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Maternal Plasma and Egg Yolk Testosterone Concentrations during Embryonic Development in Green Anoles (*Anolis carolinensis*)

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Sex steroids of presumably maternal origin have been found in avian, crocodilian, and chelonian egg yolks, and they can affect offspring morphology and behavior. The present study reports testosterone (T) levels to which embryos are potentially exposed in the green anole (*Anolis carolinensis*), a lizard with genotypic sex determination. We documented plasma and yolk T concentrations in adult females, in their developing follicles and eggs, and in freshly oviposited and incubating eggs. Plasma T was higher in reproductively active than in reproductively inactive females. Within reproductively active females, those with a single, large yolking follicle had higher plasma T than those that had one or more shelling, oviductal eggs. Individual females contributed different amounts of T to their yolks, but within females, more mature follicles or eggs consistently had higher yolk T concentrations than did less mature follicles or eggs. Similar to previous research, yolk T concentrations at oviposition were higher in male eggs than in female eggs. However, T levels during incubation did not differ by embryo sex, but rather increased in both male and female eggs. These results suggest that T plays a role in the reproductive physiology of females and potentially in the phenotypic development of their offspring. Furthermore, whereas the yolk T increase observed during follicular maturation is clearly a maternal influence, it remains unclear whether that observed during egg development (i.e., postfertilization) results from a lack of T uptake by the embryo as yolk is absorbed, from embryonic production of T that diffuses into the yolk, or from some combination of these processes. Because lizard embryos are comparatively well developed at oviposition, the assumption that yolk steroids are strictly of maternal origin may require modification, and the possibility that embryos are modulating their own steroid environment needs to be explored. © 2001 Academic Press

Key Words: **lizard; *Anolis*; maternal effects; embryonic development; testosterone.**

INTRODUCTION

The embryos of oviparous species have a prolonged period of development that occurs outside of their mothers. Consequently, it has often been assumed that epigenetic maternal effects on offspring phenotype are minimal or nonexistent compared to the potential for such effects in viviparous species. Accumulating endocrinological research has challenged that assumption, however. Experimentally elevated plasma concentrations of maternal steroids can be transferred to yolk (Arcos, 1972; Adkins-Regan *et al.,* 1995), and naturally occurring yolk steroids have been documented in birds (Schwabl, 1993; Lipar *et al.,* 1999; Sockman and Schwabl, 2000), turtles (Janzen *et al.,* 1998; Bowden *et al.,* 2001), and alligators (Conley *et al.,* 1997).

The deposition of sex steroids into yolk is an exciting discovery because of the implications for func-

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tional consequences to the developing embryo. Indeed, a link between maternal steroid deposition and offspring phenotype appears likely. Schwabl (1993) found that the social rank of juvenile canaries was positively correlated with the concentration of yolk testosterone (T) in the eggs from which they hatched. In zebra finches, females may deposit higher concentrations of androgens into their eggs when mated with more attractive males than when mated with less attractive males, thereby potentially affecting offspring growth and survival or future reproductive success via positive effects of T exposure on secondary sexual trait expression (Gil *et al.,* 1999). Furthermore, yolk estradiol concentrations vary across the reproductive season and positively correlate with a female-biased sex ratio in painted turtles (Bowden *et al.,* 2000, 2001), a species in which incubation temperature and steroids interact to determine offspring sex (e.g., Crews, 1996).

Experimental manipulations of the hormonal environment of developing embryos give further support for the idea that mothers could affect offspring phenotypes by differential steroid deposition. For example, canaries hatching from eggs injected with T begged more for food and grew faster than controls not given T injections (Schwabl, 1996a). Similarly, red-winged blackbird chicks that had received T injections in their eggs had heavier complexi (muscles that facilitate hatching and begging) than controls (Lipar and Ketterson, 2000). Finally, androgens injected into the yolks of American kestrel eggs caused delayed hatching and reduced chick growth and survival (Sockman and Schwabl, 2000). Thus, sex steroids are apparently a common component of egg yolk, and, across species, they appear to have the potential to positively or negatively affect offspring phenotype.

We are interested in how exposure to T during development affects sex differences in morphology and behavior in the green anole, *Anolis carolinensis,* a lizard with genotypic sex determination (Viets *et al.,* 1994). Previous research has documented that T is present in freshly oviposited eggs in *A. carolinensis* and that the yolk T concentration of eggs giving rise to males may be nearly twice that of eggs giving rise to females (Lovern *et al.,* 2001). The latter result must be replicated, but if true, suggests a surprising potential maternal contribution to sex differences in the early developmental environment, the consequences of which would need further exploration. Alternatively, it is possible that the embryos themselves modify their hormonal environments. Authors usually assume that yolk steroid levels result from a maternal contribution rather than from embryonic production (e.g., see studies referenced above). This assumption should generally hold true, as vertebrate embryos typically have just reached or recently completed the gastrulation stage of development at oviposition (e.g., Patten, 1971; Ewert, 1985) and thus seem unlikely to have any steroidogenic tissues. However, lizard embryos at oviposition are substantially more developed than are avian, chelonian, or crocodilian embryos (cf. Patten, 1971; Shine, 1983). Although gonads and adrenal cortices are morphologically undifferentiated in lizards at oviposition (Forbes, 1956; Austin, 1988), it is possible that steroidogenic tissue or steroid-metabolizing enzymes are nevertheless present at this early stage. Thus, sex differences in yolk T concentrations in *A. carolinensis* eggs could arise through differential maternal input of T, differential embryonic production of T or metabolism of maternal T, or some combination of these processes.

A realistic interpretation of how (and whether) embryonic exposure to T affects phenotypic development depends first on an understanding of both when embryos may be exposed to T and what the likely source (e.g., maternal or embryonic) of that T is. Additionally, because any T of maternal origin may affect the reproductive physiology of breeding females and the phenotypic development of their offspring, it would be useful to characterize the extent to which T levels change across the ovulatory cycle. Therefore, the purpose of the present study was to describe the levels of T to which *A. carolinensis* embryos and their mothers are potentially exposed. First, we examined plasma T concentrations of adult females and the yolk T concentrations of their follicles and eggs. Second, we repeated the analysis from Lovern *et al.* (2001) of yolk T concentration in male and female eggs at oviposition and extended it to examine later incubation stages.

METHODS

Animals and Housing

Field-collected adult (≥50 mm snout–vent length) male and female *A. carolinensis* were purchased from a

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commercial supplier (Fluker Farms, Port Allen, LA) and housed in the laboratory in groups consisting of one adult male and three to seven adult females. Housing enclosures consisted of 110-liter aquaria furnished with a peat moss substrate and multiple dowels and rock formations for perching, basking, and hiding. Each cage was also furnished with a small (~0.5-liter), covered plastic container filled with moistened peat moss into which females could retreat and lay eggs. To simulate breeding conditions, we housed lizards on a 14:10 h light:dark cycle using a combination of fluorescent, full-spectrum, and incandescent lights. Ambient temperature ranged from 18° at night to a gradient of 28–38° during the day, depending on location within each cage relative to a basking light. To simulate nonbreeding conditions, lizards were kept on a 10:14 h light:dark cycle, with an ambient temperature of 15° at night and 24–30° during the day. Relative humidity was kept at 70% under all conditions. Lizards had access to water in shallow dishes *ad libitum,* and their cages were misted daily. We fed lizards two (nonbreeding) or three (breeding) times per week with either mealworms or vitamin-dusted crickets. Nest boxes were checked daily for eggs when lizards were housed under breeding conditions.

Female Sample Collection

We collected blood samples from a total of 53 adult females. Forty-three of these females were sampled in June and July under breeding conditions. For comparison, a set of 10 females was sampled in October under nonbreeding conditions. All females were maintained in the laboratory for 14–28 days prior to sampling. In the field, the breeding season begins approximately in April and continues through July; thus, the conditions (breeding or nonbreeding) that females experienced in the field prior to collection matched those in the laboratory upon their arrival. All blood samples were collected from the trunk following decapitation, after which they were centrifuged, and the resulting plasma fraction was recorded to the nearest microliter and kept at −70° until analysis.

Following blood collection, the largest yolking follicle and any oviductal eggs were removed from 37 females collected under breeding conditions. Female anoles lay a series of single-egg clutches every 7–14 days, due to sequential ovulation alternately from the left and right ovaries (Smith *et al.,* 1973; Andrews, 1985). This pattern results in a linear size and age hierarchy of ova within each ovary or oviduct, with the largest (oldest) on each side the next to be ovulated or oviposited (Crews, 1980; Jones *et al.,* 1983a). Within this framework, we recognized four stages in the ovulatory cycle of breeding females (oldest follicle or egg listed first): (I) shelling egg in one oviduct, medium (3–4 mm) yolking follicle in contralateral ovary ($n = 12$); (II) shelled egg in one oviduct, large (≥5 mm) yolking follicle in contralateral ovary ($n = 8$); (IIA) shelled egg in one oviduct, shelling egg in other oviduct ($n = 7$); and (III) large (≥5 mm) yolking follicle in one ovary, previtellogenic follicles in other ovary ($n = 10$). The distinction between shelling and shelled eggs was qualitative; shelling eggs were higher in the oviduct (i.e., more recently ovulated) than shelled eggs and uncalcified areas were still visible. Stage IIA was the result of variation in female cycling patterns. Apparently, stage II females occasionally ovulated the large follicle on one side prior to ovipositing the contralateral shelled egg. The remaining 6 females sampled under environmental conditions conducive to breeding did not contain any vitellogenic follicles or oviductal eggs and were thus classified as nonreproductive. These females appeared healthy, but it was apparent that they had not recently laid any eggs, because their oviducts were regressed in comparison to those of reproductively active females.

In each stage III female, one yolk sample was collected from the single large follicle. In females from stages I, II, and IIA, yolk samples were taken from the most developed follicle or egg from each side. All samples were collected by rupture of the follicle or egg and transfer of 8–20 mg of yolk (weighed to the nearest milligram on an A-200DS balance; Denver Instrument Co.) to 1.5-ml centrifuge tubes. Thus, yolk layers that may have contained different steroid concentrations (e.g., Lipar *et al.,* 1999) were degraded prior to sampling. All samples were mixed with 500 μl dH_2O, aided by the addition of two small glass beads, and then stored at −70° until analysis.

Yolk Collection during Incubation

Eggs collected during June and July from the nest boxes in our lizard colony were randomly assigned to

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one of three yolk sampling groups: (1) day 0 of incubation (day of oviposition), (2) day 15–22 of incubation, or (3) day 32–39 of incubation. Because mean ± SE incubation time was 52.7 ± 0.3 days, the above groups consisted of yolk samples taken with 0, 29–42, and 61–74% of incubation completed. Eggs were incubated individually in small plastic cups containing a mixture of 1:1 (mass) vermiculite:dH_2O, sealed at the top with plastic wrap and a rubber band. Incubation temperature ranged from 18 to 28° on a diel cycle (following air temperature in the lizard colony under breeding conditions). At the appropriate sampling stage, 8–20 mg yolk (recorded to the nearest milligram for each sample) was removed from each egg (<10% total egg mass) with a sterile 25-guage needle and handled as described above. Needles were randomly placed within the yolks, aided by the fact that yolk volumes and distributions were not consistent across eggs. Following sample removal, we continued to incubate eggs (or began incubation of day 0 eggs) to determine hatchling sex by postanal scale dimorphism (males have enlarged postanal scales; females do not). Hatching success was 71% (48 of 68 eggs hatched). Nine males and nine females hatched from the day 0 group, five males and eight females hatched from the day 15–22 group, and eight males and nine females hatched from the day 32–39 group.

T Assays

Plasma and yolk concentrations of T were measured by radioimmunoassay (RIA) following extraction and chromatographic separation (Wingfield and Farner, 1975; Moore, 1986; Schwabl, 1993). Samples were equilibrated overnight at 4° with 1000 cpm of [^{3}H]T (NET-370, 95 Ci/mmol; NEN Life Science Products) for individual recovery determinations. 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° overnight and then centrifuged at 2000 rpm for 5 min to precipitate neutral lipids and proteins. The supernatant was dried and reconstituted in 300 μl of 10% ethyl acetate in isooctane. Plasma samples were extracted twice with 2 ml diethyl ether, dried, and reconstituted in 300 μl of 10% ethyl acetate in isooctane. To remove additional neutral lipids and to isolate T, all samples were transferred to diatomaceous earth (Celite; Sigma) microcolumns 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:distilled water (3:1 m:v) lower phase. Purified T fractions were dried, resuspended in sample buffer, and then placed overnight at 4°.

Competitive binding RIAs were performed with [^{3}H]T and T antiserum (T-3003; Wien Laboratories). The standard curve ranged from 0.5 to 125 pg and was run in triplicate. Samples were run in duplicate, averaged, and corrected for assay volume (plasma) or mass (yolk), individual recovery, and initial sample volume (mass). The 53 plasma samples were run in two assays, with four aliquots from a pooled plasma standard included in each assay to determine precision and repeatability, as measured by intra- and interassay coefficients of variation (CV). Intraassay CVs were 11 and 8%, and the interassay CV was 4%. The 110 yolk samples were run in four assays, with four aliquots from a pooled yolk standard included in each assay (intraassay CVs were 11, 6, 10, and 19%; interassay CV was 14%). Additionally, buffer standards were used to determine interassay variation among plasma and yolk assays (CV = 6%; $n = 1$ tube per assay). Average T recoveries were 86 and 71% for the plasma and yolk assays, respectively.

Statistics

Data for female plasma T concentration across condition (breeding, nonreproductive, nonbreeding) were analyzed with a Kruskal–Wallis test followed by rank-based multiple comparisons (Hollander and Wolfe, 1973), because they did not meet the assumption of normality (Kolmogorov–Smirnov test; $P < 0.01$) and data transformation did not result in a normal distribution. All other data sets were normally distributed or were log-transformed to achieve normality (Kolmogorov–Smirnov tests; all $P > 0.20$). Breeding female plasma T across reproductive stage was analyzed by one-way ANOVA followed by post hoc comparisons with the Tukey–Kramer method. Overall effects of follicle or egg maturity and individual female on yolk T (stages I, II, and IIA; stages in which two yolk samples were taken from each female) were analyzed with a repeated-measures ANOVA. Paired t tests were used within each stage. The association of yolk T concentrations between samples within each

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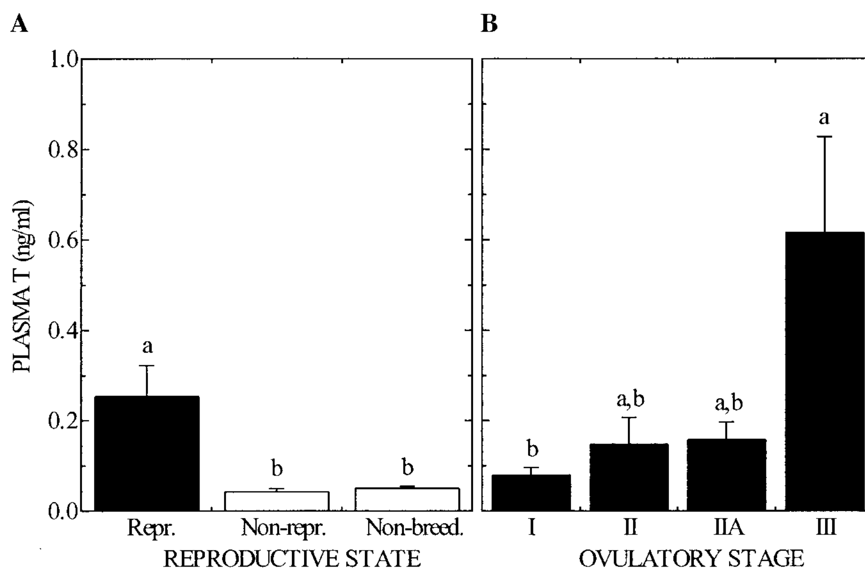

FIG. 1. Mean (+SE) plasma testosterone (T) concentrations for adult female *Anolis carolinensis* (A) by state (reproductive, nonreproductive, nonbreeding) and (B) within reproductive females by ovulatory stage. Reproductive females ($n = 37$, black bars) were actively producing eggs, nonreproductive females ($n = 6$, white bar) contained no eggs or yolking follicles even though they were being housed under conditions conducive to breeding, and nonbreeding females ($n = 10$, white bar) were sampled under nonbreeding conditions. Ovulatory stages were (I) shelling egg in one oviduct, medium (3–4 mm) yolking follicle in contralateral ovary ($n = 12$); (II) shelled egg in one oviduct, large (≥5 mm) yolking follicle in contralateral ovary ($n = 8$); (IIA) shelled egg in one oviduct, shelling egg in other oviduct ($n = 7$); and (III) large yolking follicle in one ovary, previtellogenic follicles in other ovary ($n = 10$). Different letters above bars indicate statistically different means.

female (stages I, II, and IIA), and between female plasma T and average yolk T content, was determined with Pearson's correlation procedure. Sex-specific yolk T concentration on the day of oviposition was compared by use of a one-tailed, two-sample t test with the *a priori* prediction that yolk T would be higher in eggs giving rise to males than in eggs giving rise to females (Lovern *et al.,* 2001). The effects of sampling stage, embryo sex, and the possible interaction of the two were assessed by general linear model (GLM) ANOVA. Minitab (version 10Xtra, 1995) was used for all statistical analyses. Descriptive statistics are presented as means ± SE, and the significance level required to reject the null hypothesis was set at an overall $\alpha = 0.05$. Untransformed data are presented in the figures.

RESULTS

Plasma and Yolk T in Adult Females

Female plasma T concentration was significantly affected by reproductive state ($H_2 = 10.9$, $P = 0.004$; Fig. 1A). Plasma T was fivefold higher in reproductively active compared to inactive females housed under either breeding or nonbreeding conditions. With regard only to reproductively active females, plasma T concentration also varied significantly across the ovulatory cycle ($F_{3,36} = 4.2$, $P = 0.01$; Fig. 1B). Stage III females had plasma T concentrations that were on average sixfold higher than those of stage I, II, and IIA females.

Yolk T concentrations were significantly affected by both individual female ($F_{26,53} = 3.9$, $P < 0.0005$) and relative follicle or egg maturity within females ($F_{1,53} = 21.3$, $P < 0.0005$). That is, different females contributed different amounts of T to their yolks, but regardless of ovulatory stage (I, II, or IIA), the more mature follicle or egg had more yolk T than the less mature follicle or egg (Fig. 2). Yolk T was higher in the shelling egg than in the medium follicle in 9 of 12 stage I females, it was higher in the shelled egg than in the large follicle in 6 of 8 stage II females, and it was higher in the shelled egg than in the shelling egg in all 7 stage IIA females. Furthermore, yolk T concentrations in the follicles and eggs taken from each female in stages I, II, and IIA were positively correlated ($r^2 =$

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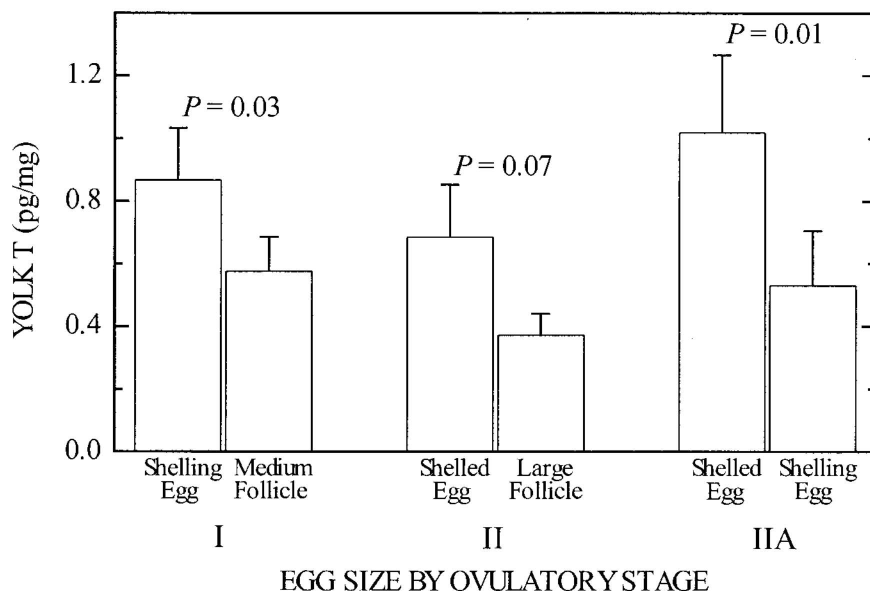

FIG. 2. Mean (+SE) yolk testosterone (T) concentrations for follicles and eggs sampled from 27 adult female *Anolis carolinensis,* separated by ovulatory stage and relative follicle and egg maturity. *P* values above bars are from paired *t* tests.

0.36, $P = 0.0009$). Finally, there was a suggestive positive association between T concentrations in the plasma and yolks of individual females ($r^2 = 0.10$, $P = 0.07$). In stage III, when plasma T concentration was highest, yolk T was also highest (1.01 ± 0.38 pg/mg, compared to 0.53 ± 0.09, 0.78 ± 0.19, and 0.72 ± 0.11 pg/mg for stages I, II, and IIA, respectively).

Yolk T during Incubation

Like Lovern *et al.* (2001), we found a sex difference in yolk T concentrations in eggs sampled on the day of oviposition. Eggs giving rise to males contained significantly higher T concentrations than those producing females ($T_{14} = 2.0$, $P = 0.033$). Yolk T concentrations in the present study are very similar to those reported by Lovern *et al.* (2001) for males and females (Fig. 3, inset). However, in the present study, which extended the analysis to include eggs sampled later in incubation, no overall effect of sex existed ($F_{1,47} = 1.2$, $P = 0.27$; Fig. 3). In contrast, we found a highly significant effect of sampling stage on yolk T concentration ($F_{2,47} = 10.8$, $P < 0.0005$), which increased as incubation progressed (Fig. 3). No interaction between sex and sampling stage was detected ($F_{2,47} = 0.1$, $P = 0.88$).

DISCUSSION

We have documented significant changes in plasma and yolk T concentrations in female *A. carolinensis* and their developing eggs, respectively, the patterns and possible functions of which are addressed in the sections that follow.

Plasma T in Adult Females

In addition to whatever role T might play in embryonic development, our results suggest that the steroid is an important component of the reproductive physiology of breeding female *A. carolinensis.* The pattern of higher plasma T concentrations in breeding than in nonbreeding females in our laboratory colony mirrors that found in females sampled in the field during the breeding and nonbreeding seasons (Lovern *et al.,* 2001). Similar seasonal changes in circulating T concentrations have been documented in females of other species (e.g., painted turtles, Callard *et al.,* 1978; red-winged blackbirds, Cristol and Johnsen, 1994; alligators, Guillette *et al.,* 1997; mountain spiny lizards, Woodley and Moore, 1999).

Although female androgen levels have received comparatively little attention, it seems clear that an-

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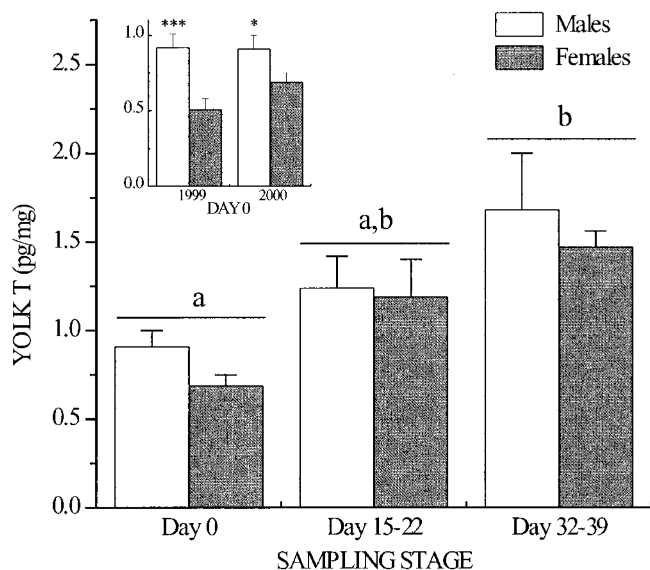

FIG. 3. Mean (+SE) yolk testosterone (T) concentrations of *Anolis carolinensis* eggs sampled at oviposition (nine males and nine females), 15–22 days into incubation (five males and eight females), or 32–39 days into incubation (eight males and nine females). Mean ± SE incubation time was 52.7 ± 0.3 days. Different letters above bars indicate statistically different means for sampling stage, collapsed across sex. Inset compares yolk T concentrations at oviposition from the present study (data collected in 2000) to those from Lovern *et al.* (2001), redrawn and reanalyzed here (data collected in 1999). Two-sample *t* tests; $^*P < 0.05$, $^{***}P < 0.005$.

drogens play a role in female reproductive biology. In females of both oviparous and viviparous species, T generally peaks around ovulation, and it may be directly or indirectly (via biochemical conversion to one of its metabolites) associated with courtship, mating, and aggressive behaviors (reviewed in Staub and De Beer, 1997). In *A. carolinensis,* previous research has demonstrated that (1) exogenous T facilitates feminine receptive behavior (e.g., Adkins and Schlesinger, 1979), at least in part due to its conversion to estradiol by aromatase (Winkler and Wade, 1998); (2) female plasma estradiol concentrations increase with the diameter of yolking follicles (Crews, 1980); and (3) receptivity occurs in females just prior to ovulation of the largest yolking follicle (Crews, 1973). Thus, changes in endogenous plasma T concentrations likely affect the probability that receptivity to male courtship will be exhibited. Our data support a role for T in facilitating receptivity in that females with a single large follicle had sixfold higher plasma T concentrations than did females with one or more oviductal eggs. Therefore, plasma T was highest in females in the present study during a time when they were also likely to have been behaviorally receptive to male courtship.

It is interesting that, although females have a large yolking follicle in two stages that we identified (II and III), plasma T was dramatically higher only when there was no oviductal egg (III). We postulate that, under typical conditions, females oviposit the oviductal egg prior to exhibiting receptive behavior (see diagram in Fig. 6 of Crews, 1980). The largest follicle in the hierarchy undergoes a rapid growth spurt near the end of the yolking phase (Jones *et al.,* 1983a), which might be coincident with the dramatic increase in plasma T observed between stage II and stage III females. It is likely that stage III females had larger follicles than did stage II females, but unfortunately our classification scheme, whereby a follicle was simply considered "large" if the diameter was ≥5 mm, did not allow us to examine this empirically. Alternatively, mean plasma T concentration in stage II females may have been sufficient to induce receptivity. Plasma T levels of stage II females were on average about twice those of stage I females, and this difference may have been biologically relevant, although it was not statistically different.

Variation in the temporal sequence of ovulation and oviposition, either among females or within females across successive cycles, led to our observation of occasional females with two oviductal eggs (stage IIA). When stage II females followed the "typical" sequence in the ovulatory cycle, they oviposited the shelled egg prior to ovulating the contralateral large follicle, thus leading to stage III. However, if stage II females ovulated the follicle first, stage IIA resulted. These females would, after the oviposition of the shelled egg, skip stage III and be classified as stage I (shelling egg, medium follicle). Regardless of how stage IIA comes about, the variation in cycling that we observed in females probably has a small, if any, effect on the overall success of females in mating and producing viable offspring. First, T levels in stage II and stage IIA females were virtually identical, suggesting that the two stages may be functionally equivalent. Second, if the comparatively high plasma T levels seen only in stage III females are necessary for inducing receptivity and subsequent mating, females that instead enter stage IIA will still likely fertilize that egg.

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Sperm from previous matings can be stored in the female reproductive tract in *A. carolinensis* (Conner and Crews, 1980), and fertile eggs have been produced by females even 4 months after their last mating (Licht, 1973).

Yolk T in Developing Follicles and Eggs

Our results demonstrate that T of maternal origin is present in developing follicles and oviductal eggs in *A. carolinensis*. Within females, yolk T levels of the largest follicle and oviductal eggs were strongly positively correlated, and average yolk T showed a suggestive positive association with female plasma T. Similarly, maternal plasma and yolk T concentrations are positively correlated in canaries (Schwabl, 1996b). One possibility that might explain why the correlation between yolk and plasma T was not stronger in the present study is that, unlike in canaries, *A. carolinensis* females have two functional ovaries (females of most avian species have a single, left ovary). Differential T levels in the simultaneously developing but temporally out of phase follicles within each ovary may therefore have increased variability found in female plasma T.

Our results further suggest that an increase in yolk T starts during follicular maturation and continues even after ovulation as the egg develops in the oviduct, given that within females the more mature follicle or egg consistently had a higher yolk T concentration than did the less mature follicle or egg. Under the assumption that the primary sources of T are the developing ovarian follicles, the result that yolk T concentrations were higher in shelled oviductal eggs than in shelling oviductal eggs particularly surprised us. The most likely explanation for this result is that even as the eggs are acquiring a shell, they are still accumulating T from the maternal environment. Although it needs to be empirically tested, this possibility is supported by the fact that the oviduct is comparatively well vascularized during the breeding season, including a direct ovarian–oviductal vascular connection in which the direction of blood flow is from the ovary to the oviduct (Jones *et al.,* 1983b).

Like Lovern *et al.* (2001), we found that yolk T concentration was higher on the day of oviposition in eggs giving rise to males than in eggs giving rise to females. The yolk T values in male and female eggs on the day of oviposition reported in the present study are extremely close to those reported by Lovern *et al.* (2001), even though the studies took place at different institutions, in different years, with animals collected from different locales and housed under different environmental conditions. To our knowledge, sex differences in yolk steroid levels so early in development have not been reported for other species, although yolk progesterone levels show a peak during late development in the eggs of males, but not those of females, in tree lizards (Jennings *et al.,* 2001). Additionally, in peafowl yolk hormone levels differ between the sexes approximately one-third of the way through incubation (Petrie *et al.,* 2001). *A. carolinensis* eggs apparently begin incubation with sex-specific yolk T concentrations, but the sex difference disappears prior to hatching. In fact, yolk T concentrations increased in both male and female eggs during incubation. This is in contrast to the pattern seen in alligators, in which yolk estradiol, T, and androstenedione all decline during incubation (Conley *et al.,* 1997). A yolk T increase during incubation could result from at least two phenomena. First, male and female embryos may produce T that, due to its lipophilic nature, passes into the yolk. Whether *A. carolinensis* embryos are capable of steroidogenesis is unknown, although it seems likely that the T increase we observed in both male and female eggs precedes morphological differentiation of the gonads and adrenal cortices, which begins approximately halfway through incubation (Forbes, 1956; Austin, 1988). Turtle embryos of both sexes can synthesize T, but only during the latter stages of embryonic development (White and Thomas, 1992). Thus, if embryo steroidogenesis is responsible for the T increase that we observed, it would have to begin early in embryonic development, prior to morphological differentiation of the primary steroidogenic organs. Second, the absolute amount of T in eggs might actually remain the same as yolk is utilized by the developing embryo, thus creating an apparent increase in concentration. We collected yolk samples rather than whole yolks (which could have addressed whether total yolk T levels change) because we needed to incubate eggs to determine embryo sex. The idea that yolk steroid content can increase during incubation is not unprecedented, however; Jennings *et al.* (2001) found that total yolk corticosterone increases during late embryonic development in tree lizards.

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This research gives us an understanding of the duration and magnitude of embryonic T exposure in *A. carolinensis,* yet little information is available on what role T might play in the development of offspring phenotype. One possibility is that yolk T is a nonadaptive consequence of maternal reproductive physiology, i.e., that T functions to associate female receptivity with ovulation, but its presence in yolk is a byproduct of that function. This possibility seems unlikely, however, given the numerous effects of yolk steroids on juvenile development documented thus far in other species. Although yolk T may in some species have an overall negative effect, correlational and experimental studies with both juvenile males and juvenile females in several species have found that it can positively affect the expression of morphology and behavior related to survival and presumably eventual reproductive success (see Introduction). Additionally, whereas the expression of sexually dimorphic behaviors in adults may not require early T exposure (Adkins and Schlesinger, 1979; Lovern *et al.,* 2001; but see Winkler and Wade, 1998), morphological sex differences might by influenced by relative T levels experienced by males and females during early development. For example, higher yolk T concentrations experienced by embryonic males around oviposition followed by higher plasma T concentrations experienced by juvenile males during posthatching development (Lovern *et al.,* 2001) could influence sexually dimorphic growth rates (Michaud, 1990) and enlargement of the dewlap (a throat fan used in communication) and its associated neuromusculature, which show sex differences in adulthood (O'Bryant and Wade, 1999; Jenssen *et al.,* 2000).

In conclusion, T likely plays a role in successful reproduction by females and in the phenotypic expression of their offspring in *A. carolinensis.* Females become sexually receptive at a time when plasma T levels peak, which facilitates mating and thus fertilization of their eggs. Subsequently, embryos are exposed to yolk T, which they may or may not add to by T production of their own during incubation. Our results challenge the assumption that yolk steroids are strictly of maternal origin, and they suggest the possibility that embryos are modulating their own steroid environment. Further work will be necessary to determine the processes that lead to the dynamic T levels that we have observed during embryonic development, and experimental manipulation of the early T environment along with quantification of the resultant phenotypic effects will be necessary to determine the consequences of embryonic T exposure and whether they are different for males and females.

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