Experimental Manipulation of Steroid Concentrations in Circulation and in Egg Yolks of Turtles

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ABSTRACT
Steroid hormones in egg yolks are increasingly recognized as an important component of maternal and offspring fitness in oviparous vertebrates. Yet, except for in birds, the mechanism by which females allocate these resources is poorly understood. We manipulated systemic levels of hormones in reproductively mature female red-eared slider turtles (Trachemys scripta elegans) with silastic implants to test the hypothesis that hormones are allocated to developing follicles as a quantitative function of circulating levels in the females. Turtles exhibited similar amounts (<1 ng/ml) of circulating steroids (dihydrotestosterone, estradiol-17β, or testosterone) in early September immediately prior to experimental manipulation. After treatment with silastic implants, circulating levels of steroids increased markedly. By the following April after hibernation, circulating levels of dihydrotestosterone had returned to preimplantation levels, but circulating levels of estradiol-17β and testosterone in estradiol-17β- and testosterone-treated turtles, respectively, remained substantially elevated through April. Focusing on testosterone, we detected nearly six-fold higher concentrations in yolk from mature follicles from testosterone-treated turtles than in yolk from mature follicles from control turtles. Our results provide support for the hypothesis that concentrations of steroids in egg yolks of turtles reflect circulating concentrations of steroids during follicular development rather than the hypothesis that females selectively allocate specific amounts of steroid hormones to each egg separately. Our findings also highlight an unambiguous physiological mechanism by which nongenetic maternal effects in oviparous species can directly influence the nutritional milieu experienced by developing embryos. J. Exp. Zool. 293:58–66, 2002. © 2002 Wiley-Liss, Inc.

Maternal allocation of resources to eggs or developing offspring is a crucial component of maternal and offspring fitness (Bernardo, ‘96; Mousseau and Fox, ‘98). In this regard, differential allocation of steroid hormones may be particularly important. For example, studies with birds have shown that: (1) hormone concentrations vary among eggs within clutches and among clutches within populations (e.g., Schwabl, ‘93; Schwabl et al., ‘97; Lipar et al., ‘99; Reed and Vleck, 2001); (2) this variation is due to circulating levels of the hormones in the females (e.g., Adkins-Regan et al., ‘95; Schwabl, ‘96a; Wilson and McNabb, ‘97); and (3) differential allocation of hormones to eggs influences hatching phenotypes (e.g., Schwabl, ‘93, ‘96b; Adkins-Regan et al., ‘95; Wilson and McNabb, ‘97; Lipar and Ketterson, 2000) and may enhance parental fitness (Gil et al., ‘99). Similar findings overall have been reported for fish as well (e.g., McCormick, ‘98).

Substantial concentrations of steroid hormones also occur in the egg yolk of alligators (Conley et al., ‘97) and turtles (Janzen et al., ‘98; Bowden et al., 2000) and may vary considerably among clutches laid by different females and among species. Of particular note, variation in allocation of steroid hormones to egg yolks may be linked to different sex-determining mechanisms in various turtle taxa and to important phenotypic variation in the offspring, including developmental rate and gonadal sex determination (Janzen et al., ‘98; Bowden et al., 2000). All these results documenting steroid
hormones in egg yolk and attendant phenotypic consequences for a variety of oviparous vertebrates thus have manifold implications regarding the potential impacts of phytoestrogens or steroid-like pollutants on embryonic development (e.g., Willingham and Crews, '99).

But what is the mechanism behind maternal allocation of steroid hormones to egg yolks in turtles? One obvious hypothesis is that hormones are allocated to developing follicles as a quantitative function of circulating levels in the females (e.g., Adkins-Regan et al., '95). In species such as turtles, where whole clutches consist of many follicles developed and ovulated simultaneously (e.g., Callard et al., '78), steroid hormone concentrations in egg yolks are relatively similar within clutches (Janzen et al., '78; Bowden et al., 2000). This result is consistent with, but not definitive proof for, the “circulating hormones” hypothesis. A contrasting hypothesis is that females selectively allocate specific amounts of steroid hormones to each egg separately, as could be true in birds where eggs are developed and ovulated sequentially (e.g., Schwabl et al., '97; Reed and Vleck, 2001).

Our objective was to test the “circulating hormones” hypothesis by manipulating systemic levels of hormones in reproductively mature female turtles. We focused on red-eared slider turtles (Trachemys scripta elegans) for several reasons: (1) they have served widely as a model species, particularly in studies of comparative physiology (summarized in Ernst et al., '94; Willingham and Crews, 2000); (2) we have previously documented substantial among-clutch variation in yolk testosterone levels in this species (Janzen et al., '98); and (3) we have shown that this variation may be causally linked to important phenotypic variation in the offspring (Janzen et al., '98). In the present study, we first assessed the efficacy of our method by measuring circulating hormone levels just prior to and at regular intervals for more than six months after experimental implantation of mature females with either dihydrotestosterone, estradiol-17\(\beta\), or testosterone. Because of results from prior research (Janzen et al., '98), we subsequently focused on the relationship between testosterone treatment and concentrations of this steroid in fully developed follicular eggs.

**MATERIALS AND METHODS**

**Experimental design**

Thirty-five gravid turtles were hand-captured at field sites in Calhoun and Jersey Counties, Illinois while making nesting forays on 7–9 June 1996 (Tucker, '97). Turtles were subsequently induced to oviposit by injection of oxytocin (10 IU/kg) to assess steroid concentrations in egg yolks (Janzen et al., '98). The spent females were transported to Iowa State University on 9 June and were held in one of three 300-gallon Rubbermaid stock tanks containing ~30 gallons of dechlorinated tap water at 27°C with a 12 hr light:12 hr dark cycle and full-spectrum light. Turtles were fed a mix of Purina Trout Chow and wild vegetation (e.g., fresh clover and goldenrod). Six adult males were trapped from the Mississippi River near the field sites on 1–8 July and were housed along with the females beginning on 15 July to promote subsequent reproductive activity. Individual turtles were identified by drilling up to four holes in different combinations of marginal scutes.

A subset of 32 female turtles was divided into four experimental groups (n=8 in each group) to investigate the relationship between systemic steroid concentrations and subsequent steroid concentrations in egg yolks. Each turtle was injected with a subcutaneous 1 ml silastic implant (polymer+ catalyst+physiological saline) containing either no steroid (i.e., control group), 2.5 mg of dihydrotestosterone, 2.5 mg of estradiol 17\(\beta\), or 2.5 mg of testosterone on 4 September and received a second implant of the same treatment on 2 October. We sampled blood (1 ml) from the brachial sinus of each turtle immediately prior to each implantation and in late afternoon every two weeks from 4 September until 31 October. Blood was collected in heparinized syringes and placed on ice until plasma was harvested.

Turtles were moved to an environmental chamber for hibernation on 31 October. The initial temperature was 21°C, which was gradually lowered to 6°C over the course of four weeks. The turtles were then held at 6°C with 8 hr light:16 hr dark in one of two 300-gallon stock tanks containing ~30 cm dechlorinated tap water. Turtles were brought out of hibernation gradually, with the temperature of the environmental chamber slowly increased beginning on 1 March 1997 until it reached 21°C on 1 April when turtles were transported to a greenhouse facility. The light and temperature treatments during hibernation are similar to those experienced by the turtles in the habitat from which they were collected (Tucker, personal observation). Blood was sampled from the brachial sinus of each turtle on 1 April, as described above. Turtles were retained under natural temperature and lighting conditions (and feeding was resumed) with the
Fig. 1. Autoradiograph of a clutch of shelled eggs in a reproductively mature female *Trachemys scripta* that died accidentally just prior to the nesting season (see MATERIALS AND METHODS for details).
intent to have them shell and oviposit eggs in late May and early June (the normal nesting period) for experimental analysis. Unfortunately, failure of the exhaust system in the greenhouse on 14–19 April caused room temperatures to rise as high as 44°C, which caused substantial mortality. This tragedy is all the more unfortunate in that one female had already shelled a clutch of eggs (Fig. 1) and the females (n=5 controls and n=4 testosterone-implanted turtles) that we autopsied within a few hours after death for analysis of yolk steroids had fully developed follicles. We did not autopsy turtles from the other two treatments, so we cannot provide information on follicle development in these cases.

**Assay validations**

**Dihydrotestosterone**

The assay for determining dihydrotestosterone in turtle plasma was based on the method described by Werawatgoompa et al. ('82). The method utilizes potassium permanganate to oxidize the 4,5 double bond of testosterone to 4,5 dihydroxy-testosterone, eliminating its affinity for the antibody and allowing the direct radioimmunoassay of dihydrotestosterone. The antibody used has a crossreactivity of 100% for testosterone, 70% for dihydrotestosterone, and <0.1% for androsterone, dehydroepiandrosterone, androstenedione, estradiol-17β, estrone, and ethiocholanolone. Duplicate samples of plasma (50 μl) were added to 950 μl of nonpyrogenic water and vortexed for 10 sec. Free dihydrotestosterone was then extracted with 3 ml of anhydrous diethyl ether. The ether fractions were decanted from the snap frozen plasma/water phase and dried under filtered air. The samples were oxidized by adding 1 ml of KMnO₄ (32 mM) for 20 min and then re-extracting with 3 ml anhydrous diethyl ether. The second ether fraction was then decanted from the snap frozen KMnO₄ reaction and dried under filtered air. Samples were dissolved in benzene:methanol (85%:15%, v:v) and purified on LH-20 columns conditioned in the same solvent. The purified fractions were then dried under filtered air.

Fractions were evaluated by adding 100 μl of assay buffer (same as above), 100 μl of antibody (diluted 1/30,000), and 100 μl of assay tracer [2,4,6,7-3H]-estradiol-17β (Amersham). The sensitivity of the assay, defined as the estradiol-17β standard which yielded 95% of the counts in the buffer control tubes, was approximately 2 pg. Precision and accuracy of the dihydrotestosterone assay were evaluated by the addition of 0.1 ng/ml or 1.0 ng/ml of dihydrotestosterone to plasma containing 0.04 ng/ml dihydrotestosterone, resulting in 0.11 ng/ml or 0.95 ng/ml dihydrotestosterone, respectively. Extraction efficiency, as determined by adding tracer to plasma samples prior to extraction and counting the fraction collected from the LH-20 column, averaged 75.5% ± 2.8%, and assay values were back corrected for this. All samples were evaluated in a single assay and the intra-assay CV was 2.3%.

**Estradiol-17β**

The antibody used has a crossreactivity of 100% for estradiol-17β, 5% for estradiol 3-methyl ether, 4% for epiestradiol, 3% for estrone, 0.3% for equilenin, and <0.1% for 2-methoxyestradiol, equilin, diethylstilbestrol, and testosterone. Duplicate samples of plasma (50 μl) were added to 950 μl of nonpyrogenic water and vortexed for 10 sec. Free estradiol-17β was then extracted with 3 ml of anhydrous diethyl ether. The ether fractions were decanted from the snap frozen plasma/water phase and dried under filtered air. Samples were dissolved in benzene:methanol (85%:15%, v:v) and purified on LH-20 columns conditioned in the same solvent. The purified fractions were then dried under filtered air.

Fractions were evaluated by adding 100 μl of assay buffer (same as above), 100 μl of antibody (diluted 1/30,000), and 100 μl of assay tracer [2,4,6,7-3H]-estradiol-17β (Amersham). The sensitivity of the assay, defined as the estradiol-17β standard which yielded 95% of the counts in the buffer control tubes, was approximately 2 pg. Precision and accuracy of the estradiol-17β assay were evaluated by the addition of 25 pg/ml or 100 pg/ml of estradiol-17β to plasma containing 4.6 pg/ml estradiol-17β, resulting in 21.9 pg/ml or 108.2 pg/ml estradiol-17β, respectively. Extraction efficiency, as determined by adding tracer to plasma samples prior to extraction and counting the fraction collected from the LH-20 column, averaged 68.5% ± 0.9%, and assay values were back corrected for this. Values for a pool sample run in all assays resulted in intra- and inter-assay CV of 5.5% and 18.3%.

**Testosterone**

Plasma testosterone was quantified using the Coat-A-Count Total Testosterone Assay Kit (Diagnostic Products Corporation, Los Angeles, CA). Precision and accuracy of the testosterone assay
were evaluated by the addition of 0.2 ng/ml, 1 ng/ml, or 8 ng/ml of testosterone to plasma containing nondetectable levels of testosterone, resulting in 0.24 ng/ml, 0.84 ng/ml, or 8.44 ng/ml testosterone, respectively. Extraction efficiency, as determined by adding tracer to plasma samples prior to extraction and counting the extract, averaged 90.2% ± 0.9%, and assay values were back corrected for this. Values for a pool sample run in all assays resulted in intra- and inter-assay CV of 4.4% and 16.0%.

RESULTS

Turtles exhibited similar amounts of circulating steroids in early September prior to initiation of the experiment (Figs. 2–4). All three focal hormones averaged <1 ng/ml in the blood at this time. Subsequently, after treatment with silastic implants and prior to hibernation, circulating levels of steroids increased markedly ($P < 0.05$) for each experimental group (four-fold for dihydrotestosterone, eight-fold for estradiol-17β, and 100-fold for testosterone) when compared to pre-implantation values or to values for the control group (Figs. 2–4). By the following April after hibernation, circulating levels of dihydrotestosterone in dihydrotestosterone-treated turtles had returned to near pre-implantation levels (1.07 ng/ml ± 0.35 ng/ml vs. 0.85 ng/ml ± 0.16 ng/ml, respectively). In contrast, circulating levels of estradiol-17β and testosterone in estradiol-17β- and testosterone-treated turtles, respectively, remained substantially elevated through April (2.23 ng/ml ± 0.89 ng/ml and 15.63 ng/ml ± 8.51 ng/ml, respectively) compared to pre-implantation levels (0.23 ng/ml ± 0.07 ng/ml and 0.87 ng/ml ± 0.76 ng/ml, respectively) and to circulating levels in April in turtles not receiving implants of these steroids (0.31 ng/ml ± 0.13 ng/ml and 1.61 ng/ml ± 0.93 ng/ml, respectively) (Figs. 2–4).

Several additional, interesting patterns emerged from the steroid treatments. First, dihydrotestosterone and estradiol-17β levels were slightly elevated in testosterone-treated turtles, especially shortly after the testosterone was implanted (Figs. 2 and 3, respectively), but these values were not significantly different from those for control turtles for any assay date ($P > 0.05$ for all comparisons). Similarly, levels of testosterone were somewhat elevated in dihydrotestosterone-treated turtles (not evident in Fig. 4 because of the scale). These values were only significantly different from those for control turtles for the 16 and 30 October assay dates ($F = 4.65$, $P = 0.05$ and $F = 9.07$, $P = 0.01$, respectively). For those two assay dates,
circulating levels of testosterone in dihydrotestosterone-treated turtles were about twice as high (\( \sim 2 \text{ ng/ml} \)) as those in control turtles (\( \sim 1 \text{ ng/ml} \)). To the contrary, control turtles and other experimental turtles generally exhibited consistent, near baseline levels of the nontreatment steroids (Figs. 2–4).

Testosterone concentrations were substantially greater in yolk from mature follicles (12–22 mm diameter) from testosterone-treated turtles (six folli-

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**Fig. 3.** Temporal changes in estradiol-17\( \beta \) in systemic circulation of reproductively mature female *Trachemys scripta* as a function of experimental treatment (dihydrotestosterone-, estradiol-17\( \beta \)-, testosterone-, or control-implanted). n=8 in each treatment group.

**Fig. 4.** Temporal changes in testosterone in systemic circulation of reproductively mature female *Trachemys scripta* as a function of experimental treatment (dihydrotestosterone-, estradiol-17\( \beta \)-, testosterone-, or control-implanted). n=8 in each treatment group.
cles from each of four females) than in yolk from mature follicles (13–20 mm diameter) from control turtles (six follicles from each of five females). That is, levels of testosterone in follicular yolk from testosterone-treated turtles were nearly six-fold higher than from control turtles (215 pg/mg ± 28 pg/mg vs. 37 pg/mg ± 8 pg/mg, respectively, P < 0.05). While substantive and highly significant, this difference is an order of magnitude lower than the corresponding comparison of circulating testosterone concentrations between these same females shortly after the experimental treatments during the previous fall (Fig. 4). Still, more than six months elapsed between the last steroid implant placement and the “final” maturation of the follicles.

DISCUSSION

This experiment produced two main results: 1) Circulating concentrations of steroid hormones increased markedly after turtles received experimental implants of particular steroids; and 2) Testosterone concentrations in egg yolks were greatly elevated in turtles that received testosterone-containing implants more than six months prior to “complete” follicular maturation. Thus, these results provide support for the hypothesis that concentrations of steroids in egg yolks of turtles reflect circulating concentrations of steroids during follicular development rather than the hypothesis that females selectively allocate specific amounts of steroid hormones to each egg separately. Taken together, these findings demonstrate the potential for using subcutaneous implants impregnated with steroids as an experimental method for manipulating yolk steroid concentrations in turtles. They also highlight an unambiguous physiological mechanism by which nongenetic maternal effects in oviparous species can directly influence the nutritional milieu experienced by developing embryos.

Of particular interest is the observation that circulating concentrations of both estradiol-17β and testosterone remained elevated nearly seven months after the last implantation of these steroids. The elevated levels of testosterone in testosterone-implanted turtles undoubtedly contributed to the marked increase of this steroid in the follicular yolk of these females. Further, the yolk testosterone levels in testosterone-implanted females were 2-4 times higher than levels observed in freshly laid eggs of Chrysemys scripta elegans females collected from the same area in a previous study (Janzen et al., ’98). In addition to or instead of embryonic physiological activity, it is conceivable that temperature-dependent differences in yolk sac aromatase activity or expression (e.g., Jeyasuria and Place, ’97), along with maternally derived differences in yolk testosterone, may function to initiate differential gonadal activation (e.g., ovary or testes) through alterations in the ratio of estrogen to testosterone in blood reaching the embryo. Such maternal transfer of a sex steroid to the egg yolk would constitute a potentially significant source of maternal influence over embryonic development and adult phenotype in oviparous species with or without temperature-dependent sex determination (e.g., Bowden et al., 2000, 2001). Our model system and technique would allow more critical experimental evaluation of this hypothesis.

Two of our steroid manipulations produced indirect effects on nontarget steroids, but only one of these effects was statistically significant. Testosterone-treated turtles had slightly elevated circulating levels of dihydrotestosterone and estradiol-17β, although these effects were apparently due to background noise. To the contrary, dihydrotestosterone-treated turtles had moderate but significantly elevated circulating levels of testosterone during two of the assay dates. This result suggests that, in response to unusually high concentrations introduced by the experimental implants, dihydrotestosterone may have impacted a testosterone negative feedback loop in the turtles. In all other cases, only circulating levels of the target steroids were elevated by the experimental implants.

In Chrysemys picta, a freshwater turtle closely related to T. scripta, elevation of both estradiol-17β and testosterone in the blood of adult females is observed in the spring at the time of maximal follicular growth, and again in the fall in association with ovarian recrudescence (Callard et al., ’78). Interestingly, testosterone concentrations are markedly higher than those of estradiol-17β during both the spring and fall elevations of circulating steroids. Research is needed to determine if T. scripta exhibits a similar pattern in nature.

We detected different circulating concentrations of the three steroids used in this study even though identical amounts of each kind were implanted in the treated turtles. We suspect that dihydrotestosterone and estradiol-17β, because they are very potent steroids, were reduced and conjugated for excretion rapidly compared to testosterone, which often naturally circulates at several orders of magnitude higher concentrations (e.g., Callard et al., ’78). Regardless, our results clearly show that levels of circulating steroids, focusing on testosterone, are reflected in concentrations of these same steroids.
in the yolk of mature follicles of turtles. Thus, the endocrinological state of the female during follicular development, to the extent that it is affected by genetic or environmental (e.g., stress) factors, can be transmitted nongenetically to the eggs that she lays.

But, is such hormonal variation in the egg yolks biologically relevant? Prior work with birds and fish has clearly demonstrated that concentrations of various hormones in the egg have profound implications for the resulting offspring (e.g., Schwabl, '93, '96b; Adkins-Regan et al., '95; Wilson and McNabb, '97; McCormick, '98; Lipar and Keterson, 2000) and may even enhance parental fitness (Gil et al., '99). Similar findings have been documented in turtles as well (Janzen et al., '98; Bowden et al., 2000, 2001). Indeed, in at least some species of turtles, concentrations of testosterone and estradiol-17β in the egg yolk are related to rate of embryonic development (Janzen et al., '98) and to sexual differentiation (Bowden et al., 2000). Such endocrinological maternal effects may in fact provide a common underlying explanation, in addition to traditional intonations of genetic effects, for well-documented, among-clutch variation in turtles for a wide variety of offspring traits (e.g., Brooks et al., '91; Packard and Packard, '93; Janzen et al., '95).

Our findings have implications in a more practical, applied context as well. Because concentrations of hormones in egg yolks have a substantive impact on offspring phenotypes in a diversity of vertebrates, it follows that exogenous substances that mimic such hormones could also have a corresponding influence on these organisms. Of special interest, given the results of this study, females could conceivably transmit so-called “environmental estrogens” such as PCBs or DDT (and its metabolites) to egg yolks during follicular development and subsequently modify the phenotypes of the resulting offspring. Indeed, environmental estrogens and other contaminants have been detected in turtle and alligator eggs (e.g., Bishop et al., '91; Heinz et al., '91), and exogenous application of those xenobiotics can result in significant sex reversal in turtles with temperature-dependent sex determination (e.g., Bergeron et al., '94; Sheehan et al., '99; Willingham and Crews, '99, 2000; but see Podreka et al., '98). And, there is increasing evidence that nongenetic environmental effects can be transmitted across generations (e.g., Francis et al., '99)! Clearly, bioaccumulation of xenobiotic hormone mimics, in conjunction with endogenous hormone levels, in female vertebrates deserves further, in-depth experimental scrutiny because there are implications for humans as well (e.g., Somawane, '95).

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LITERATURE CITED


