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RESEARCH ARTICLE

Evidence of embryonic regulation of maternally derived yolk corticosterone

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ABSTRACT

In recent years, the potential for maternal stress effects to adaptively alter offspring phenotype has received considerable attention. This research has identified offspring traits that are labile in response to maternal stress; however, an understanding of the mechanisms underlying these effects is lagging and is crucial to appreciating the significance of this maternal effect. In the present study, we sought to better understand maternal stress effects by examining the potential for embryonic regulation of corticosterone exposure, determining the phenotypic consequences of elevated corticosterone during development, and characterizing the levels of maternally transferred corticosterone in unmanipulated eggs using *Trachemys scripta*. By dosing eggs with tritiated corticosterone and tracking the steroid throughout development, we found that most corticosterone is metabolized, and less than 1% of the corticosterone dose reaches the embryo as free corticosterone. We also found that exogenous dosing of corticosterone, in concentrations sufficient to overwhelm embryonic metabolism, reduces embryonic survival and negatively impacts hatchling traits important to fitness. Our results demonstrate that concentrations of maternal corticosterone in the yolks of unmanipulated eggs are low and are significantly lower than the doses of corticosterone required to elicit phenotypic effects in hatchlings. Taken together, these results provide evidence that both the embryo and the female may minimize corticosterone accumulation in the embryo to avoid reductions in embryonic survival and negative impacts on offspring phenotype and fitness.

KEY WORDS: Embryo, Maternal effects, Stress, *Trachemys scripta*, Reptile

INTRODUCTION

Adverse environmental conditions can be perceived as stressors by vertebrates and induce complex physiological and behavioral responses intended to increase immediate survival (Wingfield et al., 1998). During reproduction, stressors experienced by a female not only influence her physiology and behavior, but can permanently alter the development and phenotype of her offspring (Harris and Seckl, 2011; Sheriff and Love, 2013). The potential for maternal stress to lead to adaptive alterations in offspring phenotype has gained considerable interest in wildlife systems within ecological and evolutionary contexts (Sheriff et al., 2017). This research has identified many aspects of the phenotype that can be altered by maternal exposure to stressors; however, an understanding of the mechanisms underlying these maternal stress effects is lagging behind and is imperative for gaining an accurate evolutionary appreciation of maternal stress effects (Groothuis and Schwabl, 2008; Sheriff et al., 2017).

Because stress can induce a wide variety of physiological and behavioral responses, it can be difficult to identify the mechanisms underlying maternal stress effects (Harris and Seckl, 2011). In vertebrates, the transfer of glucocorticoids – metabolic steroids produced in response to stressors that help mobilize energy – is one way a female's physiological state can be conveyed to, and ultimately affect, offspring (Bonier et al., 2009a,b; Sapolsky et al., 2000). Offspring can be exposed to maternal glucocorticoids through placental transfer or deposition into the yolk, and glucocorticoids can, in turn, alter offspring phenotype by directly interacting with embryonic tissues (mammals: Seckl and Walker, 2001; Seckl, 2004; Yang, 1997; birds: Hayward et al., 2006; Hayward and Wingfield, 2004; Love et al., 2005; Love and Williams, 2008; Saino et al., 2005; reptiles: Lovern and Adams, 2008; Meylan and Clobert, 2005; Sinervo and DeNardo, 1996; fish: McCormick, 1998, 1999). For example, cortisol, the primary glucocorticoid in mammals and fish, can modulate cell proliferation and differentiation and influence synaptic growth in the brain, causing permanent behavioral and cognitive disorders (Van den Bergh et al., 2005). However, the effects of maternal stress can also arise without direct exposure of the embryo to glucocorticoids. In humans, maternal stress is associated with reduced intrauterine blood flow that can reduce nutrient and oxygen delivery to the fetus, potentially underlying associations between maternal psychological state and low birth weight (Linnet et al., 2003; Teixeira et al., 1999; Van den Bergh et al., 2005). Feeding of offspring by parents is reduced in many birds during exposure to stressors, which can consequently influence offspring phenotype (Angelier and Chastel, 2009). These studies highlight how the effects of maternal stress on offspring can stem from diverse mechanisms, only some of which involve the direct exposure of embryos to maternal corticosterone.

Further complicating an understanding of maternal stress is the active role embryos and associated tissues can play in modulating exposure to maternal steroids. In some circumstances, increased steroid concentrations within the maternal environment does not result in increased steroid concentrations within the embryonic environment (Yang, 1997). The placenta has long been known to metabolize maternal steroids as they pass from the maternal environment to the embryonic environment (Diczfalusy, 1969; Burton and Jauniaux, 2015). In mammals, though some maternal cortisol is capable of crossing the placenta, it is estimated that 80–90% of the cortisol that reaches the placenta is metabolized by protective enzymes [e.g. 11β-hydroxysteroid dehydrogenase (HSD)] and converted into less active forms such as cortisone before reaching the fetus (Challis et al., 2001; Seckl and Holmes, 2007; Van den Bergh et al., 2005). Recent work has demonstrated that

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embryos of oviparous vertebrates can similarly regulate exposure to maternal steroids, including glucocorticoids, via steroid metabolism (birds: Paitz et al., 2010; Vassallo et al., 2014; von Engelhardt et al., 2009; reptiles: Paitz et al., 2012; Paitz and Bowden, 2008, 2013). For example, injections of tritiated corticosterone into quail (*Coturnix japonica*) eggs, only resulted in 0.4% of the injected dose reaching the embryo without first being metabolized (Vassallo et al., 2014). Ultimately, it appears that any effects of maternal stress arise from a complex interplay of maternal and embryonic physiology.

Oviparous species have emerged as excellent model systems for studying maternal stress effects on offspring owing to several key advantages over placental systems. Importantly, the production of glucocorticoids and the action of the hypothalamic–pituitary–adrenal (HPA) axis is highly conserved across vertebrates (Sapolsky et al., 2000). In oviparous vertebrates, maternal transfer of glucocorticoids can be quantified by the discrete amount present in the yolk at oviposition, because transfer of maternal steroids is no longer possible after the egg is laid (Love and Williams, 2008). Another key advantage is that, unlike developing fetuses in placental species, embryos develop independently from the female in oviparous species. This allows for the manipulation of embryonic glucocorticoid exposure without any confounding maternal effects stemming from the female's own physiological and behavioral responses to glucocorticoid exposure (Strange et al., 2016). Oviparous vertebrates thus provide a unique opportunity to investigate how maternal physiology (concentrations of glucocorticoids within eggs at oviposition) and embryonic physiology (modulation of glucocorticoid concentrations within eggs during development) interact to ultimately determine the phenotypic consequences of exposure to maternal glucocorticoids.

We utilized the red-eared slider turtle (*Trachemys scripta*) to study embryonic exposure to maternal glucocorticoids and integrate mechanistic and phenotypic approaches to more fully characterize the significance of maternal corticosterone regulation. Specifically, we: (1) determined whether embryos can regulate their exposure to maternal steroids by topically dosing eggs with tritiated corticosterone and tracking metabolism throughout the egg over time; (2) measured the phenotypic consequences of embryonic exposure to corticosterone by topically dosing eggs and quantifying hatchling traits important to fitness; and (3) quantified corticosterone in freshly laid, unmanipulated eggs to determine whether concentrations of maternal corticosterone approach those necessary to elicit effects in hatchlings.

MATERIALS AND METHODS

Corticosterone metabolism by the embryo

To study embryonic metabolism of corticosterone, six *T. scripta* clutches (n=58 eggs) were collected from females during the summer of 2011. Gravid females were caught in baited hoop traps and eggs were collected by inducing oviposition with an injection of oxytocin (Ewert and Legler, 1978). To characterize the movement and metabolism of corticosterone within eggs, 150,000 counts per minute (cpm) of tritiated corticosterone (Perkin Elmer, Waltham, MA, USA) dissolved in 5 µl of 70% ethanol was topically applied to the eggshell of each egg at the time of oviposition (Paitz and Bowden, 2015). Previous research demonstrates that steroid doses administered in a topical manner are readily taken into the egg (albumen, yolk and embryo) (Paitz and Bowden, 2008). After treatment, eggs were incubated at 28.5°C and one egg from each clutch (n=6) was collected every 5 days of incubation, up to day 55 of incubation, and placed into the freezer (–20°C) until analysis. Frozen eggs were separated into shell, yolk, albumen and embryo, which were weighed to the nearest 0.01 g, and each egg component was homogenized separately.

To examine the distribution of radioactivity within the egg, homogenates of each component were subjected to an ether extraction to separate free steroids from conjugated steroids (Paitz et al., 2010, 2012). An aliquot of 100 mg of each homogenate was added to 2 ml of 100% methanol, vortexed and placed at −20°C overnight. Each sample was then centrifuged for 20 min at 2000 rpm and the resulting supernatant was removed and dried under nitrogen gas. The dried supernatant was then reconstituted in 1 ml of distilled water, and free steroids were extracted from the water with 6 ml of diethyl ether (2×3 ml extractions). The aqueous fraction, which contained conjugated steroids, was held for subsequent quantification of radioactivity, while the 6 ml ether fraction was dried under nitrogen gas and reconstituted in 1 ml of 95% ethanol. Radioactivity levels were then measured in duplicate 200 µl aliquots of both the aqueous and ether fractions on a Beckman LS6500 scintillation counter (Beckman Industries, Fullerton, CA, USA). The total amount of water-soluble and ether-soluble radioactivity was calculated for each egg component.

Phenotypic consequences of corticosterone exposure

In 2013, 13 clutches of *T. scripta* eggs (n=135 eggs) were collected in the same manner as above or by excavation of freshly laid nests (<6 h from oviposition) in the field. Eggs were returned to the laboratory for corticosterone dosing and incubation. Eggs were topically dosed with 0, 0.05, 0.15 or 0.5 µg corticosterone/5 µl 90% ethanol (n=28, 35, 36 and 36 eggs in each treatment, respectively). Eggs were incubated in the laboratory at 29.4°C until hatching (Carter et al., 2016). Embryonic survival was monitored, and incubation period was measured as the number of days until pipping (first eggshell breach). Upon pipping, turtles were individually maintained, and at 10 days post-hatch, mass (g) and plastron length (mm) were measured (Carter et al., 2016). The presence or absence of carpacial scute malformations was recorded. Scute malformations included inserted or deleted scutes, asymmetrical scute distribution and 'dovetailing', where twice as many vertebral scutes are laced together.

Maternal transfer of corticosterone to the egg

We characterized maternal corticosterone in unmanipulated eggs to estimate the extent to which this maternal steroid may influence hatchling phenotype and survival in nature. This estimate was made by comparing endogenous yolk concentrations with the doses required to elicit observable effects in hatchling traits (see previous section). Because the concentrations of some maternal estrogens vary systematically in egg yolks across the nesting season (Carter et al., 2017; Paitz and Bowden, 2009, 2013), we decided to analyze corticosterone concentrations in early and late season yolks separately, in case a seasonal pattern also existed with this steroid. In 2016, one *T. scripta* egg was collected from each of N=18 early season clutches (31 May–1 June) and N=17 late season clutches (13–16 June) (see Carter et al., 2017, 2018 for more information on seasonal nesting designation). These eggs (N=35) were frozen within ∼12 h of oviposition for radioimmunoassay.

Yolk corticosterone concentrations were measured using Wingfield and Farner's radioimmunoassay (Wingfield and Farner, 1975) with a few modifications (Bowden et al., 2000; Schwabl, 1993). Previous pilot trials of this method demonstrated that concentrations of corticosterone in the yolk were very low to undetectable, so we modified our methods to include a larger initial

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yolk sample to aid in detecting corticosterone. We homogenized the yolk from a single egg and prepared a 500 mg yolk sample (in contrast to the 100 mg used in the first study). To each sample, 2000 cpm of tritiated corticosterone (Perkin Elmer) was mixed in with the yolk and refrigerated overnight at 4°C. Four standard samples were made with 1000 pg of corticosterone (Sigma-Aldrich) and two blank samples were made with distilled water. Steroids were extracted using 4 ml of 100% methanol, in two rounds of 2 ml, and samples were frozen for 24 h between rounds. After each round, samples were centrifuged at ∼2000 rpm for 15 min at 0°C and decanted into a 50 ml tube. The 4 ml methanol extracts were diluted with ∼46 ml of distilled water and run through Sep-pak columns (Waters, Milford, MA, USA). The Sep-pak column step was added to the assay to account for the larger yolk sample, to help filter out yolk components (e.g. lipids) that would later interfere with column chromatography. The Sep-pak columns were first charged with 5 ml of methanol followed by 5 ml of distilled water. Samples were loaded onto the Sep-pak column, and the steroids were extracted using 2 ml of 10% ethyl acetate in isooctane followed by 4 ml of pure ethyl acetate. We found that this modified extraction procedure provided the highest recovery of corticosterone. Samples were dried under nitrogen gas, resuspended in 500 µl of 10% ethyl acetate in isooctane, and fractionated in chromatography columns using 4 ml of the 20% fraction and 6 ml of the 50% fraction of ethyl acetate in isooctane. The 50% fraction was collected, dried under nitrogen gas, resuspended in 550 µl of phosphate buffered saline with gelatin, and refrigerated overnight. Samples were aliquoted, creating two duplicate 200 µl aliquots to run through the radioimmunoassay using an antibody specific to corticosterone (Biogenesis Inc., Kingston, NH, USA), and 100 µl was aliquoted to measure recoveries. Corticosterone concentrations were calculated based on a standard curve that ranged from 7.8 to 2000 pg. Duplicate cpm were averaged and then corrected for individual sample recovery and yolk sample mass (average recovery 68%). All samples were run in a single assay with an intra-assay variation of 8.5%.

Statistics

All statistics were conducted in SAS (v 9.4) and evaluated at an experiment-wise alpha of 0.05.

Corticosterone metabolism by the embryo

We used a linear mixed model to determine whether radioactivity counts differed with solubility (water versus ether soluble), time (days 5–55 of incubation) and their interaction across all egg components (eggshell, yolk, albumen, embryo). Clutch was included as a random effect to account for relatedness, and radioactivity counts were log transformed to meet the assumptions of normality and homoscedasticity. Egg mass was initially explored as a covariate, but it was ultimately excluded because it did not significantly contribute to the model. Tests of significance were determined using the Pillai's trace statistic.

Phenotypic consequences of corticosterone exposure

Logistical regressions (proc genmod) were used to determine whether the treatments affected embryonic mortality and the frequency of malformations in hatchlings. Because malformations presented as carpacial scute insertions/deletions of similar degree, treatment effects on the presence of malformations were analyzed as a binary trait. The models specified a binomial distribution (dist=bin) and a logistical link function (link=logit). Independent *post hoc* contrasts (contrast) were conducted to determine whether each treatment significantly differed from the control in both embryonic mortality and malformation analyses, and a Bonferroni correction was used to maintain an experiment-wise alpha of 0.05.

An ANOVA (proc mixed) was used to analyze the effects of corticosterone dose on incubation period, specifying clutch as a random effect. *Post hoc* analyses were conducted using Tukey's honest significant difference.

To determine whether exogenous corticosterone doses affected hatchling size, a MANOVA (proc GLM) was conducted using hatchling mass and plastron length. Clutch was included as a random effect. Tests of significance were determined using the Pillai's trace statistic. *Post hoc* analyses were conducted using independent contrasts (contrast) where each treatment was compared with the control, and a Bonferroni correction was used to maintain an experiment-wise alpha of 0.05.

Egg mass was initially included in all of the above models as a covariate but was not significant in any model and was therefore left out of all final models. Egg mass did not significantly differ among corticosterone dose treatments (ANOVA: $F_{3,119}$=1.4, P=0.24).

Maternal transfer of corticosterone to the egg

The primary motivation of this study was to characterize corticosterone concentrations to estimate whether endogenous concentrations of maternally transferred corticosterone might affect hatchling fitness in nature. Because maternal estrogen deposition differs across the nesting season in this species (Carter et al., 2017), we were additionally motivated to determine whether corticosterone deposition varies across the season. A one-way ANOVA (proc GLM) was used to test for a seasonal difference in yolk corticosterone concentrations.

RESULTS

Corticosterone metabolism by the embryo

Radioactivity counts throughout the egg were significantly affected by the interactive and main effects of solubility (water versus ether) and time (days 5–55 of incubation) (solubility $F_{4,88}$=686.35, P<0.0001; time $F_{40,364}$=6.58, P<0.0001; solubility×time $F_{40,364}$=7.44, P<0.0001). In all egg components, water-soluble (i.e. conjugated steroid metabolites) radioactivity was significantly higher than ether-soluble (i.e. free corticosterone) radioactivity (Fig. 1), with the albumen containing the highest water-soluble radioactivity counts (Fig. 1A). Both water-soluble and ether-soluble counts significantly changed across embryonic development. Ether solubility decreased across development to low or undetectable levels by ∼20 days of incubation in all egg components; this was especially notable in the embryo, where ether-soluble levels of radioactivity were low throughout development (Fig. 1C).

Phenotypic consequences of corticosterone exposure

We obtained 116 viable hatchlings from the initial 135 eggs dosed with corticosterone. The number of embryonic deaths in each of the 0, 0.05, 0.15 and 0.5 µg/5 µl doses were 2, 3, 3 and 11, respectively, resulting in 26, 32, 33 and 25 viable hatchlings in each treatment, respectively. More embryos died during development in corticosterone-treated eggs than in control eggs (χ^2=9.85, P=0.019). This finding was driven by the significant difference in survival between the control and the highest dose treatment (control versus 0.5: χ^2=5.88, P=0.0153); no other groups significantly differed in embryonic mortality compared with the control (control versus 0.05: χ^2=0.04, P=0.83; control versus 0.15: χ^2=0.03, P=0.86; Fig. 2A).

The frequency of scute malformations significantly increased in hatchlings from corticosterone-treated eggs (χ^2=9.76, P=0.021).

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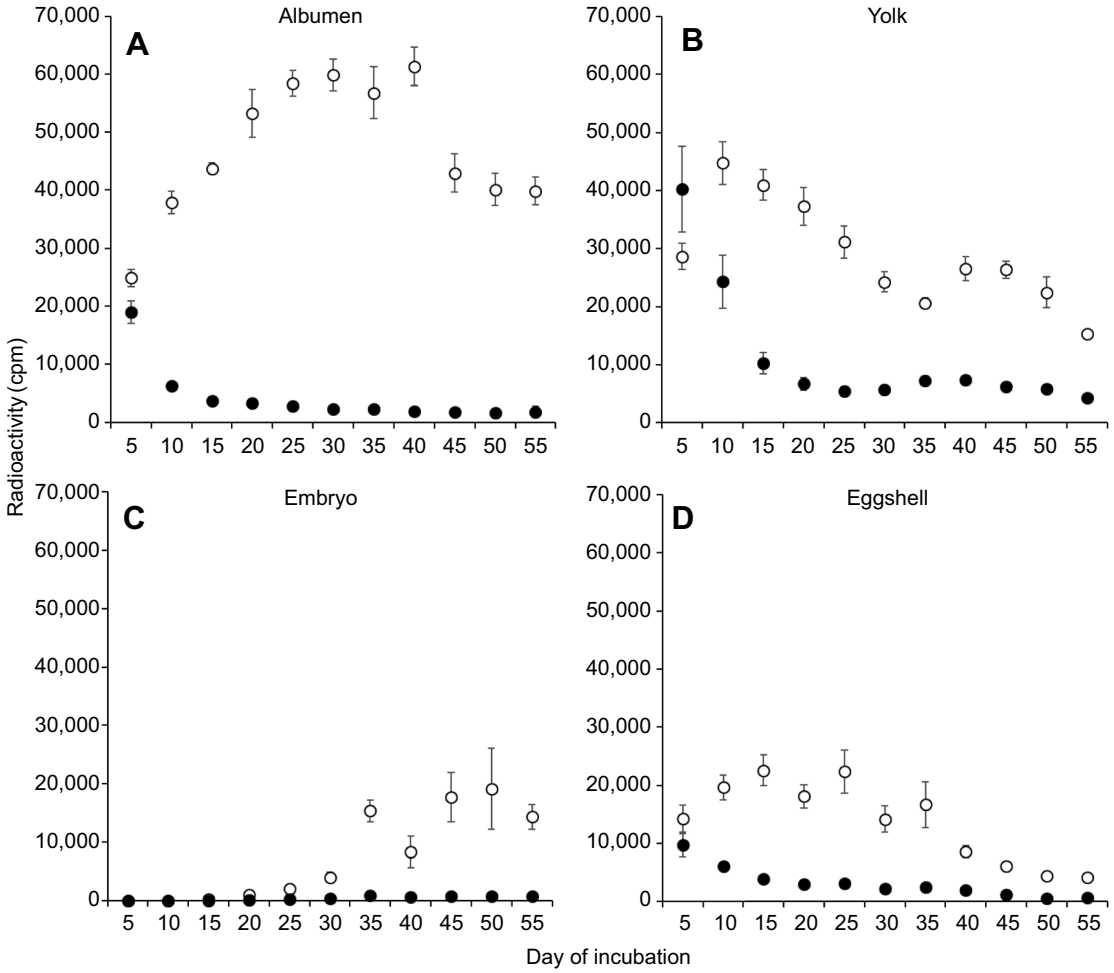

Fig. 1. Counts of radioactivity recovered from different *Trachemys scripta* egg components throughout embryonic development. Open circles are water-soluble counts (metabolites) and black circles are ether-soluble counts (corticosterone). (A) Albumen; (B) yolk; (C) embryo; (D) eggshell.

The number of hatchlings with scute malformations in each of the 0, 0.05, 0.15 and 0.5 µg/5 µl doses were 1, 8, 11 and 7, respectively. All treatments significantly differed in malformation frequency when compared with controls (control versus 0.05: χ^2=5.6, P=0.018; control versus 0.15: χ^2=9.11, P=0.003; control versus 0.5: χ^2=6.2, P=0.013; Fig. 2B).

Hatchlings from corticosterone-treated eggs were significantly smaller at hatch than control hatchlings ($F_{6,200}$=2.23, P=0.042), and this was affected by the random effect of clutch ($F_{24,200}$=22.08, P<0.0001; Fig. 2C). Mean (±s.e.m.) masses for each of the 0, 0.05, 0.15 and 0.5 µg/5 µl doses were 7.9±0.12, 7.7±0.10, 7.6±0.10 and 7.4±0.11 g, respectively. Mean (±s.e.m.) plastron lengths for each of the 0, 0.05, 0.15 and 0.5 µg/5 µl doses were 29.7±0.22, 29.4±0.20, 29.6±0.19 and 28.9±0.22 mm, respectively. Hatchlings from the highest corticosterone dose were significantly smaller than control hatchlings (control versus 0.5: $F_{2,99}$=4.16, P=0.019; control versus 0.05: $F_{2,99}$=0.5, P=0.61; control versus 0.15: $F_{2,99}$=2.28, P=0.108).

Corticosterone dose significantly extended incubation period ($F_{3,100}$=5.26, P=0.002; Fig. 2D). Mean (±s.e.m.) incubation periods for each of the 0, 0.05, 0.15 and 0.5 µg/5 µl doses were 51.6±0.32, 51.8±0.31, 52.2±0.31 and 52.6±0.32 days, respectively. Embryos from the highest corticosterone dose took longer to hatch than embryos from both the control treatment (P=0.0026) and the lowest dose (P=0.0177). No other pairwise comparisons among treatment and control groups varied significantly (P>0.05 in all cases).

Maternal transfer of corticosterone to the egg

Corticosterone was present in eggs at oviposition, but in very low concentrations (0.53±0.05 ng ml^{-1}), and did not differ between eggs laid early or late in the nesting season ($F_{1,33}$=0.003, P=0.95; Fig. 3).

DISCUSSION

Our data demonstrate that embryos are capable of rapidly metabolizing corticosterone, with less than 1% of the original dose accumulating in the embryo. By dosing eggs with concentrations of corticosterone that are high enough to (presumably) overwhelm embryonic steroid metabolism, we were able to demonstrate impacts of corticosterone accumulation on traits important to fitness: specifically, increased embryonic mortality, increased rates of scute malformations, smaller body size at hatching, and extended incubation period. Lastly, we measured maternal corticosterone concentrations in unmanipulated eggs and found very low endogenous concentrations (0.53 ng ml^{-1}). These concentrations are considerably lower than the doses required to impact offspring survival and phenotype (present study, see 'Phenotypic consequences of corticosterone exposure'). We discuss our findings in light of the underlying mechanisms and the potential selective pressures leading to these patterns more broadly.

Our findings suggest that the embryo is capable of rapidly metabolizing maternal corticosterone, and that the developmental

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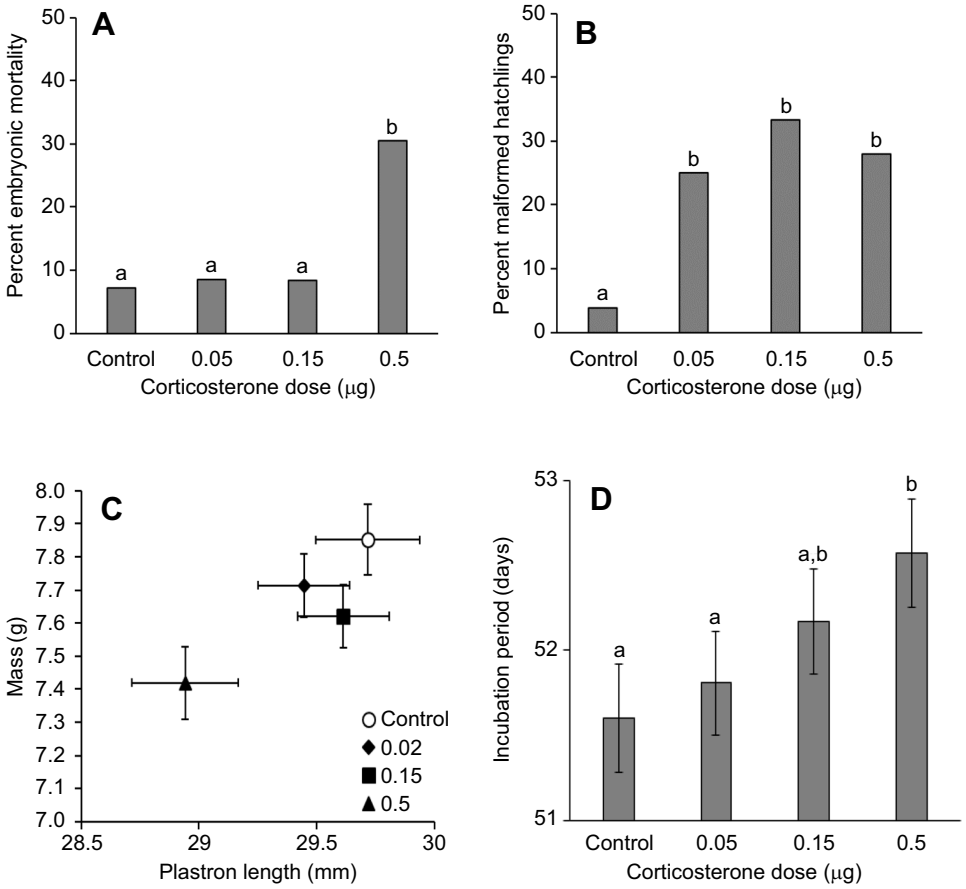

Fig. 2. Consequences of corticosterone dosing on day of oviposition. (A) *Trachemys scripta* embryos dosed with the highest corticosterone concentration has significantly higher embryonic mortality than embryos of controls and other treatments. (B) Hatchlings from eggs of all corticosterone doses had a higher frequency of scute malformations than controls. (C) Hatchlings from the highest dose were smaller at hatching. (D) Hatchlings from the highest dose experienced longer incubation periods than control or low dose hatchlings. Note that in C and D, the *y*-axis does not start at zero.

endocrine environment is active and dynamic. Corticosterone, in its free form, is an ether-soluble, lipophilic steroid that readily binds to receptors to trigger suites of physiological processes (Möstl et al., 2005). When conjugated, metabolites are water soluble and hydrophilic (Möstl et al., 2005). Although these metabolites are derivatives of corticosterone, they no longer have the same structure and are often physiologically inactive, as conjugation hinders receptor binding (Berliner and Dougherty, 1960; Strott, 2002). Hormone movement throughout the egg is facilitated by shifts in polarity (e.g. lipophilic to hydrophilic), such that metabolites accumulate in aqueous portions of the egg (e.g. albumen) and corticosterone accumulates in lipid-rich portions of the egg (e.g. yolk). In the present study, 85% of the total steroid recovered was in a water-soluble form by the end of development, and most was recovered in the albumen. This means that nearly all of the free corticosterone applied to the egg was likely conjugated into less active metabolites and stored in the albumen, away from developing embryonic tissues. Limited steroid accumulated in the embryo, and only 0.7% of the total steroid recovered was free corticosterone. These findings are comparable to a similar study in Japanese quail, where 80% of the recovered corticosterone was conjugated, and free corticosterone never reached the embryo (Vassallo et al., 2014).

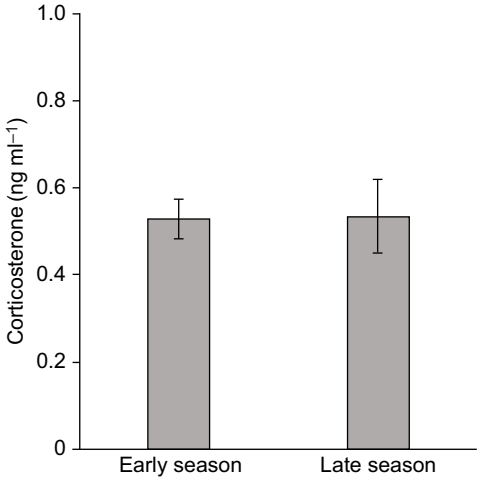

Fig. 3. Concentrations of maternally derived corticosterone in *Trachemys scripta* egg yolks in eggs are low, and do not differ between eggs laid early and late in the nesting season.

We recorded metabolism within the first 5 days of development throughout the egg, which brings the underlying metabolic pathways into question. Though we did not identify the metabolites, and therefore cannot identify the metabolic pathway(s) utilized, sulfonation likely underlies much of the metabolism in the current study (though other pathways may also be in play). Sulfonation is a highly conserved metabolic pathway active in *T. scripta* embryos (Paitz and Bowden, 2013), a primary method of glucocorticoid metabolism (Möstl et al., 2005) and the main metabolic pathway used in the placenta (Diczfalusy, 1969). The enzyme steroid sulfatase, which converts metabolites to their parent steroid, is observed in extraembryonic membranes of *T. scripta* eggs at 8 days post-lay and remains available throughout development (Paitz et al., 2017). For example, steroid sulfatase is found in many tissues from older embryos (brain, adrenal–kidney complex and liver), providing additional sites of steroid metabolism later in development (Paitz et al., 2017). Sulfotransferase, which

converts steroids (including corticosterone) into sulfonated metabolites, exhibits increasing activity over the first 10 days of development in the extraembryonic membranes of *T. scripta* (Paitz and Bowden, 2008). It is currently unknown what effects these corticosterone metabolites may have on embryonic development, but it is worth noting that estrogen sulfates can affect sex determination in this species (Paitz and Bowden, 2013). The role that females play in facilitating *in ovo* steroid metabolism (e.g. maternally derived metabolic enzymes), if any, requires further research; however, unfertilized eggs do not show metabolism of maternal steroids (Paitz and Casto, 2012), highlighting the role of the embryo in initiating metabolism. Complementing previous studies (Paitz and Bowden, 2013; Reed and Clark, 2011; Vassallo et al., 2014), our data suggest that embryos are not passive recipients of maternal steroids; rather, embryos and associated tissues actively metabolize maternal steroids to modulate the developmental environment.

Dosing eggs with high concentrations of corticosterone on the same day as oviposition significantly decreased survival and impacted multiple aspects of phenotype. Some of these effects were only observable at the highest corticosterone concertation (0.5 µg ml^{-1}) – specifically, the effects of reduced survival and hatchling size – whereas other effects, such as the increased rate of malformations, occurred at all dose concentrations. Embryonic survival is directly linked to fitness, and hatchling size is known to positively co-vary with fitness (Janzen et al., 2000). The fitness consequences of a ∼1.5-day extension in incubation period is unknown and likely negligible, particularly for a population that overwinters in the nest before emergence. The impact of scute malformations on fitness is harder to discern. Though we observe adults of reproductive maturity with scute malformations in our study population, the rate of occurrence is very low (authors' personal observation), certainly lower than the frequency observed in the hatchlings from dosed eggs in this study, suggesting a negative correlation between fitness and scute malformation. Considering the effects measured in the present study, corticosterone exposure during development seems to negatively (or neutrally) impact development and fitness in *T. scripta*, corroborating results from similar studies (Hayward and Wingfield, 2004; Saino et al., 2005). The impacts of embryonic exposure to maternal corticosterone on hatchling survival and fitness detailed here shed light on the potential selective pressures leading to and/or maintaining the steroid buffering mechanisms of the embryo.

Corticosterone was detected in unmanipulated *T. scripta* eggs at oviposition in very low concentrations (0.53 ng ml^{-1}). For comparison, Hayward and Wingfield (2004) describe corticosterone concentrations in Japanese quail eggs of 0.92±0.16 ng g^{-1}, whereas the concentrations reported here are approximately half that. Given that the lowest corticosterone dose (0.05 µg ml^{-1}) in the hatchling phenotype study only affected one of the four parameters measured (malformation), and that maternal corticosterone concentrations found in egg yolks are substantially lower than this low dose, it follows that embryos are likely capable of metabolizing the limited maternal corticosterone that accumulates in yolks in nature. This suggests that corticosterone-mediated maternal effects are limited to negligible in this species, and may also explain why other phenotypic parameters, like behavior, are not affected by maternal corticosterone (Carter et al., 2016). Of additional note, concentrations of maternal corticosterone were similar across the nesting season, unlike some other maternally derived steroids in this species (i.e. estradiol and estrone sulfate) (Carter et al., 2017).

Why are maternal corticosterone concentrations in egg yolks so low? It is possible that females may be actively buffering offspring from maternal corticosterone exposure. Though this determination is outside the scope of the present study, we outline two non-mutually exclusive hypotheses to help direct future research. First, females could reduce the accumulation of circulating plasma corticosterone in yolks during vitellogenesis through catalysis at follicles via enzymes such as 11β-HSD (Rettenbacher et al., 2013; Yang, 1997). Second, during vitellogensis and nesting, females may have a hypo-reactive HPA axis (Jessop et al., 2000, Jessop, 2001; Paitz et al., 2014; Valverde et al., 1999), reducing the amount of circulating steroid available for accumulation in the yolk. Adult female *T. scripta* are capable of mounting a stress response during the nesting season, with plasma corticosterone concentrations exceeding 3 ng ml^{-1} in many individuals and reaching up to 14 ng ml^{-1} in some (Paitz et al., 2014). These concentrations are higher than those observed in eggs (present study), supporting the hypothesis of enzymatic removal at the follicles. Moreover, these stress-induced plasma concentrations are low compared with those in other species (>40 ng ml^{-1} in *Zonotrichia leucophrys gambelii*, Breuner et al., 1999; 148.2 ng ml^{-1} in *Rattus* spp., Takahashi et al., 1998), suggesting that females may also rely on hypo-stress reactivity during nesting to limit embryonic corticosterone accumulation. Evidence of hypo-stress reactivity during nesting has been found in several turtle species (Jessop et al., 2000, 2001; Paitz et al., 2014; Valverde et al., 1999). These data provide evidence that females could play a role in buffering embryos from corticosterone exposure, but further research is needed to more comprehensively assess the degree to which females mitigate corticosterone accumulation in egg yolks and the underlying mechanism(s).

Taken together, our data demonstrate that embryos take an active role in modulating their exposure to maternal steroids during development. These buffering mechanisms may be maintained by selective pressures stemming from exposure to high concentrations of corticosterone, such as reduced survival and hatchling size, traits directly linked to fitness. Given the low concentrations of maternal corticosterone in unmanipulated yolks, it seems likely that embryos can metabolize maternal corticosterone in natural systems to avoid fitness consequences. Overall, these data highlight the importance of understanding the mechanisms underlying steroid-mediated maternal effects to gain a better appreciation of the evolutionary significance of this maternal effect.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.W.C., R.M.B., R.T.P.; Methodology: A.W.C., R.T.P.; Validation: A.W.C., R.T.P.; Formal analysis: A.W.C., R.T.P.; Investigation: A.W.C., R.M.B., R.T.P.; Resources: A.W.C., R.M.B.; Data curation: A.W.C., R.T.P.; Writing - original draft: A.W.C.; Writing - review & editing: A.W.C., R.M.B., R.T.P.; Visualization: A.W.C.; Supervision: R.M.B., R.T.P.; Project administration: R.M.B., R.T.P.; Funding acquisition: A.W.C., R.M.B., R.T.P.

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Data availability
The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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