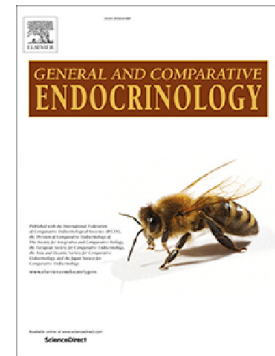


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Transgenerational effects of maternal corticosterone across early life in a viviparous snake

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Running headline: Transgenerational corticosterone effects in viviparous snakes.

Abstract

Glucocorticoids (GCs) are central mediators of vertebrate responses to intrinsic and extrinsic stimuli. Among the sources of variation in circulating GCs are transgenerational effects mediated by mothers. Here we studied potential maternal effects mediated by GCs on offspring phenotype in a live-bearing reptile, the western terrestrial garter snake (*Thamnophis elegans*). We evaluated the association between baseline corticosterone (CORT) levels during gestation (i.e., preparturition) in field-captured mothers and 1) reproductive success and offspring sex ratios, 2) birth phenotypic traits of offspring born under common-garden laboratory conditions, and 3) neonate (age 3 months) and juvenile (age 12 months) traits of offspring raised under two thermal regimes ('warm' and 'cool') during their first year of life. Reproductive success and offspring sex ratios were not associated with preparturition maternal CORT, but pregnant snakes with higher CORT levels gave birth to smaller, lighter offspring, which tended to grow faster to age three months. Neonate baseline CORT varied with preparturition maternal CORT in a sex-specific manner (positive trend for females, negative for males). Maternal CORT effects on offspring phenotype were no longer detectable in juveniles at age one year. Instead, juvenile phenotypes were most influenced by rearing environment, with offspring raised under the cool regime showing higher baseline CORT and slower growth than those raised under warmer conditions. Our findings support the notion that offspring phenotype might be continuously adjusted in response to environmental cues —both pre- and post-natal— and that the strength of maternal CORT effects declines as offspring develop and experience unique environmental challenges. Our results contribute to a growing literature on transgenerational effects of hormones and help to fill a gap in our knowledge of these effects in ectothermic amniotes.

Keywords: cross-generational effects, developmental plasticity, glucocorticoids, maternal effects, prenatal, temperature, *Thamnophis*

1. Introduction

Glucocorticoids (GCs) are central mediators of vertebrate responses to intrinsic and extrinsic stimuli, being essential for coping with environmental changes and challenges (McEwen and Wingfield, 2003; Landys et al., 2006; Guindre-Parker, 2018; Gangloff and Greenberg, In press). In the face of sudden unpredictable changes (stressors), circulating GCs become elevated above seasonal baseline levels, mediating a switch in priorities from regular day-to-day activities and functions to an emergency life-history tactic to promote immediate survival (Wingfield et al., 1998; Sapolsky et al., 2000; Landys et al., 2006). Despite the accepted importance of GCs in mediating adjustments to changing environments, the link between circulating GC levels and individual fitness metrics in natural populations remains unclear (Breuner et al., 2008; Bonier et al., 2009; Bonier and Cox, 2020; Schoenle et al., 2021). Given the central role of GCs in coping responses but the uncertainty about the adaptive significance of circulating levels, a more profound understanding of the many factors underlying the broad variation in circulating GCs remains a significant undertaking (Angelier and Wingfield, 2013; Guindre-Parker, 2018; Bonier and Cox, 2020). Importantly, the multifaceted roles of GCs in organismal function—including the regulation of energy and mineral-water balance in addition to response and recovery from stressors—need to be considered in the interpretation of GC variation in free-living vertebrates (Vera et al., 2017; MacDougall-Shackleton et al., 2019; Romero and Beattie, 2021).

Among the sources of variation in circulating GCs are transgenerational effects, especially those mediated by mothers. Maternal influences on offspring circulating GC levels can occur via heritable genetic variation (Bartels et al., 2003; Jenkins et al., 2014; Stedman et al., 2017) and/or

via diverse non-genetic mechanisms such as those mediated by nutrition, hormonal exposure, or epigenetic changes (Meaney et al., 2007; Zhang et al., 2013). GC-mediated maternal effects in wild animals have been described for diverse offspring traits, ranging from body size and condition, to different behaviors and physiological parameters, and all the way to reproduction and survival (Meylan et al., 2012; Love et al., 2013; Sheriff and Love, 2013; Sheriff et al., 2017). Although evolutionary ecologists have challenged the traditional biomedical view that elevated circulating GC levels in mothers are necessarily detrimental to offspring and have proposed alternative adaptive scenarios (e.g., maternal match hypothesis, Sheriff and Love, 2013; Sheriff et al., 2017), current evidence for beneficial effects is mixed and species-, life-history-, and context-specific (Sheriff et al., 2017). In fact, a recent meta-analysis of studies in birds, mammals, and reptiles that experimentally elevated GC levels to mimic maternal stress (or applied an ecological stressor to mothers) showed an overall trend towards potentially detrimental effects on offspring, but with much variation across studies (MacLeod et al., 2021 a).

While numerous studies have laid a foundation for our understanding of how variation in maternal GCs affect offspring phenotypes, there are yet knowledge gaps to fill (Sheriff et al., 2017; Guindre-Parker, 2018; Bonier and Cox, 2020). The effects of transgenerational GC levels on offspring GC levels remain understudied relative to other offspring phenotypic traits in wild amniotes, particularly in non-avian reptiles (Robert et al., 2009; MacLeod et al., 2021 a). Similarly, the consequences of natural variation in maternal baseline (or integrated) GC levels during gestation, rather than experimentally-induced stress levels, on offspring phenotypes have received less attention (Hau et al., 2016). Furthermore, little is still known about the context-dependence of GC-mediated maternal effects (Sheriff et al., 2017; MacLeod et al., 2021 b) and

how these effects may interact with the postnatal environment to affect variation in offspring GC levels (e.g., Love and Williams, 2008; Tilgar et al., 2016). Studies in viviparous reptile species, for which the strength of GC-mediated maternal effects is overall higher than for oviparous counterparts (MacLeod et al., 2021 a), can be particularly insightful for filling these gaps. This is especially noteworthy in that it suggests a role for prolonged embryo exposure to GCs even in cases of morphologically simple placentation, as that found in most viviparous squamates.

In this context, we studied the potential effects of preparturition maternal baseline GCs on offspring baseline GC levels and fitness-related traits during the first year of life in a live-bearing squamate reptile, the western terrestrial garter snake (*Thamnophis elegans*). Populations of the western terrestrial garter snake in the vicinity of Eagle Lake (Lassen County, California, USA) inhabit two different habitat types and correspondingly display distinct fast and slow life-history strategies (Bronikowski and Arnold, 1992; Addis et al., 2017). Lakeshore populations (hereafter L-fast ecotype) show fast growth to large adult body size, early sexual maturation with high reproductive effort, and low annual survival; populations in higher-elevation mountain meadows (hereafter M-slow ecotype) exhibit opposing traits on the pace-of-life continuum. The two ecotypes represent different genetic backgrounds thought to be driven by differing thermal regimes (Bronikowski, 2000), resource availability (Miller et al., 2011; Holden et al., 2022), and predation rates (Sparkman et al., 2013) between the lakeshore and meadow habitats. While garter snake embryos primarily derive nutrients from the yolk (lecithotrophic), there is placental transfer of gases, water, certain nutrients, and likely hormones from mothers to developing embryos, especially during later gestation (Blackburn and Flemming, 2009; Itonaga et al., 2011; Sparkman et al., 2018, and references therein).

Previous studies on GC physiology in this snake system have unveiled important patterns relevant to our present work. Females show consistent among-individual variation in circulating baseline corticosterone (CORT hereafter, the main GC in reptiles) in the laboratory (Sparkman et al., 2014; Supplemental Fig. 1). Baseline and stress-induced CORT levels show a positive correlation across individuals (Supplemental Fig. 2). Ecotypic differences in baseline and stress-induced CORT have been found in the field, with M-slow snakes showing higher levels than L-fast snakes (Robert et al., 2009; Palacios et al., 2012; Holden et al., 2022). Although the link between ecotype and CORT suggests a genetic influence, as documented for other traits in this system (e.g., growth: Bronikowski, 2000; morphology: Meulier et al., 2007; metabolism: Gangloff et al., 2020), multi-year measures showing ecotypic differences in some years, but not all, suggests environmental effects (Palacios et al., 2012; Holden et al., 2022). Regarding maternal effects, CORT-treatment of pregnant females—to mimic maternal stress during gestation—induces reproductive failure and altered behavior and size of offspring (Robert et al., 2009) and maternal physiological and behavioral phenotypes interact to shape offspring size and growth (Gangloff et al., 2018). Overall, these studies set the stage to assess the potential effects of gestational exposure to maternal baseline CORT on offspring baseline GC levels and other fitness-relevant traits through early life-history stages and in relation to postnatal environmental conditions in this viviparous snake.

In the present study, we first assess the potential effects of preparturition maternal (i.e., gestational) baseline CORT on reproductive success, offspring sex ratios, and phenotypic traits at birth (i.e., size, mass, and body condition) of offspring born under common-garden laboratory

conditions. Then, we test the potential effects of preparturition maternal baseline CORT on offspring traits (i.e., baseline CORT, growth, and survival) for offspring raised under two thermal regimes ('warm' and 'cool') that mimic those experienced by snakes of the L-fast and M-slow ecotypes in their natural habitats, respectively. These thermal treatments, applied in a laboratory reciprocal-transplant experimental design, have been used in the study of a variety of phenotypic traits in this snake species (Gangloff et al., 2015; Reding et al., 2016; Addis et al., 2017; Palacios et al., 2020). In the present work, we use this experimental design to evaluate the relative contribution of preparturition maternal baseline CORT and postnatal environmental temperature on offspring phenotypes from the two ecotypes during their first year of life.

We predicted that mothers having higher preparturition baseline CORT would give birth to offspring having higher baseline CORT levels (e.g., Hayward et al., 2006; Sheriff et al., 2010, but see Ensminger et al., 2018). Although whether maternal effects on offspring traits are beneficial or detrimental is likely to be species-specific and context-dependent (Sheriff et al., 2017), some predictions can be made based on life-history theory (MacLeod et al., 2021 a). Thus, if offspring from mothers with higher preparturition baseline CORT show smaller size, mass, or body condition at birth, and slower growth rates or survival, this would be consistent with a detrimental transgenerational effect of maternal CORT, whereas the opposite set of traits would be consistent with a beneficial maternal CORT effect. In addition, the repeated measurement of offspring at three developmental times (birth, age 3 months, and age 12 months) and under two postnatal thermal conditions (warm and cool) allows us to test the predictions that maternal baseline CORT effects would decline in strength as offspring develop (Wilson and Reale, 2006; MacLeod et al., 2021 a) and depend on the different early-life environments

experienced by offspring (Sheriff et al., 2017, MacLeod et al., 2021 b). Finally, we evaluate the potential sex-dependence of maternal baseline CORT effects, as maternally-derived GC effects often differ between female and male offspring (Robert et al., 2009; Meylan and Clobert, 2005; MacLeod et al., 2021 a).

2. Materials and methods

2.1. *Pregnant female capture and husbandry*

Between 12-27 June 2010 we collected pregnant females of each of the two ecotypes (L-fast: 22 individuals from 4 populations; M-slow: 22 individuals from 4 populations; snout-vent length [SVL] range: 415-768 mm). Pregnancy was determined by palpation of the abdomen for yolks/embryos. Pregnant females were held in temporary field housing and transported on the same date to the laboratory colony at Iowa State University, where they were housed individually in ten-gallon glass aquaria with corncob substrate and water dishes with hollow rims that provided shelter. Aquaria were placed with one end on a heating element that generated a permanent (i.e., 24 h) thermal gradient of 25-34°C to allow thermoregulation. Snakes were maintained on 12:12 light-dark daily cycles and fed thawed frozen mice once per week to satiation until parturition.

2.2. *Offspring birth and thermal treatments*

Each female gave birth on a single day between 12 August and 19 September 2010. The number of neonates, stillborns, and yolks (unfertilized eggs) delivered by each female was recorded. Live offspring ($n = 257$ neonates) were sexed by hemipene eversion and measured —mass (g) and SVL (mm)— within 24 h of birth; 11 out of the 13 stillborns could also be sexed. A subset of the neonates ($n = 192$, from 21 L-fast and 19 M-slow litters) were initially included in the laboratory reciprocal transplant experiment (see below), of which six died before the first blood sampling session. The remaining 65 neonates —from the largest litters— were released into their natal populations shortly after birth. We calculated body condition, a measure of size-corrected mass, as the residuals of the \log_{10} -mass on \log_{10} -SVL linear regression (Weatherhead and Brown, 1996). Offspring were housed individually in plastic boxes with paper substrate and a water dish that provided a retreat site. Thermal treatments were designed to mimic the differing availability of optimum temperatures in the respective habitats, where L-fast habitats are 5-10°C warmer than M-slow (Bronikowski and Arnold, 1999). Ambient room temperature was 20°C and offspring in the ‘warm’ treatment group received 16 h of supplemental heating per day, whereas those in the ‘cool’ treatment group only received 8 h per day of supplemental heating. Supplemental heating consisted of a thermal gradient (22–32°C) that allowed behavioral thermoregulation and was generated by heat tapes placed under one end of the box. Optimal and preferred field-active temperatures for this species are ~28-30°C (Peterson, 1987). Males and females from each litter were split randomly between the ‘warm’ and ‘cool’ thermal treatment groups leading to a full factorial experimental design of ecotype \times thermal treatment \times sex (Addis et al., 2017). Offspring were kept on a 12:12 L:D schedule and offered frozen/thawed pinky mice once a week. Individuals were maintained under these conditions for the duration of the

experiment, except for a period of hibernation in the dark at 4°C from January through May 2011 (full captive care details in Gangloff et al., 2015). Individual survivorship was recorded.

2.3. Blood collection and processing

We collected preparturition blood samples from adult females on 9 August 2010. Offspring blood samples were collected as neonates when approximately 3 months old (median: 97 days old, range: 71-109 days old) between 19-23 November 2010 (hereafter 3 month sampling) and again as juveniles when about 12 months old (median: 388 days old, range: 362-400 days old) between 14-19 September 2011 (hereafter 12 month sampling). Definition of age ranges for neonates and juveniles follow Miller et al., 2011. We collected blood from the caudal vein using heparin-rinsed syringes within 10 minutes of disturbance (i.e., removal of the individual from its box), a timeframe during which CORT values do not correlate with sampling time and are therefore considered as baseline (background) levels for the species (Palacios et al., 2012). Plasma was separated by centrifugation and stored at -80°C until analyses. All snakes were weighed (to the nearest 0.01 g using an electronic balance) and measured (snout-vent length [SVL] to the nearest mm) after each blood-sampling session. Treatment of all animals was in accordance with Iowa State University Institutional Animal Care and Use Committee protocol #3-2-5125-J and the California Department of Fish and Game (SC-11973).

2.4. Corticosterone radioimmunoassay

Levels of plasma CORT were determined using double-antibody radioimmunoassay kits (Catalog # 07–102103, MP Biomedical, Orangeburg, NY, USA). We followed the protocol by Robert et al., 2009, except that plasma was diluted 1:80 (instead of 1:40) because this dilution proved optimal for the range of samples in the present study. Plasma samples for adult females were run in 3 assays using 1 kit (26 Jan 2011), whereas offspring samples were run in 8 assays using 3 kits (27–30 Oct 2011). All samples were run in duplicate. Samples were re-run if the coefficient of variation (CV%) in CORT concentration between duplicates was greater than 10%, conditional on having enough plasma and kit reagents available. Mean intra-assay variation calculated from replicate samples within each assay was 10.4%. Mean inter-assay variation was 18.3%, calculated using the kit-provided low controls.

2.5. Statistical analyses

We implemented generalized linear mixed models to assess the importance of factors influencing maternal reproductive success and offspring sex ratio at birth. We defined reproductive success as live birth and failure as a stillborn or yolk (definition of failure as stillborn only did not change our results). We constructed models with a binomial error distribution (female/male or success/failure) and included the fixed effects of maternal CORT, ecotype, and the interaction of maternal CORT and ecotype. Models also included the random intercept of population of origin to account for covariance in traits for moms from the same populations. We implemented linear mixed models to assess the importance of factors influencing offspring phenotypes at birth (mass, SVL, and body condition); offspring growth to age 3 months and baseline CORT at age 3 months; and offspring growth from age 3 months to age 12 months and offspring CORT at age 12 months. As in other analyses of growth in this snake system (Addis et al., 2017; Gangloff et

al., 2018), we used specific growth rate, a measure of growth rate scaled to body size ($SGR = 100 \times [\ln(SVL2) - \ln(SVL1)]/\text{days}$). Finally, we implemented a generalized linear mixed model to assess factors influencing offspring survival from age 3 to age 12 months (our analysis here excluded the six individuals that did not survive to age 3 months), with a binomial error distribution (survival yes/no). Though offspring differed in their exact age at the 3 and 12 month samplings, there was no effect of this variation on baseline CORT (3 months: $F_{1,34.2} = 0.0018$, $P = 0.97$; 12 months: $F_{1,32.5} = 1.71$, $P = 0.20$). Models of birth phenotypes included the fixed effects of maternal baseline CORT, genetic background (ecotype), and sex. Models of phenotypes at later ages also included the fixed effect of thermal rearing treatment (warm/cool). To account for covariation of traits among siblings and among animals from the same population, all models included the random intercepts of litter and population. Although secondary to our objective of understanding GC-mediated maternal effects on offspring phenotypes, we also explored potential factors contributing to variation in preparturition maternal baseline CORT. After determining that differences in the time elapsed from capture in the field to blood collection in the laboratory did not affect maternal CORT ($F_{1,38.7} = 0.0297$, $P = 0.86$), we built a model to assess the importance of ecotype, days before parturition (Whittier et al., 1987), number of developing embryos (Holden et al., 2022), and SVL that included a random intercept for population. All CORT values were \log_{10} -transformed to meet the assumption of normally-distributed model residuals. We began by structuring models to include all highest-level interactions of fixed effects (three-way for birth phenotypes; four-way for later age phenotypes) and utilized backward selection to simplify models. We created reduced models by removing the single highest-order interaction term with the highest p-value (if $P > 0.05$). We then ran the reduced model and repeated the process, resulting in simplified models containing

the fixed effects of interest and any important interaction terms. We visually assessed distributions of model residuals and determined the relative importance of fixed effects using type III sums of squares, utilizing Wald chi-squared tests for generalized models and F-tests with corrected denominator degrees of freedom (Kenward and Roger, 1997) for linear models. Models were implemented with the lme4 package (Bates et al., 2015) and all data figures were created with ggplot2 (Wickham, 2016) in R (R Core Team, 2022).

3. Results

3.1. Birth phenotypes

Neither preparturition maternal CORT, ecotype, nor their interaction explained variation in the proportion of live offspring (i.e., reproductive success) or the within-litter sex ratio at birth (Table 1). Preparturition maternal CORT did not affect offspring SVL or mass at birth (Table 1). However, visual inspection of the relationships suggested otherwise, with four data points in the upper right corners of the corresponding scatterplots (Fig. 1A, B) appearing to influence these results. On closer examination of the dataset, we found that these points belonged to siblings from a single L-fast litter that were born large to a large mother with high CORT. Removal of that litter—which included one additional sibling (i.e., five points in total)—in order to evaluate its influence on the analyses, resulted in a clear negative effect of maternal CORT on offspring SVL ($F_{1,34.1} = 7.99$, $P = 0.008$, Fig. 1A) and mass ($F_{1,33.9} = 6.59$, $P = 0.015$, Fig. 1B) at birth. The importance of the remaining effects in these models was not affected by the removal of this litter; ecotype was not a significant predictor of variation in SVL or mass at birth, whereas males were born larger (SVL) than females irrespective of ecotype (Table 1). On the other hand, maternal

CORT and ecotype did not influence offspring body condition at birth (Table 1, Fig. 1C), but female offspring exhibited higher body condition than males (Table 1). Although not visually appearing as potentially influential points in the case of body condition (Fig. 1C), we evaluated the effect of removal of the five siblings from the litter of large offspring born to a mom with high CORT from the analysis, finding no qualitative changes in the results of the body condition analysis presented in Table 1.

3.2. Neonate and juvenile phenotypes

Preparturition maternal baseline CORT interacted with sex to explain significant, albeit small, variation in neonatal baseline CORT levels (Table 2, Fig. 2A). The model suggests a positive trend for females and a negative trend for males with increasing maternal CORT levels, although neither of these relationships was statistically significant when the sexes were evaluated in separate models (Females: $F_{1,32.5} = 1.4$, $P = 0.24$; Males: $F_{1,26.6} = 1.54$, $P = 0.23$). Offspring growth from birth to 3 months was positively influenced by preparturition maternal CORT irrespective of offspring sex (Table 2, Fig. 2B) and by the interaction of ecotype with sex (Table 2), with neonate females growing faster than males in L-fast snakes, but such pattern was not observed in M-slow snakes (Supplemental Fig. 3). Neither neonatal baseline CORT nor growth was significantly influenced by thermal rearing treatment (Table 2).

Juvenile baseline CORT levels at age 12 months and growth from 3 to 12 months of age were not influenced by preparturition maternal baseline CORT or ecotype (Table 2, Fig. 2C, D, respectively), but offspring raised under the cool rearing treatment showed higher levels of

baseline CORT and slower growth than their counterparts raised under warm conditions (Table 2, Fig. 3A, B, respectively). In addition, females had higher baseline CORT levels than males (Table 2). Finally, offspring survival from 3 to 12 months of age was not influenced by preparturition maternal baseline CORT or thermal treatment, but female snakes from the L-fast ecotype exhibited higher survivorship compared to L-fast males (92.9% and 82.5%, respectively), whereas M-slow males exhibited higher survivorship compared to M-slow females (82.9% and 63.2%, respectively; Table 2).

3.3. Variation in preparturition maternal CORT

We evaluated the potential role of different factors in explaining the observed variation in preparturition baseline CORT levels of the field-captured pregnant females that gave birth to our study subjects. Although preparturition maternal baseline CORT varied substantially among individual females by an order of magnitude (range: 16-248 ng/mL, Fig. 1), none of the evaluated effects, or their interaction, explained significant variation: maternal SVL ($F_{1,34.3} = 0.79$, $P = 0.38$), ecotype ($F_{1,16.7} = 0.47$, $P = 0.50$), days before parturition ($F_{1,36.0} = 0.27$, $P = 0.60$), number of developing offspring ($F_{1,34.2} = 0.59$, $P = 0.45$), and interactions thereof (all $P > 0.1$).

4. Discussion

Our results demonstrate the importance of preparturition maternal glucocorticoid levels in shaping fitness-related phenotypic traits —i.e., size and mass at birth, growth and circulating GCs of neonates— in the offspring of a viviparous snake. They also illustrate the complexities of

how these maternal influences interact with the postnatal environment to determine offspring phenotype during early-life ontogeny. Our findings support the notion that offspring phenotype is likely to be continuously adjusted in response to environmental cues —both pre- and post-natal— experienced during the developmental period (Sheriff et al., 2017). We found support for the prediction that the effect of preparturition maternal CORT declines as individuals develop and experience unique environmental challenges; i.e., stronger effects at birth, weaker at 3 months, and not evident at 12 months of age. In turn, postnatal environmental temperature and sex effects became more important as offspring developed. We also found some support for sex-specific effects of preparturition maternal CORT on offspring CORT at age 3 months. Below we discuss our findings within an evolutionary ecological context.

4.1. Preparturition maternal CORT, reproductive success, and sex ratios

Increased embryonic mortality due to elevation of maternal CORT has been reported in diverse vertebrate taxa (e.g., non-avian reptiles: Cree et al., 2003; Cadby et al., 2010; birds: Saino et al., 2005; fishes: Gagliano and McCormick, 2009). In accordance, an earlier study in our system found that L-fast and M-low CORT-treated females —to emulate prenatal stress— suffered increased litter failure, giving birth to a higher proportion of stillborn offspring (Robert et al., 2009). Our present results, instead, showed no association between reproductive success and maternal baseline CORT measured prenatally in gestating snakes of the two ecotypes. This finding is in line with that of a study in the oviparous lizard, *Amphibolurus muricatus*, in which maternal baseline CORT was elevated by manipulation of the maternal thermal regime (Liu et al., 2020). Together, studies in our system thus suggest that although stress-induced CORT

results in reduced reproductive success (Robert et al., 2009), variation in baseline levels do not impact this outcome (this study).

Adjustments of offspring sex ratios by maternal hormones, including GCs, have been described in all vertebrates independently of the mechanisms of sex determination (Navara, 2010). In reptiles, yolk-hormone deposition during ovarian follicle development suggests the potential for early maternal influences on offspring sex ratios; however, the extent of this phenomenon remains unclear (reviewed by Radder, 2007; Navara, 2010). Biased offspring sex ratios in relation to maternal CORT levels have been reported in some reptiles (e.g., Warner et al. 2009), but not found in others (e.g., Meylan et al., 2004; Ower et al., 2018; Liu et al., 2020). Our results are in accordance with the latter studies. Offspring sex ratios at birth were not explained by variation in preparturition maternal baseline CORT in *T. elegans*, which reinforces the finding for stress-induced CORT (Robert et al., 2009).

4.2. Preparturition maternal CORT influences on birth phenotypes

Small neonatal size or weight are common outcomes of exposure to elevated maternally-derived GCs documented in the biomedical literature (Meaney et al., 2007) and in wild vertebrates (reviewed in MacLeod et al., 2021 a). Such effects on offspring morphology at birth have also been recognized in studies involving corticosterone application to pregnant female reptiles (e.g., Meylan and Clobert, 2005; Cadby et al., 2010, summarized by Liu et al., 2020); however, whether it is body size, mass, or condition that is affected varies across species and contexts. Our results show some parallel to these findings, whereby mothers with higher preparturition baseline

CORT levels gave birth to smaller, lighter offspring, although offspring body condition was not affected (Fig. 1). Surprisingly, there was a clear exception to this pattern: a large L-fast female that gave birth to large and heavy offspring despite having one of the highest levels of preparturition baseline CORT. This adult female was the largest animal brought into the captive colony over the 48 years of sampling (SVL 768 mm, mass 221 g). Her total litter mass was also by far the highest in our sample (63 g vs. 3.32-46.8 g for the remaining females). At present, the cause of this individual departure from the pattern observed for the other snakes is not clear.

Corticosterone-mediated maternal influences on offspring size/mass/condition—as maternal effects in general—can be sex-specific. Among reptiles, cases of smaller/lighter male (e.g., Owen et al., 2018) or female (e.g., Robert and Bronkowsky, 2010) offspring being born to CORT-treated (or higher-CORT) mothers have been reported. Furthermore, the sex most affected by elevated maternal CORT can vary in different contexts within a species (e.g., maternal basking regime, Liu et al., 2019). In this light, it is interesting to note that our present results do not indicate sex-specific maternal CORT effects on birth morphology, which differs from a previous finding of shorter female, but not male, offspring in CORT-treated mothers (Robert et al., 2009). These dissimilar outcomes with respect to sex-specificity might be related to the different aspects of maternal GC phenotype studied (manipulated to mimic stress vs. naturally elevated baseline), as proposed above for reproductive success.

4.3. Preparturition maternal CORT influences on neonate and juvenile phenotypes

Increased offspring GC levels are another common outcome of elevated maternal GCs in the biomedical literature (Meaney et al., 2007). In wild vertebrates, however, transgenerational GC effects on offspring GC levels have been understudied in relation to other phenotypic traits (see Appendix 2 in MacLeod et al., 2021 a). In fact, to our knowledge, only two previous studies have evaluated transgenerational GC effects on offspring GC levels in reptiles. Experimental elevation of maternal CORT during gestation in the oviparous lizard *Sceloporus undulatus* resulted in hatchlings (4-7 day-old) with lower baseline CORT levels, yet unaltered CORT responsiveness to challenge with ACTH (Ensminger et al., 2018). The other was the study by Robert et al., 2009 in our system, finding no effect of maternal CORT treatment on offspring baseline CORT levels measured in plasma pooled for each litter at 1 month of age. Our present results show a weak, yet detectable, influence of preparturition maternal baseline CORT—in interaction with sex—on offspring baseline CORT measured at age 3 months. The trends were for male offspring CORT to decrease and female offspring CORT to increase with increasing maternal CORT (Fig. 2A). Interestingly, maternal stress during gestation in a mammal (Brandt's vole, *Lasiopodomys brandtii*) resulted in a similar result for 3 month-old offspring CORT levels (i.e., male decrease, female increase) with respect to controls (Gu et al., 2018). This sex-specific maternal effect on offspring GC levels during early development is intriguing and deserves further study.

Offspring growth from birth to age 3 months showed a positive influence of preparturition maternal CORT irrespective of offspring sex (Fig. 2B). Although also a rather weak relationship, this finding is interesting considering that neonates were measured after 3 months of being born and had thus already been exposed to external environmental factors—e.g., feeding and thermoregulating on their own—for several months. Maternally-derived GC effects on postnatal

offspring growth have been widely studied in diverse vertebrate taxa (reviewed by MacLeod et al., 2021 a). For reptiles, positive (e.g., Dantzer et al., 2013; Dupoue et al., 2016), negative (e.g., Meylan and Clobert, 2005; Hayward et al., 2006; Gu et al., 2018 for females), or no effects (e.g., Gu et al., 2018 for males) have been documented. In our study, the small body size at birth exhibited by offspring from high-CORT mothers might be compensated by the more rapid early-life growth. Such compensatory growth has been demonstrated across vertebrates, including other garter snake species such as the congener *T. marcianus* (Holden et al., 2019 and references therein).

Parturition maternal CORT effects were no longer present (or detectable) in *T. elegans* juvenile snakes at age 12 months (Fig. 2C and D). Instead, growth from 3 to 12 months and juvenile baseline CORT levels were most influenced by thermal regimes, with offspring reared in the cool treatment showing higher CORT (Fig. 3A) and slower growth (Fig. 3B) than offspring reared in the warm treatment. Temperature is a critical determinant of physiological function in ectotherms, including reptiles (Huey, 1982; Angilletta, 2009). Temperatures above and below those in the optimal range are likely to be stressful for individuals, leading to decreased performance and potential fitness costs (Huey et al., 2012; Bodensteiner et al., 2021). Furthermore, variation in baseline CORT levels of free-ranging *T. elegans* and *T. sirtalis* is in part explained by variation in ambient temperatures, with higher temperatures associated with lower CORT (Spence et al., 2021). In our system, experimental acute heat exposure (i.e., 37-38°C for 2-3 h) results in acute CORT elevation in snakes from both ecotypes (Schwartz and Bronikowski, 2013; Gangloff et al., 2016). Our present results add to the understanding of GC responses to temperature by showing that experimentally rearing offspring with decreased

daytime opportunity for thermoregulation (8 vs 16 h daily) is associated with elevated baseline CORT in juvenile *T. elegans*, irrespective of ecotype. The faster growth of offspring in the warm compared to the cool thermal regime in these individuals (i.e., 2010 cohort), has been examined extensively in the context of food consumption, metabolic rates, and the insulin-like signaling system (Gangloff et al., 2015; Reding et al., 2016; Addis et al., 2017).

Our repeated measures of offspring at birth, age three months, and at one year provide support to the hypothesis that the strength of maternal effects is likely to decline as offspring are measured at advancing stages during ontogeny (Moore et al., 2019; MacLeod et al., 2021 a). Interestingly, baseline CORT levels of offspring at 3 months of age were similar to those measured in their mothers while pregnant (median range 90-100 ng/mL), whereas levels decreased when individuals were measured as juveniles at age 12 months (median range 60-70 ng/mL, Supplemental Fig. 4). These age-related CORT concentrations are consistent with levels recorded during ontogeny in previous cohorts of laboratory-born *T. elegans* (Robert et al., 2009; Schwartz and Bronikowski, 2012). Together, these results are in line with the idea that maternal CORT levels during gestation serve as a link between the maternal and offspring environments, potentially preparing/programming offspring to match the prevailing conditions they will be born into (Sheriff et al., 2010; Sheriff and Love, 2013). As neonates grow, they might go adjusting their developmentally-plastic GC levels based on their own perception of current environmental challenges (Sheriff et al., 2017).

4.4. Variation in preparturition maternal CORT

To complement our understanding of transgenerational CORT effects, we explored potential sources of variation in preparturition maternal CORT levels, which were not experimentally manipulated. Baseline CORT in gestating snakes varied substantially among individuals, showing repeatability when measured post-parturition in these same females (Sparkman et al., 2014, Supplementary Fig. 1). Despite this consistent phenotype, maternal CORT variation could not be attributed to any of the evaluated factors. The lack of an ecotype effect did not come as a surprise—and is consistent with the absence of ecotype (and ecotype by maternal CORT) effects on early-life traits of offspring. Although when sampled in the field M-slow snakes exhibit higher baseline CORT compared to L-fast snakes in some – although not all— years (Palacios et al., 2012; Holden et al., 2021), this ecotypic pattern does not hold in captivity (Robert et al. 2009). Furthermore, pregnant females in 2010 did not show ecotypic differences in baseline CORT when sampled in the field either (Sparkman et al., 2014). Additionally, preparturition maternal CORT was not dependent on female body size, on how far advanced in pregnancy were females when sampled, and on how many embryos they were gestating. Given that females were already pregnant when captured in the field, yet unidentified environmental stimuli experienced (or perceived as stressful) by females in their native habitats might have contributed to variation in CORT. Individual differences in response to captivity could also account for some of the observed variation, as captivity is known to elevate baseline CORT in relation to field levels (Robert et al., 2009; Sparkman et al., 2014). Indeed, embryos in our study experienced elevated maternal baseline CORT, with values being intermediate between field-measured baseline and stress-induced levels. The latter provides further context for understanding the findings of our present work, as baseline and stress-induced CORT levels have different actions and can thus result in divergent, even opposing, phenotypic outcomes in offspring (Hau et al., 2016).

4.5. Potential fitness implications and future directions

Preparturition maternal CORT levels were not predictive of the proportion of surviving embryos (reproductive success) or offspring survival to age one year. However, higher preparturition maternal CORT was associated with lower birth mass and size of offspring, parameters that have been linked to reduced survival in our laboratory colony (Bronikowski, 2000; Addis et al., 2017; Gangloff et al., 2018). Furthermore, small birth size in the laboratory led to lower neonate survival in the congener *T. sirtalis* after release into the natural habitats (Jayne and Bennett, 1990). Our laboratory colony conditions, though, were designed to evaluate the effects of the two thermal regimes while avoiding many of the potential confounding factors present in natural systems (e.g., variation in food and water availability, predation, disease risk, among others). Thus, fitness outcomes in this artificial setting might not necessarily extend to those in nature. In fact, the complexity of environmental factors in natural habitats can result in *a priori* counterintuitive fitness outcomes. For instance, offspring from CORT-treated lizards (*Zootoca vivipara*) showed decreased hatchling size, hatchling body condition, and juvenile growth; yet male (albeit not female) offspring had increased survivorship when released into the wild (Meylan and Clobert, 2005). Experimental studies under natural or semi-natural (e.g., mesocosm) conditions will be necessary to better understand the adaptive potential of phenotypic traits associated with high maternal CORT during gestation (Sheriff et al., 2017). In addition, based on recent findings of the importance that water availability has on CORT levels of *T. elegans* (Holden et al., 2021), future laboratory work would benefit from water-access manipulation (e.g., Dupoué et al., 2016).

The causal mechanisms underlying the maternal-CORT effects identified in this viviparous snake cannot be determined at present given the non-manipulative nature of our approach. Regarding the patterns on birth phenotypes, two alternative yet not mutually exclusive general mechanisms could in principle be responsible: 1) a direct effect of maternal CORT on offspring traits, via the transfer of this liposoluble hormone to the yolk during ovulation and/or through the placenta during embryo development (e.g., Hayward et al., 2006; Ensminger et al., 2018), or 2) an indirect effect of maternal CORT via modification of maternal traits known to influence offspring phenotypes —e.g., body condition, nutrient transfer to yolks/embryos, thermoregulatory behavior, or an interaction of various traits (e.g., Meylan and Clobert, 2005; Cadby et al., 2010; Ensminger et al., 2018; Liu et al., 2020). Regarding the pattern for neonate growth, higher maternal CORT in *T. elegans* neonates could have resulted in enhanced growth by increasing residual yolk mobilization/utilization or by altering offspring behaviors (e.g., thermoregulation, drinking and feeding). As proposed for another viviparous snake, the aspic viper (*Vipera aspis*; Dupoué et al., 2016). Additionally, the enhanced neonate growth could have resulted through interactions with other endocrine pathways (e.g., insulin-like growth hormones; Reding et al., 2016; Adam et al., 2017). Evaluating and distinguishing among potential mechanisms would require maternal CORT manipulation, experimental designs that control for potential confounding factors, as well as the assessment of various maternal and offspring phenotypic traits.

5. Conclusions

Our study supports an important role of CORT-mediated maternal effects on offspring phenotype in a viviparous snake, complementing and expanding our understanding of CORT physiology in

this well-studied garter snake system and in vertebrates more generally. Studies in our system are among the relatively few that have tackled the question of GC-mediated maternal effects in viviparous reptiles, and viviparous snakes in particular. Results from our non-manipulative study of maternal-GC effects on offspring generally support and complement findings from experimental studies manipulating GC exposure of embryos in various taxa. In particular, they provide evidence consistent with a decline in the strength of maternal-GC effects with advancing offspring age and for developmental plasticity/flexibility of circulating GC levels of offspring during early postnatal life. Our results also suggest a cost to offspring of elevated maternal GCs during gestation, although evaluation of fitness consequences will need study under more natural settings. Overall, our results contribute to a growing literature on transgenerational effects of hormones and help to fill gaps in our knowledge of these effects in ectothermic amniotes.

CRedit authorship contribution statement

Maria G. Palacios: Conceptualization, Methodology, Investigation, Supervision, Writing – original draft. **Anne M. Bronikowski:** Conceptualization, Methodology, Investigation, Resources, Project administration, Funding acquisition, Supervision, Writing – review & editing. **Ali Amer:** Formal analyses, Visualization. **Eric J. Gangloff:** Formal analyses, Data curation, Visualization, Writing – original draft.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Model results for reproductive success, litter sex ratio, and offspring morphometric parameters at birth for garter snakes, *Thamnophis elegans*, born from field-captured mothers under common-garden conditions. Predictor variables included preparturition maternal baseline CORT levels, ecotype (M-slow and L-fast), sex, and their interactions (removed when non-significant). Models also included the random effects of maternal ID and population within ecotype. See statistical methods for further information.

Source of Variation	Reproductive success	Sex ratio	SVL	Mass	Body Condition
	$X^2 (df)$	$X^2 (df)$	$F (df_m, df_d)$	$F (df_m, df_d)$	$F (df_m, df_d)$
Maternal CORT					
Estimate (\pm SE)	0.62 \pm 0.59	0.00 ^c \pm 0.51	-14.70 \pm 7.63	-0.58 \pm 0.43	-0.0005 \pm 0.026
Test statistic	1.11 (1)	0.0003 (1)	3.48 (1, 35.2)	1.64 (1, 35.1)	0.0004 (1, 35.2)
P-value	0.292	0.985	0.070 ^a	0.209 ^a	0.985
Ecotype					
Estimate (\pm SE)	-1.33 \pm 0.83	0.19 \pm 0.29	-11.62 \pm 4.21	-0.32 \pm 0.27	0.030 \pm 0.013
Test statistic	2.60 (1)	0.44 (1)	6.99 (1, 4.3)	1.32 (1, 4.5)	4.68 (1, 4.0)
P-value	0.107	0.507	0.053	0.307	0.096
Sex					
			M > F		F > M
Estimate (\pm SE)	NA	NA	4.09 \pm 1.16	0.045 \pm 0.55	-0.019 \pm 0.0048
Test statistic	NA	NA	11.99 (1, 145.8)	0.60 (1, 143.7)	15.1 (1, 149.1)

P-value	NA	NA	0.0007	0.440	0.0002
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Abbreviations- CORT: corticosterone, SVL: snout-to-vent length, F: female, M: male, NA: not applicable.

^a These effects are significant after removal of litter RP33, see text and Fig. 1 for details

Table 2. Model results for offspring baseline CORT and growth (specific growth rate for body size) measured as neonates (3 months) and juveniles (12 months) and survival between 3 and 12 months for garter snakes, *Thamnophis elegans*, born from field-captured mothers. Predictor variables included preparturition maternal baseline CORT levels, ecotype (M-slow and L-fast), thermal treatment under which offspring were raised (cool and warm), sex, and their interactions (only shown when significant). Models also included the random effects of maternal ID and population within ecotype. See statistical methods for further information.

Source of Variation	Neonate		Juvenile		Survival
	CORT	Growth	CORT	Growth	3-12
	3 months	birth-3 months	3-12 months	3-12 months	months
	$F(df_m, df_d)$	$F(df_m, df_d)$	$F(df_m, df_d)$	$F(df_m, df_d)$	$X^2(df)$
Maternal CORT		positive			
Estimate (\pm SE)	0.093 \pm 0.19	0.679 \pm 0.012	0.16 \pm 0.11	0.024 \pm 0.017	0.99 \pm 0.97
Test statistic	0.18 (1, 34.5)	5.03 (1, 34.1)	1.87 (1, 29.9)	1.65 (1, 33.2)	1.04 (1)
P-value	0.672	0.031	0.181	0.208	0.307
Ecotype					
Estimate (\pm SE)					-2.84 \pm
	0.055 \pm 0.049	-0.020 \pm 0.012	-0.069 \pm 0.055	-0.017 \pm 0.010	0.97
Test statistic	1.16 (1, 4.52)	1.11 (1, 4.89)	1.37 (1, 4.43)	2.51 (1, 4.67)	8.58 (1)
P-value	0.336	0.342	0.301	0.178	0.003
Treatment			Cool > Warm	Warm > Cool	

Estimate (\pm SE)					-0.54 \pm
	0.025 \pm 0.028	-0.0003 \pm 0.0035	0.095 \pm 0.042	-0.035 \pm 0.005	0.43
Test statistic	0.84 (1, 138.7)	0.005 (1, 139.6)	5.36 (1, 117.2)	49.70 (1, 115.9)	1.59 (1)
P-value	0.362	0.942	0.022	< 0.0001	0.207
Sex			F > M		
Estimate (\pm SE)				-0.0079 \pm	-1.52 \pm
	0.47 \pm 0.23	-0.012 \pm 0.0046	-0.10 \pm 0.042	0.0052	0.70
Test statistic	4.03 (1, 155.1)	0.86 (1, 165.9)	5.55 (1, 132.8)	2.38 (1, 129.6)	4.68 (1)
P-value	0.047	0.355	0.020	0.125	0.030
Maternal CORT \times					
Sex	see Fig. 2A				
Estimate (\pm SE)	-0.26 \pm 0.12				
Test statistic	4.80 (1, 155.1)				
P-value	0.030				
Ecotype \times Sex		see text and Suppl. Fig. 3			see text
Estimate (\pm SE)		0.016 \pm 0.0079			2.61 \pm 0.95
Test statistic		4.13 (1, 165.7)			7.52 (1)

P-value	0.044	0.006
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Abbreviations- CORT: corticosterone, F: female, M: male.

Figure Captions

Figure 1. Scatterplots of offspring birth phenotypes as a function of preparturition maternal baseline CORT in *Thamnophis elegans* born under common-garden conditions from field-captured mothers: (A) snout-vent length, (B) mass, (C) body condition. Depicted are raw data points. Regression line and standard error of estimate are shown for cases where maternal corticosterone predicts offspring phenotypes after the exclusion of a single litter with five offspring (circled in red; see text for details).

Figure 2. Scatterplots of neonate (3 month old) and juvenile (12 month old) baseline CORT and specific growth rate as a function of preparturition maternal baseline CORT in *Thamnophis elegans* born under common-garden conditions from field-captured mothers: (A) offspring CORT at age 3 months, (B) offspring growth rate from birth to age 3 months, (C) offspring CORT at age 12 months, (D) offspring growth rate from 3 to 12 months. Depicted are raw data points. Regression line and standard error of estimate are shown where maternal corticosterone predicts offspring phenotypes (panel B) and to illustrate interaction with offspring sex (panel A).

Figure 3. Boxplots of juvenile baseline CORT and specific growth rate by postnatal thermal treatment and sex in *Thamnophis elegans* born under common-garden conditions from field-captured mothers: (A) offspring CORT at age 12 months, (B) offspring growth rate from age 3 to age 12 months. Tukey boxplots show median, interquartile range, and limits of values within 1.5 times the interquartile range.

Graphical abstract

Journal Pre-proof

Highlights

- Pregnant snakes with higher circulating CORT gave birth to smaller, lighter offspring
- Neonates of mothers with higher circulating CORT grew faster to age three months
- Neonate CORT varied with maternal CORT in a sex-specific manner
- Maternal CORT effects on offspring were not detectable by age one year
- After one year, juvenile phenotype was most influenced by temperature and sex

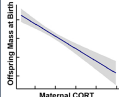
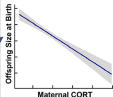
Q1: Does maternal CORT affect birth phenotypes?

Q2: How do these effects translate across early life ontogeny?

Q3: Are the effects sex or ecotype-specific?



Birth



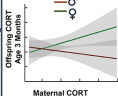
Moms with higher CORT gave birth to smaller and lighter offspring



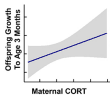
Effects of maternal CORT no longer evident
Temperature & sex became more important influences

12 months

Sex-specific effect of mom CORT on offspring CORT



Offspring from high CORT moms grew faster



3 months



Graphics Abstract

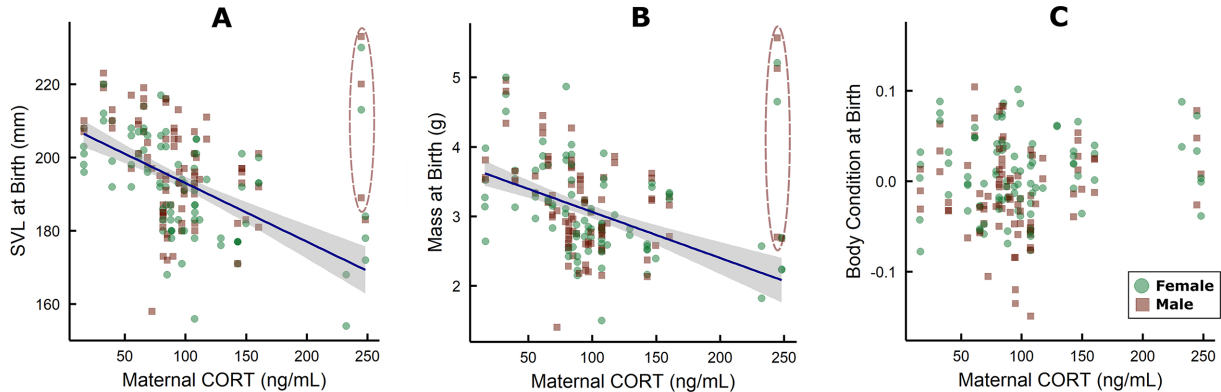


Figure 1

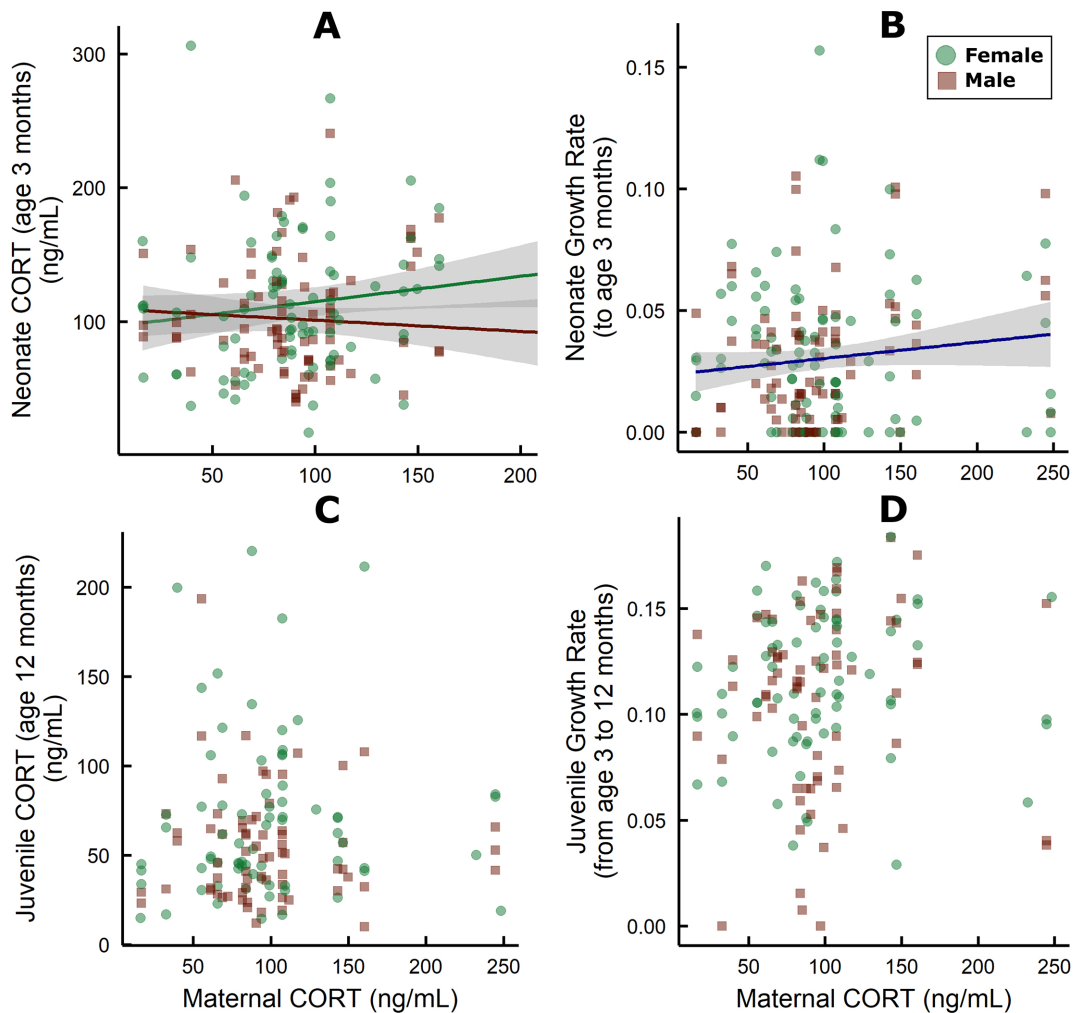


Figure 2

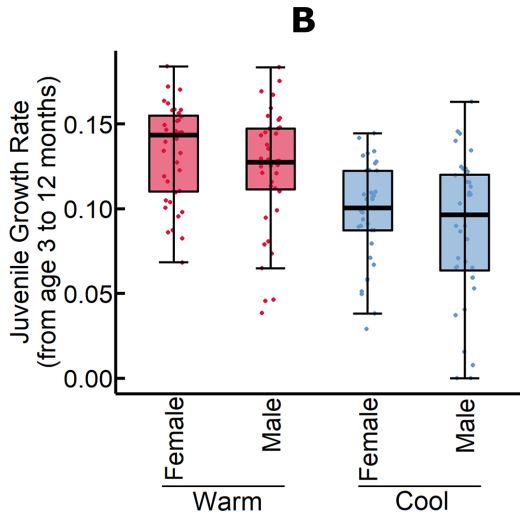
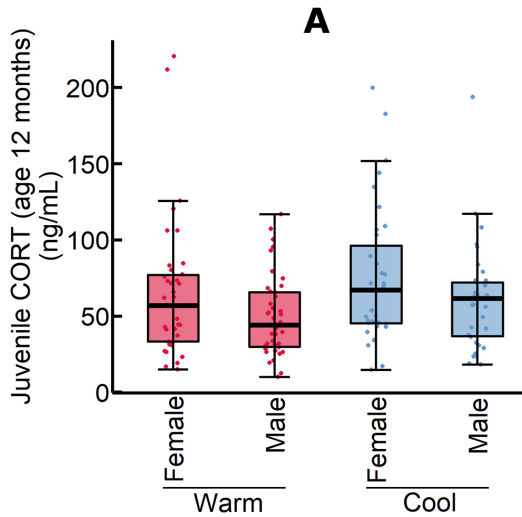


Figure 3