



Sex steroids in green anoles (*Anolis carolinensis*): uncoupled maternal plasma and yolking follicle concentrations, potential embryonic steroidogenesis, and evolutionary implications

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Abstract

The sex steroids testosterone (T) and estradiol-17 β (E2) play important roles in vertebrate reproduction and development. However, little is known about the relationship between plasma steroid levels (which can influence reproductive function) and yolk steroid levels (which can influence embryonic development) in oviparous species. Therefore, we examined the extent to which T and E2 are coupled in plasma and yolking follicles in adult females and explored the dynamics of yolk and embryo steroid content during egg incubation in green anoles (*Anolis carolinensis*). T and E2 levels were determined for the plasma and yolking follicles of breeding females and for whole embryos and yolks at several developmental stages by radioimmunoassay. Plasma and yolk concentrations of T and E2 were not correlated. On average, plasma T was only 30% that of plasma E2, but yolking follicle T was over 600% that of yolking follicle E2. Total yolk T and E2 content generally declined over the course of incubation. However, yolk T was an order of magnitude higher than yolk E2, and it showed a secondary peak in magnitude after approximately 75% of incubation was completed. Similarly, total embryonic T content rose by over 400% in the latter half of incubation whereas E2 did not change. These results demonstrate that plasma and yolking follicle steroid levels produced by breeding females can be uncoupled. Furthermore, embryos themselves may begin producing T, but likely not E2, during the latter stages of incubation. Thus, steroid exposure may be independently shaped by selection to serve both reproductive and developmental functions.
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1. Introduction

The issue of whether maternal steroids are transferred to embryos via egg yolk has been of research interest for some time, but early results were conflicting. For example, Altmann and Hutt (1938) found that injection of yolk into juvenile female chickens (*Gallus domesticus*) produced a rise in blood calcium levels equivalent to injection of estrogen, which led them to infer the presence of estrogen in the yolk. In contrast, Hertelendy and Common (1965) did not detect endogenous estrogens in

chicken yolk using thin layer chromatography, although Arcos (1972) demonstrated that radioactively-labeled sex steroids (or their metabolites) injected intravenously into laying hens crossed into their yolks. Following technical advances in steroid radioimmunoassays (e.g., Abraham et al., 1972; Wingfield and Farner, 1975), Schwabl (1993) demonstrated the presence of egg yolk steroids in canaries (*Serinus canaria*) and zebra finches (*Taeniopygia guttata*). It is now clear that steroids are a common yolk constituent not only in birds (including chickens; e.g., Elf and Fivizzani, 2002), but also in other vertebrates including fishes (e.g., *Pomacentrus amboinensis*; McCormick, 1998), turtles (e.g., *Chrysemys picta*, *Trachemys scripta*; Bowden et al., 2001), alligators (*Alligator mississippiensis*; Conley et al., 1997), and lizards (e.g., *Urosaurus ornatus*; Jennings et al., 2001).

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Although the widespread presence of steroids in yolk is clear, why they are there and what effect (if any) they have on maternal fitness remain open questions. There are at least two explanations for this phenomenon. First, females may allocate steroids to yolk as an adaptive maternal response for influencing offspring development. For example, experimentally elevated yolk androgen levels enhance nestling growth and/or survival in some avian species (Eising et al., 2001; Lipar and Ketterson, 2000; Schwabl, 1996a), but decrease it in others (Sockman and Schwabl, 2000). Such effects may be related to maternal efforts to either minimize or enhance sibling competition, depending upon the species and ecological conditions in question (Schwabl et al., 1997). Second—and alternatively—steroids may be present in yolk simply as a byproduct of the hormonal regulation of maternal physiology, due to passive uptake during yolk formation. The role that steroids play in regulating reproductive function in female vertebrates is well-established (e.g., McCarthy and Becker, 2002; Whittier and Tokarz, 1992). This issue of passive uptake versus active regulation is critical when considering the potential selective pressures influencing patterns of yolk steroid allocation and the resulting responses that have evolved for a given species (Winkler, 1993).

In the present study we used the green anole, *Anolis carolinensis*, to examine the relationship between steroid levels in female plasma and yolking follicles, as well as in egg yolk and embryonic tissue during incubation. These lizards are seasonal breeders, reproducing during an approximately 4-month long breeding season (April–July; Jenssen et al., 2001). Females alternately produce single egg clutches from their left and right ovaries at approximately 7–14 day intervals (Andrews, 1985), and sex of the offspring is determined by genotype, not temperature (Viets et al., 1994). Previous research has documented that female plasma testosterone (T) and estradiol-17 β (E2) vary across the ovulatory cycle (Crews, 1980; Jones et al., 1983; Lovern and Wade, 2001), and that these steroids play an important role in regulating female receptivity (Adkins and Schlesinger, 1979; Winkler and Wade, 1998). Females also deposit T into their yolking follicles, and this yolk T remains detectable throughout incubation (Lovern and Wade, 2001). Until the present study, whether E2 is also deposited in the yolk had not been investigated.

Specifically, in this study we: (1) examine the relationships between T and E2 concentrations simultaneously in the plasma and yolking follicles of adult females; and (2) explore the dynamics of egg yolk and embryo T and E2 content during incubation. Such information should yield insight into whether steroid transfer from mother to embryo is likely to be passive or active, and whether embryos can directly contribute to their steroid environment. If mothers have the ability to actively regulate yolk steroid deposition, then steroid

profiles in maternal circulation and those found in yolking follicles need not be associated, and therefore may be independently selected for their roles in maternal physiology and offspring development. Furthermore, if yolk and embryo steroid content change during incubation, when eggs have no further maternal contact, then an examination of the dynamics of yolk and embryo T and E2 content will yield insight into potential steroid production or metabolism by the embryo.

2. Materials and methods

2.1. Animals and housing

Adult (≥ 50 mm snout-vent length) male and female *A. carolinensis* were purchased during the breeding season from Charles Sullivan Co. (Nashville, TN) and kept in the laboratory for a minimum of two weeks prior to use. Lizards were housed in groups consisting of one male and 3–7 females in 110 L aquaria furnished with a peat moss substrate and multiple dowels and rock formations for perching, basking, and hiding. Each cage also contained a 0.5 L plastic nesting box filled with moistened peat moss. Environmental conditions were set to mimic those experienced in the field during the breeding season. We kept lizards on a 14:10 h light:dark cycle using a combination of fluorescent, ultraviolet, and incandescent lights. Ambient temperature ranged from 18 °C at night to a gradient of 28–38 °C during the day depending on location within each cage relative to a basking light. Relative humidity was kept at 70%. Lizards had access to water in shallow dishes ad libitum and their cages were misted daily. We fed lizards three times per week with mealworms or vitamin-dusted crickets. Lizards remained reproductively active for the duration of this study, as evidenced by the observation of courtship behavior, copulation, and egg production within the colony. The females taken from the colony and used in the study all had active, yolking follicles (see below).

2.2. Female plasma and yolking follicle collection

A subset of females from our breeding colony ($n = 11$) was sacrificed to collect both blood and yolk samples for determination of T and E2 concentrations. Blood samples were collected from the trunk following decapitation. Plasma fractions obtained following centrifugation were measured to the nearest μ L and individually stored at -80 °C until analysis. Immediately following blood sampling, the actively yolking follicle (the largest one; Crews, 1980; Jones et al., 1983) was removed from each female, weighed to the nearest mg, and stored at -80 °C until analysis. Sampled follicles spanned the range of female yolk deposition, from just

beginning to accumulate yolk (under 35 mg total mass, 3.5 mm diameter) to just prior to ovulation (over 125 mg total mass, 6.0 mm diameter) (Crews, 1980; Jones et al., 1983). Thus, our results are not biased by stage in the ovarian cycle.

2.3. Yolk and embryo collection during incubation

Eggs were collected from our breeding colony within 24 h of oviposition and placed individually in 266 mL plastic cups in a vermiculite:dH₂O mixture (1:1 m:v). Each cup was sealed with plastic wrap and a rubber band and placed in an incubator that averaged 28 °C (range 27–29 °C). We randomly assigned eggs to be sampled at 8, 16, 24, or 32 days of incubation ($n = 8$ per group). Because hatching under these conditions typically occurs at 34 days, sampling occurred after approximately 25, 50, 75, and 100% of embryonic development had been completed. On the appropriate sampling day, eggshells were cut open and the yolk and embryo were gently teased apart. The embryo was weighed to the nearest mg, decapitated, and immediately frozen in dry ice to prevent potential enzymatic breakdown of steroids. Then the yolk was weighed to the nearest mg and both the yolk and embryo were transferred to –80 °C until analysis. Embryo samples from day 32 of incubation could be sexed by external morphology (post-anal scale size). However, sample sizes were small (four of each sex), showed no statistical differences in mass or steroid content, and were therefore pooled for analyses.

2.4. Radioimmunoassays

Plasma, yolk, and embryo levels of T and E2 were measured by radioimmunoassay (RIA) following extraction and chromatographic separation (Painter et al., 2002; Schwabl, 1993; Wingfield and Farner, 1975). Plasma samples were thawed and mixed with 0.5 mL dH₂O to provide sufficient volume for extraction. Yolk samples were thawed, homogenized (to circumvent any possible hormonal layering effects; Lipar et al., 1999), and 5–30 mg was mixed with 1.0 mL dH₂O for extraction. Whole embryos were placed on ice and homogenized in 1.5 mL 0.1 M phosphate buffered saline (PBS). All samples were equilibrated overnight at 4 °C with 1000 cpm of ³H-T (NET-370, 95 Ci/mmol) and 1000 cpm of ³H-E2 (NET-317, 90 Ci/mmol) from NEN Life Science Products for individual recovery determinations.

Extraction procedures varied depending upon the sample tissue. Plasma samples were extracted twice with 2 mL diethyl ether, dried with nitrogen gas, and reconstituted in 500 µL of 10% ethyl acetate in isooctane. Yolk samples were extracted twice with 3 mL petroleum ether:diethyl ether (30:70 v:v), dried with nitrogen gas, and reconstituted in 1 mL 90% ethanol. The extracted

samples were stored at –20 °C overnight, then centrifuged at 2000 rpm at 0 °C for 5 min to precipitate neutral lipids and any proteins that were extracted by the ether. The supernatant was dried and reconstituted in 500 µL of 10% ethyl acetate in isooctane. Embryonic tissue homogenates were kept on ice during extractions. They were extracted three times with 5 mL diethyl ether and centrifuged after each extraction at 3000 rpm at 4 °C for 10 min. The ether extracts were combined, dried under nitrogen gas, reconstituted in 1 mL 90% ethanol, and stored overnight at –20 °C. The following day, samples were centrifuged at 2000 rpm at 0 °C for 10 min, and the supernatant was further purified of neutral lipids with a 2 mL hexane wash. We then washed the hexane with 1 mL 90% ethanol to recover any steroids that may have come out with the hexane, and the ethanol fractions were combined, dried under nitrogen gas, and reconstituted in 500 µL of 10% ethyl acetate in isooctane.

To remove additional neutral lipids and to isolate T and E2, all samples were transferred to diatomaceous earth (Celite, Sigma) columns for chromatographic separation. Columns consisted of a Celite:ethylene glycol:propylene glycol upper phase (6:1.5:1.5 m:v:v) and a Celite:dH₂O (3:1 m:v) lower phase. Neutral lipids and dihydrotestosterone were eluted with 1.5 mL isooctane and 1.5 mL 10% ethyl acetate in isooctane, respectively, and discarded. T and E2 were eluted with 2 mL of 20% and 2.5 mL of 40% ethyl acetate in isooctane, respectively, and saved. Samples were dried under nitrogen gas, resuspended in PBS, and placed overnight at 4 °C.

Competitive binding RIAs were performed using the appropriate tritiated steroid tracer (see above) and antisera from Wien Laboratories for T (T-3003) and Biogenesis for E2 (7010-2650; formerly known as 1702 from Arnel). The standard curves for both hormones ranged from 0.5 to 125 pg and were run in triplicate. Samples were run in duplicate, averaged, and adjusted for individual recovery and initial sample volume (plasma) or mass (yolk, embryo). The female plasma and yolking follicle samples were run in one assay (intra-assay coefficient of variation, CV, was 3.8% for T and 5.9% for E2). The yolk and embryo samples from incubating eggs were randomized across sampling stage in two assays (intra-assay CV's were 10.8 and 9.4% for the T assays and 11.1 and 3.4% for E2; inter-assay CV's were 7.6% for T and 11.2% for E2).

2.5. Statistics

We used nonparametric statistics followed by rank-based post hoc comparisons (Hollander and Wolfe, 1973) and sequential Bonferroni corrections (Rice, 1989) when appropriate, because most of the data sets were not normally distributed and could not be transformed to produce normal distributions (Kolmogorov–Smirnov tests; $p < 0.01$). Descriptive statistics are presented as

means \pm SE, and the significance level required to reject the null hypothesis was set at an overall $\alpha = 0.05$. The T data from egg yolk and embryonic tissue during incubation contained one outlier in each age class (>2 standard deviations higher than the mean T level); these data points were winsorized (Sokal and Rohlf, 1995; p. 407) for presentation in Figs. 2 and 3. Because statistical analyses were rank-based, our treatment of outliers did not affect their outcome.

3. Results

3.1. T and E2 in female plasma and yolking follicles

Reproductively active females had detectable T and E2 circulating in their plasma and deposited into their yolking follicles (Fig. 1). Plasma E2 concentration was significantly higher than plasma T concentration (Wilcoxon matched pairs; $W = 4$, $p = 0.011$). In contrast, yolk T concentration was significantly higher than yolk E2 concentration ($W = 50$, $p = 0.037$). Nine of 11 females had higher plasma E2 than plasma T concentrations ($\chi^2 = 4.45$, $p = 0.035$), whereas 8 of 11 follicles from these same females had higher yolk T than yolk E2 concentrations ($\chi^2 = 2.27$, $p = 0.13$). Spearman rank correlation tests indicated no significant correlations between plasma and yolk T and E2 (all $p > 0.10$).

3.2. T and E2 in yolk and embryonic tissue during incubation

Yolk mass significantly decreased during incubation (Kruskal–Wallis test; $H = 20.3$, $p < 0.001$; Fig. 2A), as did total T and E2 content (T: $H = 18.8$, $p < 0.001$; E2: $H = 15.1$, $p = 0.002$; Fig. 2B). Overall, yolk T content was an order of magnitude higher than yolk E2 content (113 ± 40.8 vs. 11.6 ± 2.4 pg). Considering individual samples, yolk T was higher than yolk E2 in 30 of 32 eggs ($\chi^2 = 24.5$, $p < 0.001$). Furthermore, whereas E2 con-

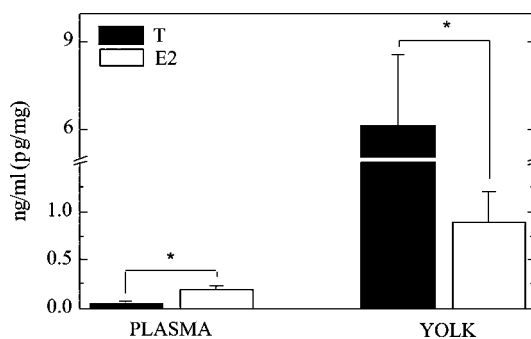


Fig. 1. Testosterone (T) and estradiol-17 β (E2) concentrations in the plasma (ng/ml) and yolking follicles (pg/mg) of reproductively active female green anoles ($n = 11$). * $p < 0.05$ following Wilcoxon matched pairs tests.

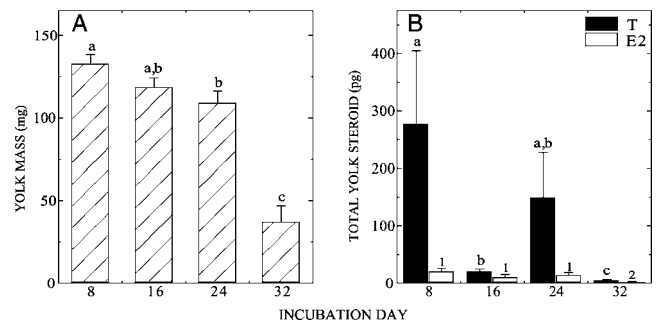


Fig. 2. (A) Yolk mass and (B) yolk testosterone (T) and estradiol-17 β (E2) content during incubation of green anole eggs. Sampling occurred on day 8, 16, 24, or 32 after oviposition, or after approximately 25, 50, 75, and 100% of incubation had been completed (hatching occurs around day 34). Bars with different letters (yolk mass, yolk T) or numbers (yolk E2) are significantly different following Kruskal–Wallis tests and subsequent rank-based multiple comparisons.

centration in the yolk showed a steady (albeit low magnitude) decline in incubation, T content was extremely dynamic. Yolk T sharply declined between days 8 and 16, sharply increased between days 16 and 24, and once again sharply declined between days 24 and 32 (Fig. 2B).

Embryo mass significantly increased during incubation ($H = 28.8$, $p < 0.001$; Fig. 3A). Similarly, embryonic T content remained low during the first half of incubation at days 8 and 16 and significantly increased during the latter half of incubation at days 24 and 32 ($H = 15.1$, $p = 0.002$; Fig. 3B); embryonic E2 content did not change ($H = 3.1$, $p = 0.38$; Fig. 3B). At days 8 and 16, roughly half (56%) of embryos had higher T than E2 content ($\chi^2 = 0.25$, $p = 0.62$). However, by days 24 and 32, 88% of embryos contained more T than E2 ($\chi^2 = 9.0$, $p = 0.003$).

4. Discussion

Our results suggest two main points. First, T and E2 concentrations in the plasma of reproductively active

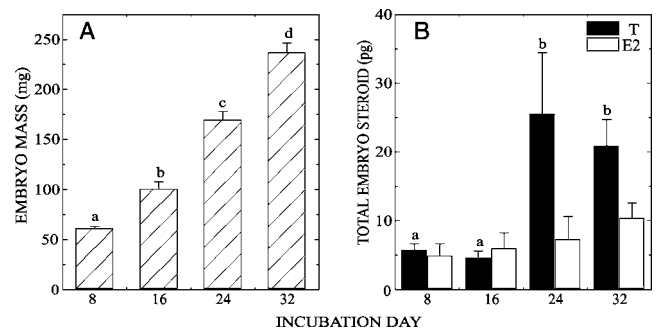


Fig. 3. (A) Embryo mass and (B) embryo testosterone (T) and estradiol-17 β (E2) content during incubation of green anole eggs. See Fig. 2 for sampling details. Bars with different letters are significantly different following Kruskal–Wallis tests and subsequent rank-based multiple comparisons.

female green anoles do not necessarily mirror the T and E2 concentrations found at the same time in their yolking follicles. We found that T and E2 actually showed opposite patterns in the plasma and yolk. Plasma T concentration was only 30% that of plasma E2, but yolking follicle T concentration was over 600% that of yolking follicle E2. Furthermore, plasma and yolk steroid levels were not correlated for either T or E2. Second, although the E2 content in egg yolks and embryos was relatively low and stable throughout incubation, the T content was much higher and dynamic. We found that egg yolk T showed two obvious peaks—one at day 8 of incubation and a secondary one at day 24 of incubation. Embryonic T remained low at days 8 and 16 of incubation, but it increased by over 400% at days 24 and 32. These data suggest that plasma and yolking follicle steroid levels produced by breeding females can be uncoupled. They also are consistent with the idea that maternally derived yolk T is metabolized early in incubation, but that it starts being produced by the embryos around day 24, some of which diffuses into the yolk thus explaining the secondary peak in yolk T content.

Hormone levels in female plasma and follicles have also been measured in other species, and often these levels are related. For example, Schwabl (1996b) found that maternal plasma T and the yolk T levels of her eggs are positively correlated in canaries. Similarly, Adkins-Regan et al. (1995) found that E2 levels in the plasma and yolk of quail (*Coturnix coturnix japonica*) are positively related, both naturally and when artificially elevated via maternal implants. T levels in plasma and yolking follicles can be elevated via maternal implants in turtles as well (Janzen et al., 2002). These results demonstrate that within individual females steroid levels found in plasma and yolk tend to covary, but they do not necessarily demonstrate that such covariance is unregulated. Several studies have suggested that there can be an active, “buffering” effect whereby hormone concentrations found in plasma and yolk, although sometimes positively related, may be one or more orders of magnitude different (e.g., Painter et al., 2002; Wilson and McNabb, 1997). More research is necessary to determine how such regulation could occur, but different lipid profiles between plasma and yolk coupled with the lipophilic nature of sex steroids, or differences in the distribution of steroid-binding proteins and in their affinities for androgens and estrogens, could possibly play important roles in producing tissue-specific steroid levels (e.g., Jennings et al., 2000).

Our results suggest that green anole embryos likely start producing T sometime after 50% of incubation has been completed. It will be necessary to conduct assays investigating steroid metabolism directly before we can conclude that embryonic tissue is steroidogenic, but we have shown that T increases in both yolk and embryo during incubation. Several additional pieces of evidence

support the possibility that such dynamics are due to embryo steroidogenesis. First, increases late in incubation in yolk E2 have been reported in chickens (Elf and Fivizzani, 2002) and in yolk corticosterone in tree lizards (*Urosaurus ornatus*; Jennings et al., 2001); the authors in each of these studies interpret this as evidence for embryo steroidogenesis. Similarly, in a previous study we found that the concentration of yolk T increases in green anole eggs during incubation (Lovern and Wade, 2001). At the time we were not able to rule out the possibility that T concentration was increasing simply due to yolk absorption, because we collected yolk biopsies rather than whole yolks. However, the consistency in the pattern of results in the two studies provides support for the idea that the total yolk T dynamics demonstrated in the present study are the result of steroid production by embryos. Second, embryo steroid production is known to occur in both avian and reptilian taxa (e.g., Ottinger et al., 2001; White and Thomas, 1992), although it has not yet been specifically examined in *A. carolinensis*. Third, the rise in embryonic T that we observed occurred around the time that the gonads would have been differentiating (Austin, 1988; Forbes, 1956), suggesting a possible functional relationship between gonadal development and steroid production.

Previous research with green anoles has demonstrated the importance of T in regulating female reproductive behavior. Plasma T levels are higher in the breeding than nonbreeding season, and within the breeding season they are highest around the time of ovulation when females are likely to be most behaviorally receptive to male courtship (Lovern et al., 2001; Lovern and Wade, 2001). While plasma E2 levels play the primary role in activating receptive behavior (Crews, 1980; Jones et al., 1983), circulating T facilitates receptivity via local aromatization to E2 (Winkler and Wade, 1998).

Steroids likely influence embryonic development in green anoles as well, although there is still much research to be completed. For example, maternally derived yolk T levels are higher in recently ovulated eggs containing male embryos than in those containing female embryos, but this sex difference disappears during incubation (Lovern and Wade, 2001, 2003). It is hypothesized that this sex difference is observed because eggs containing relatively high T content are more often fertilized by y-bearing sperm (producing males) and those with relatively low T content are more often fertilized by x-bearing sperm (producing females) (Lovern and Wade, 2003). Such an effect could occur, for example, if different steroid profiles lead to changes in the female reproductive tract, which in turn differentially affect the ability of x- and y-bearing sperm to reach and fertilize the egg (reviewed in James, 1996; Krackow, 1995). Other influences of maternally derived T are possible as well (e.g., effects on survival, somatic growth, or phenotype differentiation; see Section 1), particularly

given that yolk T allocation can vary tremendously among females (Lovern and Wade, 2001). As incubation progresses, embryos likely become steroidogenic, which could influence subsequent development. For example, both male and female lizards develop hemipenes (bilateral penises), which females lose prior to hatching (Dufaure and Hubert, 1961). The sexual differentiation of these structures occurs during the latter half of incubation, when we saw dynamics in both yolk and embryo T content in the present study. If there is a male-biased sex difference in T production by embryos, then T may be responsible for the development of hemipenes and associated structures in males and the relative lack of T may result in their regression in females. Although we did not see a sex difference in yolk or embryo T content in the present study in day 32 embryos (the only class for which we knew embryo sex), examination of a larger sample, particularly during the later stages of incubation, is necessary.

Yolk steroid levels have now been described in numerous taxa, making it possible to look for general patterns shared across species. Perhaps the most striking pattern to emerge so far is the variation in the T:E2 ratio observed across taxa. The yolk T:E2 ratio tends to be >1 in bird and lizard eggs (e.g., Lipar et al., 1999; Schwabl, 1993; present study), but <1 in alligator and turtle eggs (e.g., Bowden et al., 2001; Conley et al., 1997; Elf et al., 2002). These trends may be indicative of functional differences in steroid roles that have evolved in different clades. For example, one exciting possibility is that the T:E2 ratio relates to the mode of sex determination. Birds and most lizards have genotypic sex determination, and the T:E2 ratio (>1) as well as experimental manipulations of steroid levels in these species suggest that T may be more effective than E2 in affecting early development (e.g., Lipar and Ketterson, 2000; Schwabl, 1996a; but see Adkins-Regan et al., 1995). In contrast, sex is determined in alligators and most turtles by the incubation temperature experienced by embryos, and the T:E2 ratio (<1) as well as experimental manipulations of yolk E2 suggest that this steroid plays an important role in the sex-determining process (e.g., Bowden et al., 2000; Wibbels et al., 1994). Alternatively, steroid ratios may be more closely tied to phylogenetic history. An interesting way to address this question is to examine T:E2 ratios in closely related species that possess different sex-determining mechanisms. Research thus far suggests that sex-determining mode may in fact be more explanatory than phylogeny. Different turtle species show wide variation in yolk steroids, with the largest degree of variation occurring across species with different sex-determining modes (Janzen et al., 1998).

In this study, we have documented that plasma and yolk follicle T and E2 levels can vary independently in breeding female green anoles, and that changes in

yolk and embryo steroid content during incubation likely reflect differences in embryonic steroid production. We believe these results demonstrate that maternal and embryonic steroid exposure may be independently shaped by selection to serve both reproductive and developmental functions, as discussed above. This does not imply that the pattern we have documented is static; it is quite possible that at different times of the year, or under different environmental conditions, relationships among plasma, yolk, and embryo steroid content could vary. Fascinating studies from numerous species demonstrate multiple levels of influence on maternal yolk steroid allocation and embryonic development, including seasonal effects (e.g., Bowden et al., 2000), social effects (e.g., Müller et al., 2002; Whittingham and Schwabl, 2002), and abiotic effects (e.g., environmental contaminants; Whittingham and Crews, 2000). Indeed, the possibility for such influences is what makes this area of research so compelling, ultimately linking maternal and developmental conditions—and all of the factors which influence them—to offspring phenotypic differentiation.

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