and field. Boxplots represent CORT estimates from our laboratory experiment, including the ~23.5 °C laboratory control. The lines within the boxes represent medians, box limits depict the first and third quartiles, and box whiskers are 1.5 times the interquartile range. The overlaid scatterplot represents the relationship between CORT and T_B of 10 active lizards in the field.

(pooled CV = 69.1; CV values for each species and treatment are given in Table 3).

3.2. Field study

Mean T_B of the field-sampled *E. multicarinata* was 23.61 °C (range = 19.4–29.5 °C) and mean (±s.e.) plasma CORT concentration was 108.84 ± 47.84 ng/ml (Fig. 3). Mean CORT concentration did not differ between *E. multicarinata* sampled in the field and laboratory ($t = 0.75$, $df = 10.55$, $P = 0.4715$). However, field CORT estimates were more variable than those from the laboratory (field $s^2 = 22885.95$, laboratory $s^2 = 6716.097$, $F_{9,57} = 3.41$, $P = 0.0041$). Neither linear ($F_{1,8} = 0.169$, $P = 0.6918$) nor quadratic (T_B: $F_{1,7} = 0.1479$, $P = 0.7119$, T_B^2: $F_{1,7} = 0.0018$, $P = 0.9669$) regression found a significant effect of T_B on plasma CORT in the field collected lizards (Fig. 3). Even so, CORT from the field-sampled *E. multicarinata* appeared similar to, but more variable than, CORT from the laboratory lizards (Fig. 3).

4. Discussion

Temperature broadly affects the biology of ectotherms and exposure to extreme T_B can by highly detrimental, if not fatal (e.g., Angilletta, 2009; Bradshaw, 2003; Chown and Terblanche, 2007). An important component of the response of vertebrate ectotherms to dangerous situations is increased production of GC hormones such as CORT that act to initiate an emergency physiological state commonly referred to as the stress response (Bradshaw, 2003; Selye, 1950). The stress response might be adaptive when vertebrate ectotherms are exposed to non-optimal T_B, potentially inducing animals to seek thermal shelter. Even so, the effects of temperature on CORT in vertebrate ectotherms, such as reptiles, are not well understood. Results from our experiment suggest that T_B directly affects CORT in northern and southern alligator lizards. Temperature thus appears to be an important factor affecting the physiological stress response in these lizards.

Our measurements for the T_B of active *E. multicarinata* concord with previous observations (Brattstrom, 1965; Cunningham, 1966; Kingsbury, 1994; Sheen, 2001) confirming the biological relevance of our experimental temperature treatments. The 20 °C and laboratory control [~23.5 °C] treatments modeled average thermal conditions experienced by these lizards (Cunningham, 1966; Kingsbury, 1994; Sheen, 2001; Stewart, 1984), while the other treatments modelled progressively more extreme conditions. While both species have been observed with active T_B of approximately 10 and 35 °C (Cunningham, 1966; Kingsbury, 1994; Sheen, 2001; Stewart, 1984), we did not observe lizards with T_B this extreme. Even so, these treatments should represent biologically-relevant extreme temperatures that are occasionally encountered by active alligator lizards.

E. multicarinata displayed generally higher CORT levels and a greater response to ACTH than *E. coerulea*, but the CORT response of each species to $T_B \geqslant 20$ °C was similar. Because all animals were acclimated under common-garden laboratory conditions for at least 10 months prior to the onset of experiments, any species-specific differences in the CORT response likely reflect genetic or developmental differences rather than short-term/reversible phenotypic plasticity to their capture environments. In both *E. coerulea* and *E. multicarinata*, CORT levels were lowest when lizards were exposed to 20 °C and elevated at higher temperature (plateauing at 28 °C). 20 °C approximates, but is slightly below, the average activity temperature observed in both species (Cunningham, 1966; Kingsbury, 1994; Stewart, 1984; and present study). Because prior work suggests that the optimum temperature in *E. multicarinata* is 28 °C (Kingsbury, 1994; Licht, 1967), the result that 20 °C induced the lowest CORT levels is somewhat surprising. While 28 °C maximizes ATPase activity (Licht, 1967), it is possible that this is not the optimum temperature for the entire organism. However, both laboratory experiments (Licht, 1964) and field observations (Kingsbury, 1994) have shown that *E. multicarinata* thermoregulate to ~28 °C when able. Moreover, we observed *E. coerulea* thermoregulating to approximately 28 °C in the laboratory (27.7 °C when the lights and heater were on). These results suggest that alligator lizards seek out T_B that induce CORT levels elevated above the minimum, but more frequently experience T_B that induce reduced CORT levels.

CORT levels increased with temperature above 20 °C in both species, but only *E. multicarinata* displayed increased CORT levels at 10 °C. This result supports the hypothesis that the thermal CORT response is adapted to the respective thermal environments of these species. Exposure for a longer period or to a colder temperature might significantly elevate CORT in *E. coerulea* as in *E. multicarinata.* Even so, variation in the thermal CORT response could partially explain the biogeographic differences between *E. coerulea* and *E. multicarinata*. Even if 5 h exposure to 10 °C does not stress *E. multicarinata*, *per se*, maintaining a CORT response is energetically expensive and many features of this response (e.g., inhibition of foraging or courtship) incur additional costs (Bradshaw, 2003; Nelson, 2011; Norris, 2007). Because *E. multicarinata* and *E. coerulea* are ecologically similar (Brattstrom, 1965; Stebbins, 2003), they presumably are under intense competition with each other. Competitive exclusion would explain why, even though their tolerances are similar, these species are rarely found at the same location (e.g., Armstrong and McGehee, 1980; Hutchinson, 1959). If *E. multicarinata* incur CORT-induced costs at cold temperatures and *E. coerulea* do not, presumably as a result of general adaptation to cooler environments, then *E. coerulea* will be competitively superior in these environments. The potential effect of the thermal CORT response on the competitive landscape also has implications for how species are affected by climate change. In species where temperature affects CORT, exposure to novel thermal environments will alter the physiological stress experienced by populations. Subtle species-specific differences in the stress response might affect the competitive landscape sufficiently that species go locally extinct through displacement well before environmental temperatures exceed critical thermal limits.

Because CORT was elevated, our results suggest that 5 h exposure to temperatures ≠ 20 °C in *E. multicarinata* and >20 °C in *E. coerulea* may challenge homeostasis. However, no temperature treatments induced CORT levels as high as ACTH challenge in either species. Temperature treatment therefore failed to induce a maximal CORT response (Klukowski, 2011; Phillips and Klukowski, 2008; Romero and Wingfield, 1999). Given that alligator lizards can be observed active at all of our experimental temperatures in nature (Cunningham, 1966; Kingsbury, 1994; Stewart, 1984) and they had the capacity to further elevate CORT concentration in response to additional stressors, the CORT response that we observed likely allowed maintenance of homeostasis (Bradshaw, 2003; Romero et al., 2009). Because CORT is an important regulator of intermediary metabolism (Nelson, 2011; Norris, 2007) and metabolic rate increases with body temperature in ectotherms (Angilletta, 2009), CORT levels might increase with temperature > 20 °C to facilitate an elevated metabolic rate. Thus, the CORT response that we observed at these temperatures might be a fully adaptive response and not indicative of stress, *per se*. However, this does not explain the elevated CORT levels that we observed in *E. multicarinata* exposed to 10 °C. Moreover, longer exposure to 10 °C or 35 °C, or exposure to even more extreme temperatures, can be fatal and thus likely challenges homeostasis

beyond the counteracting ability of CORT. Further work examining the exact physiological effects of these thermally-induced increases in CORT is needed to tease apart these possibilities.

In addition to having higher mean CORT levels in our laboratory experiment, *E. multicarinata* displayed more variation in CORT than *E. coerulea*. The average CV for CORT in reptiles in response to stressors ranges from 55.9 to 96.2 (Cockrem, 2013). Thus, *E. coerulea* displayed slightly below-average variation in CORT whereas *E. multicarinata* displayed above-average variation (Table 3). If this variation is heritable and additive, the CORT response of *E. multicarinata* to temperature will have greater evolutionary potential than that of *E. coerulea* (Falconer and Mackay, 1996). This could have important implications for the ability of these animals to invade new thermal habitats or to respond to impending climate change. An alternate explanation for the difference in variance that we observed is that these variances reflect how the animals were sampled. Most of the *E. coerulea* examined were collected from a single site, whereas the *E. multicarinata* examined were collected over a broad geographic range, and thus might be expected to be more variable. However, *E. multicarinata* clade of origin (Feldman and Spicer, 2006) did not affect CORT, suggesting that the CORT response has not diverged among *E. multicarinata* clades. Furthermore, the *E. multicarinata* that we assayed for our field study displayed greater CORT variation than those from our laboratory experiment, even though the field-study lizards were sampled from a single county. These observations suggest that natural populations of *E. multicarinata* display high variance in their CORT response.

While our experimental data demonstrate an effect of T_B on CORT, we did not detect a correlation between T_B and CORT in field-active *E. multicarinata*. This lack of correlation likely resulted from the small sample size of our field study and the high variance of CORT in *E. multicarinata*. Moreover, many additional factors can affect CORT in the field (e.g., time of day, nutritional status, previous predator encounters, etc, Bradshaw, 2003; Nelson, 2011; Romero, 2004) but were controlled in the laboratory. It is therefore not surprising that we were unable to detect a correlation between T_B and CORT in the field. Even so, mean CORT of *E. multicarinata* in the field and laboratory did not differ and the variance that we observed in the laboratory was within the bounds observed in the field. These observations suggest that our laboratory CORT results likely apply to natural populations.

Our data provide evidence that CORT and T_B are causally related in alligator lizards and not simply correlated (e.g., Fig. 1). A causal relationship between CORT and T_B can be inferred because CORT levels predictably changed with temperature even though the order that the lizards were exposed to each temperature treatment was randomized and treatment order had no effect on CORT. In other reptile species, experimentally elevated CORT alters thermoregulatory behavior, either increasing heat seeking (Belliure et al., 2004; Preest and Cree, 2008) or cooling behaviors (Belliure and Clobert, 2004), depending on species. By contrast, we demonstrate that T_B directly affects CORT levels in *E. multicarinata* and *E. coerulea*. T_B also affects CORT in garter snakes (Schwartz and Bronikowski, 2013) and Children's pythons (Dupoué et al., 2013). The causal pathway between CORT and T_B might thus function in both directions in reptiles. If so, this could represent an important regulatory feedback loop that could partially control behavioral thermoregulation. Exposure to extreme T_B could increase CORT thereby inducing thermoregulatory behaviors (heating or cooling) as appropriate. This might generally be a negative feedback loop, with high T_B inducing cooling behaviors through elevation in CORT. Alternatively, a positive feedback loop might help maintain high CORT levels during energetically demanding periods, such as the reproductive season. Given the effects of CORT and T_B on metabolism (Norris, 2007; Preest and Cree, 2008; Squires, 2003), such a system might maximize the availability of free energy. Further work is necessary to understand how T_B, CORT, and thermoregulatory behavior interact, and the potential importance of this interaction as a regulatory mechanism.

To conclude, T_B and CORT are causally related in northern and southern alligator lizards. The exact relationship between T_B and CORT is species specific and the thermal CORT response appears to be adapted to the thermal environment of each species. Physiological stress likely plays an important role in the thermal ecology of these species and may affect the outcome of their competitive interactions in different thermal environments. Although poorly explored to date, causal relationships between T_B and CORT may be common in vertebrate ectotherms. If so, the thermal CORT response of species will affect how they are geographically distributed and how they respond to impending climate change.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2014.07.004.

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