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Newly Deposited Maternal Hormones can be Detected in the Yolks of Oviductal Eggs in the Green Anole Lizard

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Abstract

Studies often examine egg yolks after oviposition with the goal of drawing conclusions about maternal allocation of gonadal steroid hormones and how it may affect offspring development. However, these hormones might originate from a few sources, including the ovary, blood plasma, or the embryo itself. The goal of this study was to investigate whether maternal steroids can enter oviductal eggs. In Experiment 1, gravid female green anole lizards were injected with 1 μ Ci 3H-T. Plasma, ovarian follicles (separated into yolking and non-yolking samples), and shelled oviductal eggs were collected at times ranging from 15 min to 24 hr after treatment. Main effects of tissue, time, and an interaction between them all existed on recovered 3H-hormone corrected for tissue mass. Of particular interest, there was a decrease in plasma with coincident increase in eggs. In Experiment 2, females were injected with doses ranging from 0.01 to 0.45 μ Ci of 3H-T per gram body weight. Across tissues, 3H-hormone levels corrected for mass were greater with increasing doses. Values also differed among tissues and an interaction was detected. Within each dose, plasma and non-yolking follicles generally had higher concentrations of 3H-hormone than did yolking follicles and oviductal eggs. However, at and after 6 hr, eggs had higher total radioactivity levels than both yolking and non-yolking follicles had (not corrected for mass). The results indicate that steroids can cross through relatively well-formed shells before oviposition, suggesting a way in which maternal hormones might influence developmental factors after yolk deposition.

Maternal hormones have been detected in the egg yolks of many vertebrates, including birds (Schwabl, '93; Schwabl, '97; Cariello et al., 2006), reptiles (Conley et al., '97; Bowden et al., 2001; Rhen et al., 2006), and fish (McCormick, '98; Manire et al., 2004). In birds, these hormones, especially yolk testosterone (T), have been implicated in influencing a variety of aspects of development, including offspring sex, growth, and behavior (Adkins-Regan et al., '95; Schwabl, '96; Bowden et al., 2000; Rutkowska and Cichon, 2006; von Engelhardt et al., 2006). Behaviors affected by maternal allocation of sex steroids include embryonic vocalizations, begging and social behaviors of chicks, as well as food acquisition and sexual behavior in adulthood (Strasser and Schwabl, 2004; Daisley et al., 2005; Boncoraglio et al., 2006; Partecke and Schwabl, 2008). Most of the studies have examined T in multi-egg clutches after eggs have been laid. A variety of patterns have been detected, making it difficult in some cases to draw firm conclusions. For example, across avian species, yolk T can increase, decrease, or remain the same with laying order (reviewed in Groothuis et al., 2005).

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More information on mechanisms associated with deposition of maternally derived steroids would therefore be useful to understanding their influences on behavior and other factors. For example, the steroids could passively diffuse from maternal plasma into yolks of developing follicles, or some sort of active regulation could occur for the two tissues, either together or independently (reviewed in Groothuis and Schwabl, 2008). Additionally, the source of these gonadal steroid hormones present in the yolk is not clear. They certainly accumulate during yolk deposition in the ovary (from thecal and granulosa cells), but they may also be produced by developing embryos, and/or come from the mother's plasma after the egg has been ovulated (reviewed in Moore and Johnston, 2008). This study was designed to specifically address the potential for passive diffusion at two stages of development.

Green anoles (*Anolis carolinensis*) are ideal for this work. They are seasonally breeding lizards from the southeastern United States. Unlike most of the species studied for maternal hormone deposition, in green anoles one egg is oviposited at a time and the yolking follicles begin to mature from alternating ovaries. Thus, it is possible to find a female with one largely shelled egg and a yolking ovarian follicle on one side and a recently ovulated follicle that is beginning to be shelled on the other. Eggs are laid approximately every 7–10 days (Lovern and Wade, 2003a). A sex difference in yolk T exists at oviposition (before gonadal differentiation) and while the eggs are still in the oviduct, such that male eggs have higher levels than female eggs (Lovern et al., 2001; Lovorn and Wade, 2003b). Gonadal differentiation occurs around 10 days after oviposition (Holmes and Wade, 2005), and T levels extracted from embryos triple between 16 and 24 days after oviposition, consistent with the idea that embryos begin to synthesize the steroid during this period (Lovern and Wade, 2003a). As it is unlikely that yolk hormones are derived from the embryo at earlier stages of development, it is reasonable to hypothesize that the observed sex difference is because of a maternal contribution.

The goal of this study was to investigate the potential for maternal transfer of T at two developmental stages—into yolking follicles and oviductal eggs that already have formed some shell. To the best of our knowledge, the only experiment directly measuring the transfer of maternal hormone in a similar manner was done in Japanese quail. It involved injecting females with 3H-T and quantifying it in the yolk after oviposition (Hackl et al., 2003). Unlike birds, reptile eggs spend a few days to a few weeks in the oviduct while they are being shelled (Moore and Johnston, 2008). Thus, a longer period exists in reptiles during which maternal hormones might enter the yolk and potentially influence offspring development. We conducted two experiments to assess the time course and dose response of maternal transfer of T into ovarian follicles and oviductal eggs.

METHODS

Animals

Male and female green anole lizards were purchased from Charles Sullivan Co. (Nashville, TN) during the breeding season (May and June). Animals were group-housed such that several females and one male were in 21-gallon aquaria with peat moss as substrate. Water dishes were provided, and rocks and sticks were available for perching and basking. Cages were misted daily with water, and the lizards were fed crickets and mealworms three times a week. Full spectrum lighting and heat lamps were provided over each cage. Ambient temperature ranged from 28°C during the day to 19°C at night with a 14:10 h light/dark cycle. Relative humidity was kept at approximately 70%. All procedures adhered to the Michigan State University Institutional Animal Care and Use Committee and NIH guidelines.

Experiment 1

The abdomens of females were palpated to determine approximate stages of follicle/egg development. Reproductively active animals were weighed and injected subcutaneously with 1 μCi 3H-T (80.4 Ci/mmol in ethanol; PerkinElmer, Boston, MA) added to 19 μL of phosphate-buffered saline (average dose = 0.4 $\mu\text{Ci/g}$ bodyweight). Survival times of 15 or 30 min and 1, 3, 6, 12 or 24 hr were randomly chosen ($n = 3$ to 5 for each tissue and time point; Table 1). The females were then rapidly decapitated, and the following tissues were collected: blood from the exposed neck, yolk follicles, remainder of the ovaries (non-yolk follicles) and any oviductal eggs. All these eggs had thick, opaque shells. A few eggs in earlier stages of shelling were discovered; however, the sample size was far too small to analyze in a meaningful way. Those data are therefore not reported. Entire yolk follicles and non-yolk follicles were weighed, homogenized in 7 mL of Ultima Gold (PerkinElmer, Shelton, CT), and counted on a Beckman LS 6500. Oviductal eggs were weighed, and the entire contents of the egg (minus the shell, but including any embryos present) were placed in scintillation fluid, homogenized, and counted. Empty eggshells were weighed and the value was subtracted from the total to determine the quantity of tissue evaluated. Collected blood was centrifuged and an aliquot (20 μL) of the plasma was added to the same amount of Ultima Gold, vortexed, and analyzed using the liquid scintillation counter. To facilitate comparisons across tissues, plasma volumes were converted into units of mass (analysis of several anole plasma samples produced an average of 1.1 g/mL).

These procedures were validated by homogenizing a known amount of 3H-T with tissues similar to experimental tissues, including plasma, yolk follicles, and oviductal eggs. On average, 99%, 84% and 99% of the added radioactivity was recovered, respectively.

Experiment 2

As in the previous experiment, females containing large yolk follicles and/or oviductal eggs were weighed and subcutaneously injected with 0.01, 0.02, 0.11, 0.22 or 0.45 μCi of 3H-T per gram body weight. Based on the results from Experiment 1 (see below), they were individually maintained for 12 hr ($n = 4$ to 5 animals per dose; Table 2). The females were then rapidly decapitated, and the tissues were collected and analyzed as in Experiment 1.

Statistical Analysis

For both experiments, mixed-model ANOVAs were used to analyze potential differences in tissue mass and total 3H-hormone levels across the reproductive tissues (within animals) and among the survival times or doses (between animals; Experiments 1 and 2, respectively). Analyses were also conducted for 3H-hormone levels after correction for body mass across plasma, yolk follicles and non-yolk follicles, and oviductal eggs. Significant interactions were broken down further with one-way ANOVAs, followed by Tukey–Kramer post-hoc tests or planned pairwise comparisons, as appropriate.

RESULTS

Experiment 1

Although the mass of the tissue types differed from each other ($F_{2,44} = 54.88$, $P < 0.001$), there was no effect of injection time ($F_{6,22} = 0.343$, $P = 0.906$) or interaction between them ($F_{12,44} = 0.64$, $P = 0.793$) on this variable. The average mass (with standard error) of oviductal eggs was 126.5 ± 10.0 mg. Yolk follicles and non-yolk follicle samples were 65.47 ± 8.93 mg and 6.45 ± 0.49 mg, respectively.

The total radioactivity present in the three reproductive tissues (not including plasma because only a small aliquot was counted) differed from each other ($F_{2,44} = 54.0$, $P < 0.001$).

An effect of time ($F_{6,22} = 4.56$, $P = 0.004$) and an interaction between the two variables ($F_{12,44} = 15.25$, $P < 0.001$) were also detected. Overall, the amount of radioactivity in the tissues differed, regardless of injection time (all $P < 0.025$; planned pairwise comparisons). Importantly, by 6 hr after treatment, the highest levels detected were from oviductal eggs. Data from analyses within tissues and survival times are indicated in Table 1.

When 3H-hormone levels were corrected for mass, main effects of tissue type ($F_{3,66} = 116.698$, $P < 0.0001$) and survival time ($F_{6,22} = 9.076$, $P < 0.0001$; Fig. 1) were still detected. We also observed an interaction between these two variables ($F_{18,66} = 19.29$, $P < 0.0001$). Due to this interaction, we examined each tissue individually. All tissues other than yolking follicles ($F_{6,22} = 1.149$, $P = 0.368$) showed a significant change in 3H-hormone levels over time. In plasma, it decreased over the first four time points ($F_{6,22} = 17.68$, $P < 0.0001$). An opposite pattern existed in oviductal eggs, in which 3H-hormone levels increased over time with a peak at 12-hr post-injection ($F_{6,22} = 9.268$, $P < 0.0001$). Non-yolking follicles showed an increase in 3H-hormone levels at 1 hr and then a gradual decrease over time ($F_{6,22} = 3.186$, $P = 0.021$).

Within each time point evaluated, 3H-hormone levels corrected for mass varied across tissues (all $F > 4.56$, $P < 0.024$; Fig. 2). At 15 min, 30 min, and 1 hr, levels in plasma values were higher than all other tissues (Tukey–Kramer, all $P < 0.05$). At 3 hr, plasma values were higher than oviductal eggs ($P < 0.05$). At 6 hr, both plasma and non-yolking follicles were significantly higher than eggs and yolking follicles (all $P < 0.05$). At 12-hr post-injection, plasma 3H-hormone levels were higher than yolking follicle values ($P < 0.05$). Finally, at 24-hr post-injection, each of the other three tissues had significantly higher 3H-hormone values than the yolking follicle (all $P < 0.05$).

Experiment 2

Similar to Experiment 1, the mass of the three different reproductive tissues differed from each other ($F_{2,32} = 74.61$, $P < 0.001$), but as expected there was no effect of dose on tissue mass ($F_{4,16} = 0.378$, $P = 0.821$) or an interaction between the two ($F_{8,32} = 1.02$, $P = 0.439$). Oviductal eggs weighed 129.58 ± 8.21 mg on average. Yolking and non-yolking follicles were 84.14 ± 11.00 mg and 7.26 ± 0.54 mg, respectively.

The total radioactivity present in the three reproductive tissues differed among the tissues ($F_{2,32} = 74.44$, $P < 0.001$) and the doses ($F_{4,16} = 21.53$, $P < 0.001$), and an interaction between tissue and dose was detected ($F_{8,32} = 15.77$, $P < 0.001$). Data breaking down this interaction are presented in Table 2. Consistent with Experiment 1, oviductal eggs contained more radioactivity than both yolking and non-yolking follicles at all but at the lowest dose.

Across tissues, 3H-hormone levels corrected for mass were greater with increasing doses ($F_{4,16} = 9.96$, $P = 0.0003$; Fig. 3). Values also differed among tissues ($F_{3,38} = 8.90$, $P < 0.0001$) and a tissue $\times$ dose interaction was detected ($F_{12,48} = 2.68$, $P = 0.0076$). Interestingly, yolking follicles were the only tissue in which increasing doses did not produce significantly greater levels of 3H-hormone ($F_{4,16} = 2.33$, $P = 0.100$; all others $F > 4.45$, $P < 0.0132$). In addition, significant differences among the tissues for the 0.01-, 0.02-, and 0.22- $\mu\text{Ci/g}$ injections were observed (all $F > 6.327$, $P < 0.0135$). Although some details differ within specific doses, overall plasma and non-yolking follicles had higher levels of 3H-hormone than did yolking follicles and oviductal eggs (Fig. 3). Specifically, at 0.01 $\mu\text{Ci/g}$, plasma and non-yolking follicles had more 3H-hormone than yolking follicles (Tukey–Kramer; all $P < 0.05$). In addition, at that dose, non-yolking follicle levels were higher than those in eggs. At 0.02 $\mu\text{Ci/g}$, plasma and non-yolking follicle values were higher than those for both egg and yolking follicles (all $P < 0.05$). Finally, at 0.22 $\mu\text{Ci/g}$, the 3H-hormone levels in non-yolking follicles were higher than in yolking follicles. Significant

differences across tissues were not observed at 0.11 and 0.45 $\mu\text{Ci/g}$ although patterns were similar to the other doses and Experiment 1 (which used an average dose of 0.4 $\mu\text{Ci/g}$).

DISCUSSION

The results of this study demonstrate that it is possible for maternal hormones to enter the egg much later than generally considered. On average, total recovered radioactivity levels were higher in oviductal eggs than in yolking and non-yolking follicles in both Experiments 1 and 2. This pattern is clear by 6-hr post-treatment, and even greater at 12 hr. In non-yolking follicles, total levels do not change over that time, and while they vary between 30 and 44 nCi in yolking follicles, they increase from 5 to 108 nCi in oviductal eggs. Concentrations of radioactivity in the various tissues quantified (overall radioactivity corrected for tissue mass, which also allows comparisons with plasma) are consistent with this idea. First, the opposing patterns of time course data in plasma and oviductal eggs (Experiment 1) suggest that transfer from the former to the latter could occur in green anole lizards. Second, concentrations of 3H-hormone in yolking follicles did not vary over time (Experiment 1) or dose (Experiment 2), contrasting with those from oviductal eggs, which showed both time- and dose-dependent increases. Thus, the results are consistent with the idea that maternal plasma may even have a greater influence on gonadal steroid levels in oviductal eggs than on yolking follicles. This idea, of course, does not preclude the possibility that gonadal steroids are directly deposited into the yolk from ovarian sources, as in other species (reviewed in Moore and Johnston, 2008).

In green anole lizards, male eggs have higher levels of T than female eggs at the time of oviposition and even a few days before (Lovern et al., 2001; Lovorn and Wade, 2003a,b). Because anole embryos appear not to produce T until after the egg has been oviposited, the source is likely maternal (Lovern and Wade, 2003a). In addition to the possibility of deposition into follicles as they yolk, the present data suggest that this sex difference in hormone concentration could arise from the mother's plasma T while the egg is in the oviduct. Similar to anoles, sex differences in egg yolks have been documented in a few other organisms. For example, male chicken eggs have higher levels of T when the social status of the mother is taken into account (Muller et al., 2002). It has also been suggested that maternal allocation of steroid hormones can influence the sex ratio in reptiles with temperature sex determination, such that higher levels of estradiol in the yolk decreases the number of males in a clutch (Bowden et al., 2000).

A variety of studies have examined the role of maternal hormones present in egg yolks (Hackl et al., 2003; Hayward and Wingfield, 2004; Love et al., 2005; Williams et al., 2005). However, relatively few have investigated the potential for maternal transfer of steroid hormones into yolks before oviposition. In one study, estradiol injections or implants given to female Japanese quail produced similar levels in her plasma and yolks of eggs subsequently laid (Adkins-Regan et al., '95). In another study, gravid red-eared slider turtles were given implants of testosterone, which resulted in higher levels of T in the yolks of mature follicles (Janzen et al., 2002). Similarly, experimentally increased maternal corticosterone appeared to transfer to oviductal eggs in tree lizards (Moore and Johnston, 2008).

Although available data are limited, our results together with those discussed above suggest that maternal steroids absorbed into oviductal eggs may influence the development of a variety of organisms. Unlike green anoles, in those species which lay multiple-egg clutches, the potential consequences may be complicated, as both male and female embryos would likely be affected at similar developmental stages. In birds, however, these influences may not be as dramatic as in other taxa. Birds typically have fairly short egg retention times; for

those commonly studied it is less than a day, suggesting limited (although some) potential for transfer of gonadal hormones into oviductal eggs (Moore and Johnston, 2008). In contrast, squamate reptiles undergo approximately 40% of the embryonic development inside the oviduct (Shine, '83). Even those reptiles that do not have as much development before oviposition, such as turtles, can retain their eggs for longer periods if conditions are not optimal (Shine, '83). In parallel, monotremes have internal gestation lengths of approximately 20 days, which may allow for some hormone transfer (Temple-Smith and Grant, 2001). The transfer of hormones during the time that the egg spends in the oviduct could be important for organizing any number of functions, including the morphology and physiology of organs at critical stages of maturation/differentiation and behaviors that appear at almost any stage after hatching.

Although we injected 3H-T into the gravid female anoles, it is possible that the steroid was further metabolized either by the mother or by the embryo. Embryos are known to metabolize steroid hormones after they enter the yolk (Paitz and Bowden, 2008; von Engelhardt et al., 2009). Such metabolism, as well as the potential for direct diffusion of other hormones into oviductal eggs, could be investigated in future studies. Based on previous studies (see Introduction), our goal was to use 3H-T as a model for gonadal hormone exposure. However, given the general similarity in structure across steroid hormones, the data of this study are consistent with the idea that other steroids from maternal plasma, including glucocorticoids, could contribute to embryonic exposure.

In summary, by examining both yolking follicles and oviductal eggs, we identified the later stage as a second time when circulating maternal steroids might affect embryonic development. We demonstrated that yolking follicles are not substantially influenced by hormone from maternal plasma, suggesting that steroids deposited at this early stage of development come directly from the ovary. In contrast, eggs with substantial shells can absorb maternal steroids while in the oviduct. Depending on the female's condition, this period could simply increase the concentration of existing yolk steroids or it could alter their relative concentrations of particular ovarian and/or adrenal hormones. In either case, the change in individual steroids from what was initially deposited into the yolk before the ovulation of these eggs might be important for particular aspects of embryonic development.

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Figure 1.

^3H -hormone levels in each tissue assayed after varying survival times in Experiment 1. Note the coincident decrease in plasma levels and increase in oviductal eggs. Sample sizes ranged from three to five individuals at each time point.

Figure 2.

Selected data from Experiment 1 highlighting relative levels of 3H-hormone accumulation across tissues. Data at 15 and 30 min were similar to the 1-hr point, and those at 12 hr showed a similar pattern to Experiment 2, and are thus not included here. Different letters indicate significant differences between tissues within time points ($P < 0.05$).

Figure 3.

^3H -hormone levels in all tissues assayed in Experiment 2. The top panel contains the lowest two doses, whereas the bottom panel shows the higher doses. The data were split into two different graphs to facilitate viewing differences in the lower doses, which are compressed if a single axis is used. Different letters indicate significant differences within each dose.

Table 1

Effect of time post-injection on radioactivity recovered from reproductive tissues [nCi; means (SE)].

Time (min)	n	Non-yolking follicle	Yolking follicle	Oviductal egg	F-, P-value
15	4	10.75 (2.87) ^a	37.11 (5.38) ^{§b}	5.42 (0.80) ^{§a}	36.0, <0.001
30	4	16.19 (4.91) ^{§b}	27.84 (2.42) ^{§a}	9.06 (2.72) ^{§b}	15.02, 0.005
60	5	25.08 (9.94)	41.52 (6.08) [§]	24.99 (5.22) ^{§\$}	3.87, 0.067
180	3	19.75 (3.50)	43.96 (9.37) [§]	63.58 (8.44) ^{§#}	5.86, 0.065
360	4	15.53 (2.36) ^a	29.75 (4.88) ^{§b}	69.52 (8.04) ^{§#c}	30.00, 0.001
720	5	7.34 (0.90) ^a	31.70 (7.47) ^{§b}	108.39 (21.14) ^{§c}	21.95, 0.001
1440	4	5.32 (1.59) ^a	13.29 (4.20) ^{§a}	83.71 (5.83) ^{§#b}	156.36, <0.001
F-, P-value		1.88, 0.131	2.88, 0.032	13.52, <0.001	

Main effects of time and tissues, as well as an interaction between them, were detected. Different lower case letters indicate significant differences between tissues within a time point; different symbols indicate significant differences between times within a tissue (planned comparisons, LSD).

Table 2

Effect of dose on radioactivity recovered from reproductive tissues [nCi; means (SE)].

Dose (μCi/g)	n	Non-yolking follicle	Yolking follicle	Oviductal egg	F-, P-value
0.01	4	1.67 (0.25) ^{§a}	6.40 (1.18) ^{§b}	15.09 (3.78) ^{§b}	11.83, 0.008
0.02	5	3.29 (0.17) ^{§a}	11.37 (2.75) ^{§b}	41.87 (4.28) ^{§c}	71.6, <0.001
0.11	4	15.08 (2.18) ^{§a}	48.75 (8.53) ^{§b}	167.82 (9.13) ^{§c}	127.71, <0.001
0.22	4	46.91 (15.30) ^{§a}	97.73 (16.04) ^{§a}	353.77 (73.55) ^{§b}	16.66, 0.004
0.45	4	90.56 (34.08) ^{§a}	167.40 (27.75) ^{§b}	612.20 (108.43) ^{§c}	27.54, 0.001
F-, P-value		5.59, 0.005	22.55, <0.001	19.71, <0.001	

Main effects of time and tissues, as well as an interaction between them, were detected. Different lower-case letters indicate significant differences between tissues within each dose; different symbols indicate significant differences between doses within each tissue (planned comparisons, LSD).