RESEARCH ARTICLE

Maternal stress alters the phenotype of the mother, her eggs and her offspring in a wild-caught lizard

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Abstract

1. While biomedical researchers have long appreciated the influence of maternally derived glucocorticoids (GCs) on offspring phenotype, ecologists have only recently begun exploring its impact in wild animals. Interpreting biomedical findings within an ecological context has posited that maternal stress, mediated by elevations of maternal GCs, may play an adaptive role preparing offspring for a stressful or rigorous environment. Yet, the influence of maternal stress on offspring phenotype has been little studied in wild animals.

2. We experimentally elevated GCs to ecologically relevant levels (mimicking increases in maternal stress hormones following a nonlethal predator encounter, a heat challenge, or a chasing or confinement stressor) in female eastern fence lizards *Sceloporus undulatus* during gestation. We tested the hypothesis that maternally derived stress hormones themselves are sufficient to alter offspring phenotype. Specifically, we examined the effects of experimentally elevated maternal GCs on fitness-relevant traits of the mother, her eggs and her subsequent offspring.

3. We found that daily maternal GC elevation: (a) increased maternal antipredator behaviours and postlaying glucose levels; (b) had no effect on egg morphology or caloric value, but altered yolk hormone (elevated GC) and nutrient content; and (c) altered offspring phenotype including stress-relevant physiology, morphology and behaviour.

4. These findings reveal that maternally derived GCs alone can alter offspring phenotype in a wild animal, changes that may be mediated via maternal behaviour, and egg hormone and nutrient content. Understanding the ecological consequences of these effects under different environmental conditions will be critical for determining the adaptive significance of elevated maternal GCs for offspring.

KEYWORDS
corticosterone, developmental plasticity, maternal programming, maternal stress effects, prenatal stress, *Sceloporus undulatus*

1 | INTRODUCTION

When faced with environmental challenges or changes, activation of the hypothalamic–pituitary–adrenal (HPA) axis and subsequent release of glucocorticoids (GCs) is central to organisms' coping and responding appropriately (McEwen & Wingfield, 2003; Romero, Dickens, & Cyr, 2009). The release of GCs can thus mediate the relationship between the environment experienced by an individual and their behaviour, physiology and fitness (Boonstra, 2013; Romero et al., 2009; Wingfield & Sapolsky, 2003). There is a growing body of experimental evidence showing that circulating maternal GCs during gestation can alter offspring phenotypes (reviewed in Love, McGowan, & Sheriff, 2013; Meaney, Szyf, & Seckl, 2007). The biomedical community has explored the relationship between elevated maternal GCs and offspring with regards to human health, with resulting phenotypic responses commonly characterized as negative side effects to elevated maternal stress (Meaney et al., 2007; Seckl & Meaney, 2004; Weinstock, 2005). An alternative, ecologically driven hypothesis suggests that exposure to maternal GCs in utero or in ovo may adaptively prepare offspring for their future environment (Meylan & Clobert, 2005; Sheriff, Dantzer, Love, & Orrock, 2018; Sheriff & Love, 2013). For example, offspring of GC-treated mothers fled towards refuge in response to acute stress more often than those from control mothers in the common lizard *Lacerta vivipara* (De Fraipont, Clobert, John-Alder, & Meylan, 2000). As such, the influence of elevated maternal GCs on offspring phenotype, which we define as maternal stress effects (sensu Sheriff et al., 2017), has been proposed as a factor influencing ecological and evolutionary processes (Meylan, Miles, & Clobert, 2012; Sheriff, 2015; Sheriff et al., 2018).

The effect of maternal stress on offspring phenotype has been proposed to function primarily via direct effects of exposure of the developing embryo to maternally derived GCs (Sheriff et al., 2018). However, elevated maternal GC levels may also indirectly alter offspring phenotype via changes in maternal physiology, behaviour, thermoregulation, and/or hormone and nutrient allocation to developing embryos (Cottrell, Holmes, Livingstone, Kenyon, & Seckl, 2012; Meylan, Belliure, Clobert, & de Fraipont, 2002; Sheriff et al., 2017). For example, in song sparrows *Melospiza melodia*, risk-induced increases in maternal GCs reduced female body condition and altered their nesting behaviour and incubation time, ultimately reducing their reproductive investment and output (Travers, Clinchy, Zanette, Boonstra, & Williams, 2010; Zanette, White, Allen, & Clinchy, 2011). It has also been shown in many bird taxa that females with elevated GCs lay eggs with reduced yolk mass (indicating reduced nutritional value; Henriksen, Groothuis, & Rettenbacher, 2011), although little is known about maternal GC-induced shifts in nutrient transfer (Fowden & Forhead, 2009). Yet, few studies have holistically examined how elevated maternal GCs may alter maternal behaviour and energetics, hormonal and nutrient allocation to developing embryos, and offspring phenotype.

Here, we examined the effects of experimentally increased maternal GCs on mothers, their eggs and their offspring using wild-caught eastern fence lizards *Sceloporus undulatus*. This species is subject to a variety of stressors, including fluctuating temperatures, competition for resources and mating opportunities, and predation risk (Cooper, Burghardt, & Brown, 2000; Jensen, 2008; Thawley & Langkilde, 2017). The latter likely imposes an important selective pressure, particularly within sites invaded by predatory red imported fire ants *Solenopsis invicta* (Freidenfelds, Robbins, & Langkilde, 2012). These ants are a major threat to the lizards due to their high density, active prey-searching mode, swarming attack behaviour and ability to fatally envenomate animals much larger than themselves (Haight & Tschinkel, 2003; Holtcamp, Grant, & Vinson, 1997; Langkilde, 2009b). This is significantly different from the hunting strategies of visual predators (e.g., snakes and birds) with which these lizards evolved (Curio, 1976; Duncan, 1966; Parmley & Parmley, 2001). Hatchling lizards are vulnerable to attack by both invasive and native ants, but are particularly at risk from red imported fire ants; exposure to just five of these ants for 30 s can result in mortality (Gifford, Robinson, & Clay, 2017; Langkilde & Freidenfelds, 2011). Lizards can increase their chances of surviving noxious encounters by behaviourally responding to fire ant attack (Freidenfelds et al., 2012; Langkilde, 2009b). Interactions with fire ants can elevate basal corticosterone levels (CORT; a key GC in lizards; Graham, Freidenfelds, Thawley, Robbins, & Langkilde, 2017), and increased circulating CORT concentrations can affect fitness-relevant behaviour, allowing lizards to avoid and escape fire ant attack (Langkilde, Thawley, & Robbins, 2017; Trompeter & Langkilde, 2011). However, these fire ant-induced responses may make lizards more susceptible to predation by birds (Thawley & Langkilde, 2017).

We experimentally elevated maternal CORT during gestation in lizards from sites with long histories of fire ant invasion to test the hypothesis that elevated maternal CORT is a mechanism driving changes in offspring phenotypes. We applied exogenous CORT daily at concentrations that mimic the natural CORT response of lizards to a nonlethal interaction with fire ants (Graham et al., 2017; Trompeter & Langkilde, 2011). We examined the influence of this daily ecologically relevant elevation of maternal CORT on the mothers and their eggs and offspring. We predicted that elevated maternal CORT would result in:

1. An increase in maternal antipredator behaviour, indicated by increased fleeing/escape behaviour and time spent hiding, and a concurrent reduction in time spent basking;
2. An increase in maternal energy mobilization, indicated by elevated plasma glucose, given that changes in behaviour and CORT are predicted to be energetically costly (Landys, Ramenofsky, & Wingfield, 2006);
3. An increase in yolk CORT and a decrease in testosterone levels, and a reduction in nutrient allocation and individual egg size as mothers reduce investment in reproduction;
4. An increase in hatchling HPA axis reactivity, indicated by increased baseline CORT and responsiveness to an ACTH challenge (Hayward, Richardson, Grogan, & Wingfield, 2006; Sheriff, Krebs, & Boonstra, 2010), decreased offspring glucose levels, as maternal stress has been shown to programme glucose intolerance and

decreased pancreatic glucose production (Lesage et al., 2004), and decrease haematocrit levels as a proxy of physiological condition (Owen, Sogge, & Kern, 2005; Sheriff et al., 2010);

5. A decrease in hatchling size (mass, length and relative hindlimb length; Saino, Romano, Ferrari, Martinelli, & Møller, 2005), more prevalent fire ant-specific antipredator behaviours (increased time spent off the ground and response to tactile stimulation mimicking ant attack) and more prevalent visual predator antipredator behaviour (greater response to an approaching novel object and decreased activity; sensu Sheriff & Love, 2013).

2 | MATERIALS AND METHODS

2.1 | Study system

Gravid female eastern fence lizards were collected in late April and early May from Geneva State Forest, Conecuh National Forest and Blakeley State Park, southern Alabama, in 2015 (CORT-treated [stressed] S = 13, control [C] = 16) and 2016 (S = 15, C = 20). These sites, similar in habitat and abiotic factors (Langkilde, 2009a; MacLeod, Freidenfelds, Leighton, & Langkilde, unpublished data), are located within a mixed temperate forest dominated by *Pinus palustris* (longleaf pine) and *Quercus* (oak) trees, interspersed with clearings in the form of meadows and roads. All three sites were invaded by red imported fire ants between 69 and 85 years ago (Callcott & Collins, 1996).

2.2 | Adult capture and housing

Immediately upon capture we obtained a blood sample from the postorbital sinus of each female (time to bleed = 160 ± 8 *SE* seconds). Glucose was recorded immediately with a whole blood glucometer (One Touch Ultra2; LifeScan Inc., Milpitas, CA), and blood samples were placed on ice until centrifugation; plasma was separated and frozen within 4 hr of collection for later CORT analysis. The glucometer was validated by pooling serum of *S. undulatus* and separated into four fractions, 30 µl each, which were treated as independent samples. The samples were measured with the glucometer prior to spiking and values recorded (294.25 ± 3.86 mg/dl). The samples were then spiked with 5 µl of 900 mg glucose drawn from a commercially available glucose standard (900 mg/dl Glucose Standard, YSI 1531; YSI, Yellow Springs, OH). This amount was chosen to reflect the range of glucose values measured in this species. The spiked samples were measured using the glucometer (347.75 ± 4.19 mg/dl). Average recovery of the glucose spike was 91.51 ± 1.10% (Cooper, Khosravan, Erdmann, Fiene, & Lee, 2006; Trompeter & Langkilde, 2011). Females were toe-clipped for individual identification and measured for mass (0.01 g), SVL (mm), tail length (TL, mm) and right hindlimb length (HL, mm). Lengths were taken by pressing the ventral side of the lizard against a clear ruler; hindlimb length was measured from the intersection of the first and second toes to the anterior seam of the hip and body wall (see Langkilde, 2009b supp materials for a photograph). Gravidity was determined via abdominal palpation

and scored on a scale from 1 to 4, with females scoring an average of 2.9 at capture (1: nonyolked follicles; 2: developing yolked follicles; 3: large preovulatory follicles; 4: oviducal eggs, Graham, Freidenfelds, McCormick, & Langkilde, 2012). Females were temporarily housed at Auburn University's Solon Dixon Forestry Education Center for 2–13 days prior to transport by minivan (individually housed in cloth bags to reduce stimulation, within a climate-controlled box) to the Pennsylvania State University (16 hr).

Females were housed in pairs in plastic bins (46 × 40 × 30 cm, $L × W × H$) until oviposition (McCormick, Robbins, Cavigelli, & Langkilde, 2017). Bins were furnished with a hollow perch for basking and shelter, a water bowl, moist sand and a 60-W incandescent light bulb above the perch to provide heat for 8 hr a day (c. 32°C, 0800–1600 hr). The room was kept at c. 21°C and overhead lights provided ambient lighting on a 12:12 light:dark schedule (0700–1900 hr). Food (crickets *Acheta domestica*, dusted with vitamins and minerals) was provided every other day, and water was available ad libitum.

2.3 | Experimental procedures

2.3.1 | Maternal hormone application

Females were randomly assigned to one of two groups: CORT treatment (N = 28) or control (N = 36). At the beginning of the experiment, females in these two treatments did not differ in morphology or physiology: mass ($F_{1,56}$ = 0.196, p = 0.659), SVL ($F_{1,56}$ = 0.242, p = 0.625), TL ($F_{1,56}$ = 0.400, p = 0.530), relative HL ($F_{1,56}$ = 1.522, p = 0.220), capture CORT ($F_{1,75}$ = 0.010, p = 0.920), capture glucose ($F_{1,63}$ = 1.805, p = 0.184) or stage of gravidity (χ^2_4 = 4.638, p = 0.327). We placed a coloured dot of nontoxic nail polish on each female's back to visually indicate her treatment (Pure ICE, New York, NY). CORT-treated females received a daily transdermal application of 0.2 µl/g lizard of 4 mg CORT in 1 ml of sesame seed oil vehicle (0.8 µg CORT/g lizard; Trompeter & Langkilde, 2011); control females received the corresponding volume of oil vehicle only. The CORT or control solution was applied to the middle of the females' backs daily from capture until laying between 1930 and 2030 hr, after the room lights had turned off and lizards had ceased activity, avoiding the need to handle the lizards (number of CORT applications: 9–55; avg = 31.5 ± 1.6 applications). Thirty minutes after dosing, plasma CORT concentrations (Trompeter & Langkilde, 2011) reflect those measured 30 min after a lizard is exposed to a variety of stressors including a nonlethal attack by fire ants (Graham et al., 2017), following a 30-min heat challenge (lizards brought to panting; Simpson, Telemeco, Langkilde, & Schartz, 2018), a chasing stressor (lizard chased around a plastic tub; Trompeter & Langkilde, 2011) and a restraint stressor (held within a cloth bag; Graham et al., 2012). The dose-elevated CORT levels in females return to baseline levels within 3.5 hr (MacLeod, Sheriff, Ensminger, Owen, Langkilde, in press), creating a more realistic exposure than sustained high CORT levels that pellet or silastic implants may provide (Sopinka et al., 2015). CORT treatment did not affect baseline maternal CORT measured 3 days

postlaying ($F_{1,44}$ = 8.021, p = 0.304), supporting the transient, acute nature of this treatment. We chose this method of GC elevation over exposure to a natural stressor as it allowed us to examine the effect of maternal CORT directly without the confounding effect of individual perception of risk (Meylan & Clobert, 2005) or other effects (e.g., fire ant envenomation).

2.3.2 | Maternal behaviour and glucose

Maternal behaviour was recorded daily starting 1 week after capture (to allow lizards to acclimate to captivity) until oviposition (length of observations = 2–23 days; avg = 16.1 ± 1.6 *SE* days). Observations occurred hourly from 1000 to 1500 hr, and the observer was blind to female treatment. During observations, we recorded the location of each lizard in its home bin as: on the perch under the heat lamp (basking), under the perch (seeking refuge), in the corner of the bin, or along the wall of the bin (torso within 2 cm of the wall; thigmotaxis), or in the open. Glucose was measured 3 days postlaying (Stoot et al., 2014).

2.3.3 | Egg collection and incubation

Bins were checked for eggs daily at 0700, 1200 and 1700 hr. Checks were done visually so as to not disturb females and included identification of nesting tunnels, eggs (visible through the bottom of the transparent bins), and mass loss and abdomen collapse of females. Once found, eggs were immediately measured (mass, length, width) and then transferred to small plastic containers (11 × 7.5 cm *D × H*, <5 eggs per container) filled with moist vermiculite (*c*. −200 kpa) and sealed with plastic wrap (Langkilde & Freidenfelds, 2011; Warner, Radder, & Shine, 2009). Eggs were incubated at 30 ± 1°C until hatching (*c*. 45 days; Langkilde & Freidenfelds, 2011). Containers were rotated within the incubator every day to avoid potential within-incubator effects of position. After 38 days, containers were checked twice daily (0930 and 1700 hr) for hatchlings.

For clutches with more than 8 eggs, two eggs were randomly selected at laying and frozen at −20°C (maternal treatment: CORT = 6, control = 7). One egg per pair was used to measure yolk hormone concentrations and the other egg per pair to measure nutrient content (as described in Yolk composition assay). Hatch date was recorded and incubation length was calculated. Clutch size was recorded but was not significantly affected by maternal treatment ($F_{1,60}$ = 3.049, p = 0.086). Dosing started postfollicular development and resorption of eggs by this species has been heavily argued against in the literature (Blackburn, 1998). Clutch size will thus not be discussed further.

2.3.4 | Hatchling morphology and housing

Upon hatching, hatchlings were toe-clipped for individual identification and measured for mass, SVL, TL and HL as described in Adult capture and housing (2015: *S* = 60, *C* = 62; 2016: *S* = 10, *C* = 27). Sex was determined by the presence (male) or absence (female) of enlarged postanal scales (Haenel & John-Alder, 2002). Hatchlings were housed in same-clutch groups within plastic bins (46 × 40 × 30 cm, *L × W × H*) and fed fruit flies *Drosophila melanogaster* daily, dusted with vitamin and mineral powders (Herptivite and Ultrafine Calcium with vitamin D; Rep-Cal, Los Gatos, CA) 1–2 times per week. Water was available ad libitum.

2.3.5 | Hatchling blood sampling

At 4–7 days of age, hatchlings were divided into two groups and sampled for one of either baseline CORT (*S* = 10, *C* = 12) or CORT response to an ACTH challenge (*S* = 5, *C* = 8; one hatchling per clutch for each measure). Due to our inability to take repeat blood samples from hatchlings because of their small size, we could only use a single hatchling for each measurement type. We had initially planned to measure CORT at multiple time points post-ACTH injection; however, sample sizes were not large enough and we were only able to use measurements taken 60-min post-ACTH injection and present these data here (resulting in smaller samples sizes for this measure than for baseline CORT). Blood samples were collected immediately after decapitation following euthanization with isoflurane. To measure baseline CORT, blood samples were obtained within 188 ± 3 s of capture), which should be fast enough to avoid any potential effects of isoflurane (Romero & Reed, 2005). To measure CORT response to an ACTH challenge, hatchlings were injected interperitoneally with 0.1 IU ACTH $\mu l^{-1}\,g^{-1}$ lizard within 113 ± 11 s of capture (adrenocorticotropic hormone from porcine pituitary, A6303; Sigma-Aldrich, St. Louis, MO; Klukowski, 2011) and blood samples were obtained 60 min later. Haematocrit levels were measured after both baseline and all post-ACTH blood samples (including from hatchlings not included in the 60-min post-ACTH results) were centrifuged (*S* = 24, *C* = 29). Plasma was collected and stored at −20°C for 4 months while in the field and then at −80°C at Pennsylvania State University until assayed.

2.3.6 | Hatchling behaviour

At hatching, one hatchling from each clutch was randomly selected for use in behavioural trials (*S* = 22, *C* = 37) and subsequently housed individually in a plastic bin (42 × 27 × 27 cm, *L × W × H*). Behaviour trials began when hatchlings were 4–7 days of age. Ten minutes prior to the start of the trial, individual housing bins were moved to a recording area where a camera (Microsoft LifeCam HD-6000 attached to laptop) was suspended above the bin, and the bin was surrounded by black cloth preventing visual disturbance by the investigator or other lizards. Hatchlings were recorded undisturbed for 30 min to determine baseline behaviour. We analysed the footage with JWATCHER (v1.0; Macquarie University, Sydney, Australia) to quantify the time spent: basking on the perch, under the perch, on the edge and corner of the bin, in the open and distance moved (using a 1 cm × 1 cm grid overlay during video analysis).

Immediately following baseline behaviour measures, a novel object—a red-tipped stick—was advanced towards the front of the hatchling to estimate flight initiation distance. The experimenter was hidden behind the black cloth and used the camera with live feed to locate the lizard. Three minutes after this novel object test, we touched a monofilament line against the back of the hatchling for 30 s (mimicking tactile stimulation from ants, which are a predator of juvenile lizards; Thawley & Langkilde, 2017) and recorded whether hatchlings fled or not. All behavioural trails were run twice, on separate days, for each hatchling.

2.4 | Laboratory assays

2.4.1 | Plasma corticosterone assay

Adult and hatchling plasma CORT was measured via a commercially available enzyme immunoassay (Corticosterone High Sensitivity EIA, Immunodiagnostic Systems LTD., Fountain Hills, AZ). This assay has been previously validated for use in this species (Trompeter & Langkilde, 2011). We assayed samples in duplicate following the manufacturer's guidelines. Plasma (1–5 µl) was brought to a final volume of 50 µl with assay buffer, and final CORT concentrations corrected for the dilution factor. Sample volume did not significantly explain variation in final CORT concentrations ($F_{1,50}$ = 1.077, p = 0.304) and samples fell in the detectable range of the standard curve. The mean intra- and interassay coefficients of variation for adult CORT were 4.05% and 10.7%, respectively, and for hatchling, CORT were 2.43% and 9.6%, respectively.

2.4.2 | Yolk composition assay

Yolk CORT and testosterone concentrations were measured at the St. Louis Zoo's Endocrinology Lab. While frozen, eggshells were removed and whole yolk (given these contain no identifiable albumen; Uller, Astheimer, & Olsson, 2007), were shipped on dry ice to the zoo for extraction and assay. To extract, egg yolk (0.05 ± 0.001 SE g) was diluted with 50 µl of phosphate-buffered saline (PBS) and homogenized using a vortexer. The sample was incubated at 37°C for 1 hr and 100 µl of absolute ethanol was added. The sample was homogenized again by vortexing and then centrifuged for 10 min at 13,000 *g* to precipitate solids. The resultant supernatant was frozen at −80°C until assayed. Yolk CORT and testosterone were quantified using commercially available EIA kits (Arbor Assays DetectX Corticosterone EIA Kit, Product # K014; Arbor Assays DetectX Testosterone EIA Kit, Product # K032). The assays were validated via assessing parallelism and quantitative recovery. Samples fell within the detectable range of the standard curve. For both assays, samples were diluted 1:10 with assay buffer, and all assays were run according to kit directions. All samples were assayed in duplicate and corrected for dilution and were run on one plate with mean intra-assay variation of duplicate samples of 9.71% for CORT and 8.39% for testosterone.

Egg nutrient composition was assessed on a Direct Detect infrared spectrometer (EMD Millipore, Billerica, MA) for the concentrations of proteins and lipids, as carbohydrates represent a very minor yolk constituent in reptiles (Noble, 1991; Speake & Thompson, 1999; White, 1991). Eggs were weighed before and after lyophilization to quantify water content and then homogenized. Egg yolk (3.749 ± 0.008 SE mg) was diluted with 0.3 ml of PBS (for protein assay) or chloroform/methanol (2:1, v/v; for lipid assay) following EMD Millipore guidelines and standard lipid determination protocol (Folch, Lees, & Sloane-Stanley, 1957). Samples were vortexed for 20 min and then immersed in a sonicating water bath for 20 min. The solution was then filtered through a 0.2-ʋm filter (Pall Cooperation Acrodisc, Product # 4192, Port Washington, NY), and the resulting supernatant was read on the Direct Detect infrared spectrometer against a bovine serum albumin (protein) and cholesterol (lipid) standard (Guisande & Harris, 1995). The assays were validated via assessing parallelism and quantitative recovery. All samples were assayed in duplicate in one run with mean intra-assay variation of duplicate samples of 6.08%.

Total caloric content was derived using the Parr Oxygen Combustion Vessel 1108, digital thermometer 6775, plain jacket calorimeter 1341 and standard calorimetry procedures (Ballinger & Clark, 1973). Briefly, eggs were lyophilized for 24 hr, the shell and membrane were removed, and the yolk was weighed to the nearest 0.0001 g. The dry sample was loaded into the vessel, which was pressurized to 30 atm O_2, and combusted. The change in water temperature was recorded and caloric values were calculated using standard equations for kilojoule per gram of dry yolk.

2.5 | Statistical analysis

Body condition was calculated as the residuals of the log mass on log SVL, and relative HL was calculated as the residuals of hindlimb length on SVL (Du, Robbins, Warner, Langkilde, & Shine, 2014; Graham et al., 2012; Langkilde, 2009b; Schulte-Hostedde, Zinner, Millar, & Hickling, 2005). Maternal behaviours were analysed as individual data points (hourly point samples) and plotted as per cent of observations determined from multiple point observations (averaged across all observations; Figure 1), while hatchling behaviour was analysed as timed duration as recorded from video footage and plotted as minutes (Figure 5). All analyses were conducted using JMP PRO (v12; SAS Institute Inc., Cary, NC) and R (v3.2.3; R Foundation for Statistical Computing, Vienna, Austria) with the LME4 package (Bates, Mächler, Bolker, & Walker, et al., 2015). Models with multiple related variables were first run using a multivariate ANOVA (MANOVA, in JMP), and individual differences were then assessed using linear mixed models (LMM) or logistic regression (LR).

We used a mixed model LR (R) to test the effects of maternal treatment (control/CORT treatment) on individual maternal behaviour, which were recorded as binary variables, and the
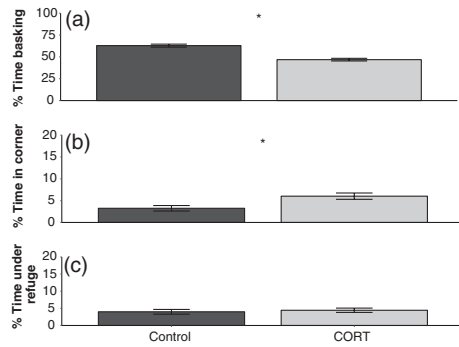
FIGURE 1 Percentage of observations that control (C = 13) and corticosterone-treated (S = 13) females were observed (a) basking, (b) in the corner of their home bin and (c) under the refuge of their home bin. Bars represent means ± 1 SE. *p < 0.05

response of hatchlings to tactile stimulation. Maternal identity (ID) or hatchling ID was included as a random factor respectively and maternal treatment was included as a factor. We used LMMs (JMP) with random slopes and intercepts to test the effects of maternal treatment on egg morphology, egg incubation length, hatchling haematocrit levels, hatchling morphology, hatchling gross distance travelled, individual hatchling baseline behaviour and hatchling flight initiation distance from a novel object. Maternal treatment was included as a factor in all cases. Hatchling identity was included as a random effect in hatchling behaviour trials (hatchling gross distance travelled, individual hatchling baseline behaviour and hatchling flight initiation distance from a novel object) to control for the fact that we took multiple measures per individual, and maternal ID was included as a random effect of all analyses of hatchling traits to control for nonindependence due to measuring multiple eggs or hatchlings from the same clutch (egg morphology, egg incubation length, hatchling haematocrit levels, hatchling morphology). For hatchling tail length, SVL was included as a covariate to isolate differences in tail length from differences in overall length. We used linear models (LM; JMP) with random slopes and intercepts to test the effects of maternal treatment on maternal postlay glucose, clutch mass, egg protein and lipid concentrations, egg hormone concentrations, egg caloric value, and hatchling baseline CORT and stress responsive CORT levels. Maternal treatment was included as a factor in all cases. In the model of clutch mass, we included the number of eggs in the clutch as covariate as this can affect clutch mass (Williams, 1994).

Site of capture, duration of CORT treatment, length of maternal observation, time in laboratory prior to oviposition, egg container, hatchling sex, maternal gravidity at capture and hatchling trial number (first or second trial) for hatchling behaviours were included initially but did not significantly explain variation in dependent variables in any of our models (p > 0.200); thus, these were omitted from the final models to preserve degrees of freedom. Effect sizes were calculated as Cohen's d. Results are represented as mean ± 1 SE.

3 | RESULTS

3.1 | Maternal behavioural and physiological responses

CORT treatment significantly altered maternal behaviour in their home bins (MANOVA: $F_{7,1871}$ = 10.984, p < 0.001). CORT-treated females spent 15.2% less time on the basking perch and 3.2% more time in the corner of their home bin compared to control females (LR: $\chi^2_{1,876}$ = 3.185, p = 0.001, d = 9.942, $\chi^2_{1,876}$ = 2.960, p = 0.003, d = 4.095, respectively; Figure 1a,b) but females in these treatments did not differ in their time spent along the edge, under the refuge or in the open (LR: $\chi^2_{1,876}$ = −0.442, p = 0.659, d = 3.652; $\chi^2_{1,876}$ = −1.321, p = 0.186, d = 0.68, Figure 1c; $\chi^2_{1,876}$ = 0.635, p = 0.526, d = 2.779, respectively). CORT-treated females had significantly higher glucose levels 3-days postpartum compared with control females (glucose level: S = 315.67 ± 20.35 mg/dl, C = 273.78 ± 7.74 mg/dl; LM: $F_{1,46}$ = 8.031, p = 0.006, d = 0.843).

3.2 | Egg phenotype

CORT treatment did not alter egg morphology (MANOVA: $F_{4,58}$ = 1.336, p = 0.336). Total clutch mass and individual egg length, width and mass were similar between the CORT-treated females and control females (LMM: $F_{1,60}$ = 0.245, p = 0.622, d = 0.128; $F_{1,173}$ = 0.069, p = 0.794, d = 0.04; $F_{1,173}$ = 1.175, p = 0.282, d = 0.166; $F_{1,173}$ = 1.158, p = 0.283, d = 1.165, respectively). Eggs from CORT-treated females had 127% higher yolk CORT concentrations (LM: $F_{1,12}$ = 9.467, p = 0.011, d = 186; Figure 2a) and 32% lower protein concentrations (LMM: $F_{1,12}$ = 10.049, p = 0.009, d = 1.965; Figure 2c) but similar yolk testosterone, lipid concentrations, water amount and caloric value (LM: testosterone: $F_{1,12}$ = 0.001, p = 0.971, d = 0.022; lipid: $F_{1,12}$ = 3.160, p = 0.103, d = 3.16; water: $F_{1,12}$ = 0.544, p = 0.476, d = 0.544; caloric: $F_{1,12}$ = 0.342, p = 0.569, d = 0.342, respectively; Figure 2b,d,e) (caloric value: S = 23.44 ± 0.27 kJ/g yolk, C = 23.68 ± 0.27 kJ/g yolk). CORT treatment did not alter incubation length ($F_{1,153}$ = 1.982, p < 0.168, d = 0.227), but there was a significant interaction between year and treatment ($F_{1,153}$ = 14.212, p < 0.001, d = 0.609) whereby eggs from CORT-treated females had a significantly shorter (5 days) incubation time in 2015 ($F_{1,121}$ = 60.382, p < 0.001, d = 1.419) but a similar incubation time in 2016 ($F_{1,31}$ = 1.877, p = 0.198, d = 0.521), compared to eggs from control females.
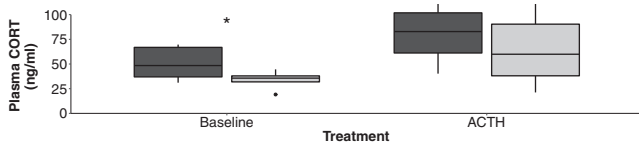
3.3 | Hatchling phenotype

Hatchlings from CORT-treated females had 42% lower baseline plasma CORT than did hatchlings from control females (LM: $F_{1,20}$ = 8.29, p = 0.01, d = 1.293; Figure 3). Sixty minutes post-ACTH injection hatchlings from CORT-treated females had similar CORT levels to those from control females (LM): $F_{1,10}$ = 0.398, p = 0.546, d = 0.446).

Hatchlings from CORT-treated females tended to differ morphologically compared to hatchlings from control females, although these differences did not achieve statistical significance (MANOVA: $F_{4,181}$ = 2.108, p = 0.082). These hatchlings had 3% longer SVL (LMM: $F_{1,159}$ = 4.235,
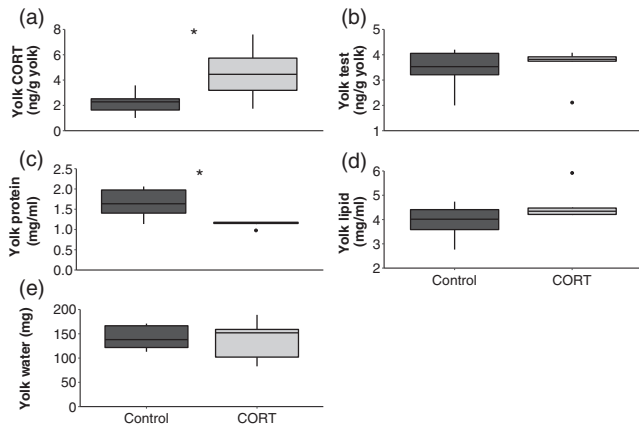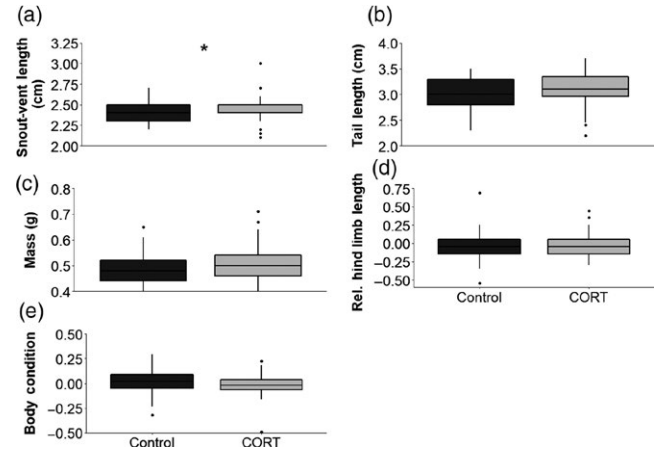
FIGURE 2 Yolk (a) corticosterone (CORT), (b) testosterone (Test), (c) protein, (d) lipid concentrations and (e) water mass for eggs from control and CORT-treated females (CORT and testosterone: $C = 7$, $S = 6$; protein, lipid and water: $C = 8$, $S = 5$). Box plot represents median, 25–75 quartile range and range. *$p < 0.05$

FIGURE 3 Plasma corticosterone (CORT) levels at baseline and in response to the ACTH challenge of hatchlings from CORT-treated and control females (baseline: $C = 12$, $S = 10$; ACTH: $C = 5$, $S = 6$). Box plot represents median, 25–75 quartile range and range. *$p < 0.05$

FIGURE 4 Morphology of hatchlings from control females ($C = 90$) and corticosterone-treated ($S = 70$): (a) snout–vent length, (b) tail length, (c) mass, (d) relative hindlimb length and (e) body condition. Box plot represents median, 25–75 quartile range and range. *$p < 0.05$

4 | DISCUSSION

We investigated the intergenerational effects of increased physiological stress by experimentally elevating maternal GCs during gestation in wild-caught eastern fence lizards and demonstrate effects on maternal behaviour and glucose, egg investment and offspring phenotype. Elevated maternal GCs altered multiple aspects of hatchlings' physiology, morphology and behaviour, suggesting potentially fitness-relevant intergenerational effects of maternal stress. Our results also suggest potential mechanisms for these changes to offspring traits such as the combination of direct exposure to maternally derived CORT (via the demonstrated increases in egg CORT) and indirect effects of maternal GC elevation via GC-induced changes in maternal behaviour and glucose and subsequent nutrient allocation to eggs. This demonstrated effect of increased GCs during gestation at the maternal, egg and offspring level is important as it provides a holistic understanding of the multilevel impacts of gestational stress. We explore each of these findings within an ecological context below.

4.1 | Maternal phenotype

Gravid females treated with CORT spent less time on the basking perch and more time in the corner of their home bin (Figure 1). Given our CORT treatment only elevated CORT for 3.5 hr (MacLeod et al., in press) and was applied in the evening, the change in maternal behaviour, measured during the day, occurred when female CORT was likely at baseline levels. This suggests that these are baseline behavioural shifts females undergo when they experience a repeated stressor. The decrease in time spent on the basking perch may have implications for avoiding predation and for thermoregulation. Elevated plasma CORT has been shown to increase an animal's perception of predation risk (i.e., increase in "fear") and their

$p = 0.045$, $d = 0.33$; Figure 4a) but were similar in tail length, mass, relative HL and body condition compared to those from control females (LMM: $F_{1,159} = 0.820$, $p = 0.369$, $d = 0.145$, $F_{1,159} = 0.046$, $p = 0.829$, $d = 0.03$; $F_{1,159} = 0.030$, $p = 0.957$, $d = 0.028$; $F_{1,159} = 2.135$, $p = 0.150$, $d = 0.234$, $S = -0.021 \pm 0.021$, $C = 0.015 \pm 0.0174$, respectively; Figure 4b–e).

Hatchlings from CORT-treated females had altered baseline behaviour compared to hatchlings from control females (MANOVA: $F_{6,88} = 2.578$, $p = 0.033$). Hatchlings from CORT-treated females spent more time hiding under the basking perch (LMM: $F_{1,58} = 5.960$, $p = 0.021$, $d = 0.669$; Figure 5a), but a similar amount of time basking on top of the perch, in the centre of the bin, and at the edges of the bin (LMM: $F_{1,58} = 1.622$, $p = 0.214$, $d = 0.346$, Figure 5b; $F_{1,58} = 0.007$, $p = 0.934$, $d = 0.023$, $S = 3.627 \pm 1.145$ min, $C = 3.747 \pm 0.880$ min; $F_{1,58} = 2.228$, $p = 0.149$, $d = 0.409$, $S = 22.382 \pm 1.998$ min, $C = 18.077 \pm 2.073$ min, respectively). Fewer hatchlings (18.5% fewer) from CORT-treated females responded to tactile stimulation mimicking encounters with ants (LR: $\chi^2_{58} = 4.302$, $p = 0.038$, $d = 2.9$; Figure 5c). All hatchlings, regardless of treatment, travelled similar distances during behavioural trials and exhibited similar flight initiation distances when approached by a novel object (LMM: $F_{1,58} = 0.058$, $p = 0.811$, $d = 0.066$, $S = 146.566 \pm 41.609$ mm, $C = 134.279 \pm 29.529$ mm; $F_{1,58} = 0.527$, $p = 0.596$, $d = 0.211$, $S = 0.848 \pm 0.435$ cm, $C = 1.378 \pm 0.309$ cm, respectively).
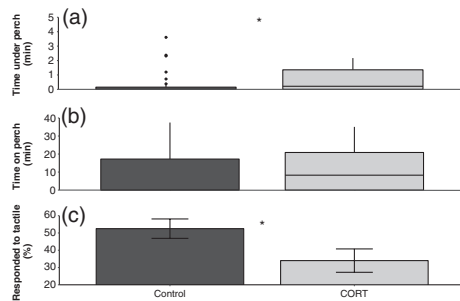
FIGURE 5 Behaviour of hatchlings from corticosterone-treated and control females showing per cent of time, during a 30-min observational trial, spent (a) under and (b) on the basking log and (c) the response rate to tactile stimulation (C = 37, S = 22). Box plot represents median, 25–75 quartile range, and range and bars represent means ± 1 SE. *p < 0.05

antipredator behaviours, such as hiding or fleeing (Langkilde, 2009b; Langkilde et al., 2017; Thaker, Lima, & Hews, 2009; Trompeter & Langkilde, 2011). By reducing the time spent on their basking perch and increasing time in the corner of their home bin, our data potentially suggest that CORT-treated females may reduce predation risk from visual predators (Belliure & Clobert, 2004; De Fraipont et al., 2000; Treit & Fundytus, 1988), in particular birds and snakes that are common predators to most lizards (Cooper et al., 2000; Jensen, 2008). While the increase in time spent in the corner is small, it is a doubling of that activity and, it reduces their access to direct radiant heat (the top of the basking perch was 7–8°C warmer than off the perch), a behaviour that may come at a cost to thermoregulation (Belliure & Clobert, 2004). Reduced body temperature in lizards can slow egg and hatchling development (Parker & Andrews, 2007). Thus, the altered thermoregulatory behaviour of females may provide an indirect mechanism for maternal GCs to alter offspring phenotype and warrants further exploration.

In addition to behavioural changes, adult females that were treated with CORT also exhibited higher plasma glucose levels 3 days postlaying compared to control females, suggesting that glucose levels were higher throughout the treatment period (e.g., Hällgren & Berne, 1983). This may have occurred due to changes in the homeostatic set point of glucagon or insulin, altered receptor sensitivity to these hormones (Hällgren & Berne, 1983; Kahn, Goldfire, Neville, & De Meyts, 1978) or GC-induced insulin resistance (Rizza, Mandarino, & Gerich, 1982). Elevated glucose levels could serve to promote survival in high predation risk environments by increasing energetic substrate for antipredator behaviours (Sapolsky, Romero, & Munck, 2000). Greater glucose levels during gestation may also serve to compensate for the potential negative effects of reduced basking behaviour on hatchling body size (Mitanchez, 2010; Parker & Andrews, 2007; Salmanzadeh, 2012).

4.2 | Egg phenotype and composition

CORT treatment had no effect on total clutch mass or individual egg morphology and mass. We expected a reduction in total clutch mass,

given the energetic costs associated with elevated CORT and the concurrent behavioural and physiological shifts we found in mothers. Previous studies have shown that increased maternal GCs can decrease egg size and mass (Henriksen, Groothuis, et al., 2011; Mileva, Gilmour, & Balshine, 2011). Our findings may differ from those previous because the weight maintenance diet in the laboratory offset the potential CORT-associated energetic costs (French, DeNardo, & Moore, 2007); a conclusion supported by our finding of similar postlaying maternal body condition between the treatments (MacLeod et al., in press).

Maternal CORT treatment resulted in eggs with higher yolk CORT and similar testosterone concentrations (Figure 2). The transfer of hormones from the mother to eggs has been well demonstrated in avian and aquatic organisms (Hayward & Wingfield, 2004; Saino et al., 2005; Sopinka, Capelle, Semeniuk, & Love, 2017) but less so in reptiles (but see Johnston, 2009; Lovern & Adams, 2008; Painter, Jennings, & Moore, 2002). Elevated maternal GCs typically increase egg GCs (Hayward & Wingfield, 2004; Saino et al., 2002), as we documented, but decrease egg testosterone levels (Henriksen, Groothuis, et al., 2011; Okuliarová, Šárniková, Rettenbacher, Škrobánek, & Zeman, 2010). While both CORT and testosterone are known to alter hatchling development and phenotype (Andersson, Uller, Lõhmus, & Sundström, 2004; Mileva et al., 2011; Schwabl, 1996), in our study any hormone-induced changes in hatchling phenotype are likely driven by differences in egg CORT given that testosterone levels did not differ between the treatments (although we cannot rule out effects of oestrogen or other hormones).

Eggs from CORT-treated females had similar caloric content, despite the known energetic costs of elevated GCs in mothers (Henriksen, Groothuis, et al., 2011; Mileva et al., 2011). Nutrient allocation to eggs was, however, affected by maternal stress: Eggs from CORT-treated females had lower protein to lipid ratios resulting from lower protein concentration and a nonsignificant trend towards higher lipid content in yolk compared to control eggs (Figure 2). Yolk proteins are implicated in embryonic and hatchling developmental size, with lower protein levels resulting in smaller hatchlings (Roulston & Cane, 2002; Shepherd, Crowther, Desai, Hales, & Ozanne, 1997). However, this is contrary to our findings of increased length of offspring and no change in mass or body condition. This discrepancy may be explained by reduced incubation length yet similar caloric content of eggs due to CORT treatment. Although we found that CORT treatment reduced incubation length in 2015 only, the lack of difference in 2016 was likely due to smaller sample sizes that year.

Increased CORT has been suggested to increase basal metabolic rate (Damjanovic et al., 2009; Wack et al., 2012). The increased CORT in eggs of CORT-treated mothers could have increased metabolic rate of embryos via increased heart rate (Monk et al., 2000; Owen et al., unpublished data). As birds and reptiles have been shown to hatch after a relatively fixed number of heart beats (Du, Radder, Sun, & Shine, 2009; Giesing, Suski, Warner, & Bell, 2011), this increased heart rate could increase the developmental rate of the offspring, allowing them to hatch earlier. Additionally, shorter

incubation length may allow more energy to be allocated to embryonic development as opposed to energetic maintenance costs (Du et al., 2009) resulting in larger offspring despite decreased protein content of the eggs.

4.3 | Hatchling phenotype

Hatchlings from CORT-treated females had baseline CORT concentrations a third lower than those of hatchlings from control females, while CORT concentrations post-ACTH were similar in both groups. The similar post-ACTH CORT concentrations may be due to greater ACTH responsiveness in hatchlings from CORT-treated females (S = +91%, C = +60% greater ACTH-induced CORT compared to baseline levels; Figure 3); however, we could not directly test for this. Maternal stress often results in offspring with greater baseline and stress-induced plasma GC levels (Meaney et al., 2007; Sheriff et al., 2010), but in systems where prey have frequent, nonlethal interactions with predators (that significantly increase GC levels), there may be selection for lower initial baseline GC levels to keep CORT from reaching deleterious concentrations (Romero et al., 2009). This may be particularly important in systems where these nonlethal interactions result in significant wounding of prey and require a highly functional immune system, such as is the case in our study sites, which have been invaded by fire ants (Freidenfelds et al., 2012). We suggest that the lower initial baseline CORT and similar peak ACTH-response levels in hatchlings may (a) act to protect lizards from the deleterious consequences of chronic nonlethal interactions with fire ants that could potentially increase baseline CORT levels beyond a performance threshold (e.g., homeostatic overload; McEwen & Wingfield, 2003; Romero et al., 2009) and (b) allow hatchlings to still mount an appropriate acute CORT response to fire ant attack and other stressors (Langkilde et al., 2017).

Hatchlings from CORT-treated females were longer (SVL; Figure 4) but had a similar tail length, relative HL, body condition (residuals of log mass/log SVL) and physiological condition (proxied by haematocrit) compared to hatchlings from control females. While the change in SVL (0.6 mm, 3% increase) was small, it is similar to previous work (SVL – 0.7 mm, 3% increase, Ferguson & Fox, 1984) that has shown such changes can increase survival of hatchlings in this and other lizard species. Previous work from our laboratory has also shown the antipredator benefits of small changes in morphology (Langkilde, 2009b). Further, larger hatchlings may have a greater competitive advantage and growth rate throughout their development, which often results in larger adults (Sorci, Clobert, & Belichon, 1996). A larger body size may also confer an advantage at these sites by diluting effects of fire ant envenomation (Hayes, Lavín-Murcio, & Kardong, 1995).

Hatchlings from CORT-treated females spent more time hiding and were less likely to respond to tactile stimulation mimicking ant attack, compared to hatchlings from control females (Figure 5). These findings do not support our prediction that elevated maternal CORT would result in hatchlings that spent more time up off the ground and that would be more responsive to ant-like stimuli (Langkilde, 2009b; Trompeter & Langkilde, 2011). The increased time hidden and lower response rate to tactile stimulation to ant-like stimuli could be adaptive for visual predators such as snakes and birds by maintaining crypsis, but potentially detrimental to avoiding ant predation (Thawley & Langkilde, 2017). Further investigation is needed to elucidate the potential effects of predator-induced maternal stress from predators with different hunting modes on offspring phenotype.

5 | CONCLUSIONS

We show that maternal exposure to GCs affects the mother, her eggs and her subsequent offspring. Our results build on previous research that has demonstrated the effect of maternal stress in inducing alterations to offspring phenotype (reviewed in Love et al., 2013). The concurrent effects of GC manipulations on maternal behaviour and egg GC levels and other aspects of egg composition suggest that direct and indirect exposure to maternal GCs likely mediated the observed changes to offspring phenotype. Although we did not test the outcomes of our documented phenotypic changes under different environmental conditions (sensu Sheriff et al., 2018), prior information about the adaptive significance of these traits suggests that they may better match offspring to specific environments (Parker & Andrews, 2007; Romero et al., 2009; Thawley & Langkilde, 2017). A better understanding of all (direct and indirect) consequences of maternal stress effects on offspring will help to illuminate the evolutionary potential and ecological consequences of maternal GCs for adapting an offspring to a changing environment.

AUTHORS' CONTRIBUTIONS

D.C.E., T.L. and M.J.S. conceived the ideas and designed methodology; D.C.E., K.J.M. and, D.A.S.O. collected the data; D.C.E. analysed the data; D.C.E. wrote the manuscript and all authors provided edits.

DATA ACCESSIBILITY

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