

Maternally Derived Yolk Hormones Vary in Follicles of the Painted Turtle, *Chrysemys picta*

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ABSTRACT The transfer of hormones from a female to her offspring is known to occur in egg laying vertebrates, and the potential for these early, maternally derived hormones to influence sex determination in reptiles with temperature-dependent sex determination is intriguing. In the present study, we examine variation in the concentrations of progesterone, testosterone, and estradiol among three follicle size classes within a female painted turtle (*Chrysemys picta*) and among females across four periods that span the pre- to post-nesting season. Females were collected, and both follicles and shelled eggs (when present) were harvested for hormone analysis. Progesterone levels did not vary seasonally. However, the concentration of progesterone did vary among and within follicle classes, and was primarily dependent upon ovulatory state: Recently ovulated follicles (as yolks within shelled eggs) contained significantly more progesterone than unovulated follicles. Concentrations of testosterone were low and did not vary either among size classes or across the season. Estradiol levels decreased with increasing follicle size and were higher later in the nesting season. Thus, hormone concentrations varied among follicle sizes and states but in patterns that differed among hormones. This variation has the potential to influence sex determination. *J. Exp. Zool.* 293:67–72, 2002. © 2002 Wiley-Liss, Inc.

In egg laying vertebrates, hormones can pass from the female to her offspring through the transfer of yolk. This transfer of maternal hormones could have direct implications for offspring phenotype and fitness, but few studies have examined this potential. In red-winged black birds (*Agelaius phoeniceus*), increased yolk testosterone has been linked to an increase in the size of the hatching muscle, which in turn could result in decreased hatching time, thus resulting in reduced stress on the hatching and an increase in hatching success (Lipar and Ketterson, 2000). In reptiles with temperature-dependent sex determination (TSD), variation in yolk hormone levels among clutches may mediate maternal effects on sex determination, perhaps allowing female condition to alter offspring sex ratio (Conley et al., '97; Roosenburg and Niewiarowski, '98; Bowden et al., 2000, 2001). In eggs from clutches of the painted turtle (*Chrysemys picta*) incubated at the local pivotal temperature (the temperature that results in a 1:1 sex ratio), we previously demonstrated seasonal variation in clutch sex ratios and in the ratio of estradiol to testosterone. More male-biased clutches were produced early in the season when the estradiol:testosterone ratio was low, and more female-biased clutches were produced late in

the season when the ratio was high (Bowden et al., 2000). We have since replicated the correlated seasonal shifts in both hormones and sex in a second year (Bowden et al., 2001).

Partly in pursuit of an understanding of these seasonal shifts in hormone levels, we previously determined that there are hormonally heterogeneous layers within the yolk of *C. picta* and the red-eared slider turtle, *Trachemys scripta elegans* (Bowden et al., 2001). In both species, estradiol was more concentrated in the internal portions of the yolk, while progesterone and testosterone were most concentrated in the external portion of the yolk. We assessed hormone levels across the nesting season in *C. picta*. Estradiol levels were significantly lower in all layers of eggs laid early in the nesting season when compared to eggs laid later in the nesting season and, again, estradiol was most concentrated in the inner layers of yolk, but significantly so only in late season eggs (Bowden et al., 2001).

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The distribution of hormones in the yolk corresponds with the female ovulatory and hormonal plasma cycle. Mature females begin enlarging follicles in the late summer or early fall and undergo a period of quiescence from late fall through the winter. In the spring, females undergo a second period of follicle maturation prior to the start of ovulation in mid- to late May. Nesting begins approximately two weeks later followed in mid-June to early July by the ovulation and laying of a second clutch for many females (Moll, '73; Christiansen and Moll, '73; Callard et al., '78).

As expected from this seasonal pattern of folliculogenesis, female plasma hormones also cycle seasonally. In the fall, when follicle maturation begins, plasma levels of estradiol and testosterone are high and then decline in the winter during gonadal quiescence. In the spring, progesterone and testosterone levels peak as ovulation begins; as they decline, estradiol levels rise and vitellogenesis occurs. This cycle is repeated if more than one clutch is to be ovulated in a season (McPherson et al., '82; see also Fig. 1 in Bowden et al., 2001). Thus, hormone concentrations in the yolk appear to reflect circulating plasma levels. Indeed, the artificial elevation of circulating estradiol in female Japanese quail (*Coturnix japonica*) did result in elevated estradiol concentrations in the yolk (Adkins-Regan et al., '95).

In the present study of *C. picta*, we compare yolk hormone levels both among the various size classes of follicles in a given female and within a follicle size class among females collected at different periods in the nesting season. We then compare these levels with the earlier studies on seasonal variation in plasma hormones and on the layering of hormones within the yolk.

MATERIALS AND METHODS

Female *C. picta* were collected from Crooked Creek Lake, Brown County, IN during the summer of 2000. During each of four periods, two females were collected to represent the pre-ovulatory condition (11 May), the first ovulatory event (25 May), the second ovulatory event (22 June and 3 July), and the post-ovulatory condition (8 September). These eight females were brought into the laboratory and killed by decapitation.

Both ovaries were removed as were any shelled eggs present. Ovarian mass was determined, and presence or absence and number of corpora lutea were noted. Follicles were counted and classified by size. Follicles were classified into three, nonover-

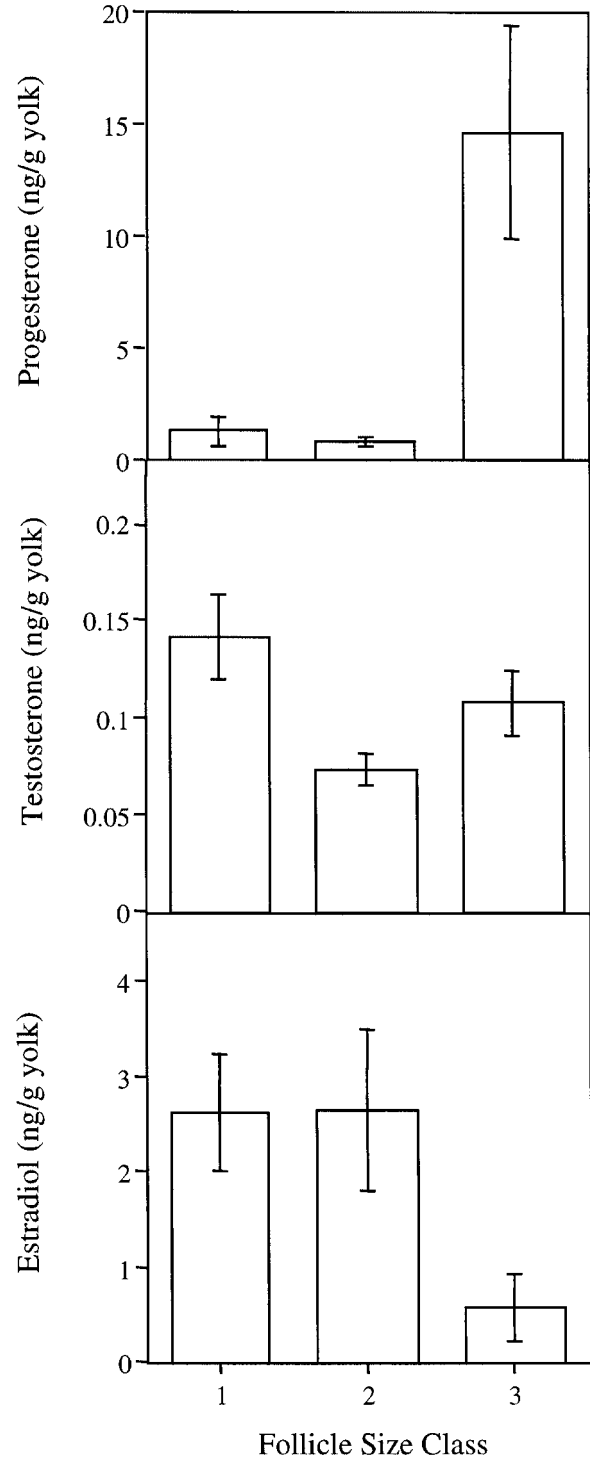


Fig. 1. Variation in yolk hormone concentrations (mean \pm 1 SE) across follicle size classes for female *Chrysemys picta*.

lapping size classes: class 1 consisted of follicles between 5–10.9 mm in diameter; class 2 of follicles between 11–16 mm; and class 3 of follicles greater

than 16 mm and yolks in shelled eggs. This size grouping approximated that used by Moll and Legler ('71) for a neotropical slider turtle (*Trachemys scripta ornata*). Depending upon presence in a female, one to three follicles of each size class were collected and frozen at -20°C for subsequent yolk hormone analysis. Each female also had numerous follicles less than 5 mm in diameter that were not assayed. This study was carried out in accordance with the Bloomington Institutional Animal Care and Use Committee guidelines (study $\epsilon 00-007$).

Radioimmunoassay

We used a competitive-binding steroid radioimmunoassay (RIA) to measure levels of progesterone, testosterone, and 17β -estradiol in *C. picta* yolk samples from ovarian follicles and from yolks from eggs (i.e., ovulated follicles). To prepare yolk samples for the assay, one or two yolks per follicle class per female were homogenized, and a sample of approximately 50 mg of yolk was collected from each and suspended in 500 μl of dH_2O . We followed the RIA procedure of Wingfield and Farner ('75) (see also Bowden et al., 2000, 2001): 2,000 cpm of tritiated progesterone, testosterone, and 17β -estradiol (New England Nuclear, Boston, MA) was added to each sample to serve as a tracer. Samples were then vortexed and allowed to equilibrate overnight at 4°C . The hormones were then extracted from the samples using petroleum and diethyl ethers and reconstituted in 90% ethanol (Schwabl, '93). The reconstituted samples sat overnight to allow for sedimentation of neutral lipids and then were centrifuged and decanted. The supernatant was then dried and resuspended in 10% ethyl acetate in isooctane in preparation for column chromatography. The columns consisted of a celite:ethylene glycol:propylene glycol upper phase and celite:water lower phase. Samples were directly applied to the columns, and hormone separation was completed by eluting each fraction with a unique ethyl acetate:isooctane ratio (2% for progesterone, 20% for testosterone, and 40% for 17β -estradiol). Fractionated samples were dried under nitrogen gas and resuspended in phosphate buffer. Hormone concentrations were measured by competitive-binding radioimmunoassay using antibodies specific for each hormone of interest (antibodies for progesterone and testosterone from Wien Laboratories, Succasunna, NJ; antibody for 17β -estradiol from Arnel, New York, NY).

Yolk samples were run in duplicate, and hormone concentrations were compared to a standard curve that ranged from 3.91 to 1,000 pg for progesterone and from 1.95 to 500 pg for testosterone and 17β -es-

tradiol. Recovery values averaged 27.4% for progesterone, 74.3% for testosterone, and 63.1% for estradiol. All samples were run in a single assay. The intra-assay variation, calculated as the coefficient of variation for the standards, was 19.95% for progesterone, 7.19% for testosterone, and 9.39% for 17β -estradiol.

Statistics

Initial associations were examined graphically using StatView. All analyses were performed in SPSS 10 for Macintosh. Due to the non-normal distribution of samples, we performed Mann-Whitney U tests to examine whether hormone concentration was affected by changes in follicle size and the ovulatory status of the follicle. A Kruskal-Wallis analysis of variance on ranks was run to examine differences among follicle classes for estradiol.

RESULTS

Not every female contained follicles from each size class. One of the two females collected on 25 May (first ovulation) had follicles from class 2 and 3, and shelled eggs. Neither female collected on 11 September (post-ovulation) had follicles in the largest class; one had no follicles larger than 12.8 mm, the other had no follicles larger than 9.5 mm. Corpora lutea were present in each of the females collected on 25 May and 22 June/3 July. The females collected during the first ovulatory event each had a single set of corpora lutea that matched the number of shelled eggs present. Females collected during the second ovulatory event each had two sets of corpora lutea, the larger (presumably more recent) set matched the number of shelled eggs present. The females from 8 September had no evident corpora lutea—any that had been present earlier presumably had regressed (Moll, '73).

Mean progesterone concentrations (\pm SE) were 1.22 ± 0.67 ng/g in class 1 follicles, 0.76 ± 0.21 ng/g in class 2 follicles, and 14.53 ± 4.72 ng/g in class 3 follicles (Fig. 1). Progesterone concentrations were highest in follicles greater than 16 mm in diameter when compared to all smaller follicles (Mann-Whitney U: $Z = -3.170$; $P = 0.001$; Fig. 2). Ovulated follicles (present as egg yolks) contained significantly higher levels of progesterone than unovulated follicles (Mann-Whitney U: $Z = -3.065$; $P < 0.0001$).

Mean testosterone concentrations (\pm SE) were 0.14 ± 0.006 ng/g in class 1 follicles, 0.007 ± 0.002 ng/g in class 2 follicles, and 0.11 ± 0.004 ng/g in class 3 follicles (Fig. 1). Testosterone levels did not vary significantly among follicle sizes (Mann-Whitney U: $Z = -0.410$; $P = 0.693$; Fig. 3), nor were

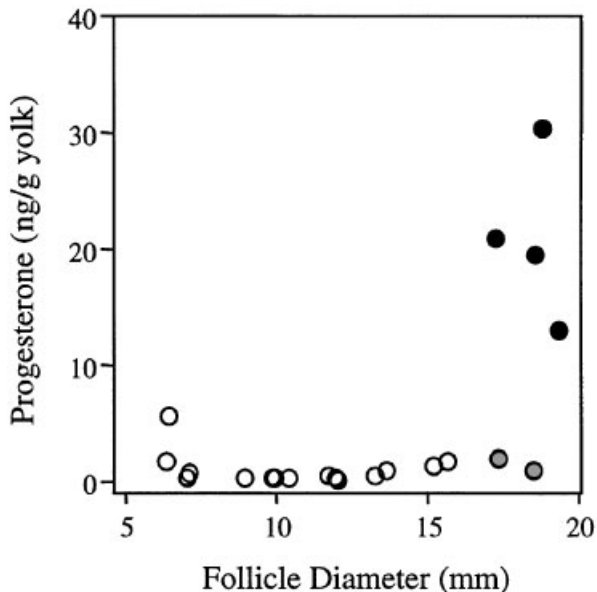


Fig. 2. Concentration of progesterone in the yolk of *Chrysemys picta*. Open circles represent preovulatory follicles <16 mm in diameter, gray circles represent preovulatory follicles >16 mm in diameter, and closed circles represent ovulated follicles.

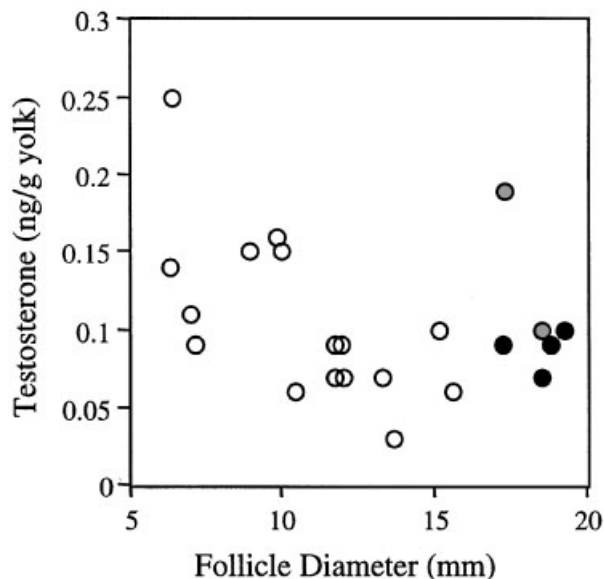


Fig. 3. Concentration of testosterone in the yolk of *Chrysemys picta*. Open circles represent preovulatory follicles <16 mm in diameter, gray circles represent preovulatory follicles >16 mm in diameter, and closed circles represent ovulated follicles.

they influenced by ovulatory state (Mann-Whitney U: $Z = -2.397$; $P = 0.013$; Fig. 4).

Mean concentrations of estradiol (\pm SE) were 2.35 ± 0.61 ng/g in class 1 follicles, 2.74 ± 0.84 ng/g in class 2 follicles, and 0.60 ± 0.36 ng/g in class 3 follicles (Fig. 1). Estradiol levels were lowest in follicles greater than 16 mm in diameter when compared to all smaller follicles (Mann-Whitney U: $Z = -2.397$; $P = 0.013$; Fig. 4). Ovulatory state did not significantly affect estradiol concentration (Mann-Whitney U: $Z = -1.320$; $P = 0.195$). Follicle class 3 was marginally statistically different from classes 1 and 2 (Kruskal-Wallis: $\chi^2 = 5.810$; $P = 0.055$; $df = 2$; Fig. 1). Estradiol levels in each of the four eggs representing first clutches (mean = 0.11 ng/g of yolk) were lower than in any of the four eggs representing second clutches (mean = 1.67 ng/g of yolk). Due to the small sample size, this difference was not statistically analyzed.

DISCUSSION

The frequency of clutches laid annually by *C. picta* varies geographically from one or two clutches per season for northern populations (Moll, '73; Snow, '80; Congdon and Tinkle, '82) to as many as four clutches per season at the southern end of the range (Moll, '73). The presence of two sets of corpora

lutea in each of the two females collected on 22 June/3 July confirms that at least some females locally do lay two clutches per season.

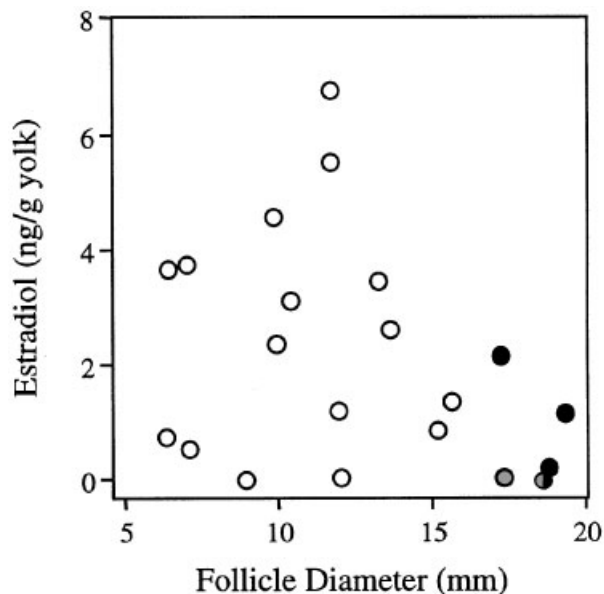


Fig. 4. Concentration of estradiol in the yolk of *Chrysemys picta*. Open circles represent preovulatory follicles <16 mm in diameter, gray circles represent preovulatory follicles >16 mm in diameter, and closed circles represent ovulated follicles. The gray and black circle represents two overlapping points; one preovulatory, the other ovulated.

The presently observed pattern of follicle class distribution across the season corresponds to a previous account for *C. picta*. Congdon and Tinkle ('82) found that females began maturing at least two sets of follicles in the fall prior to ovulation, with the majority of the yolk being allocated to the first set to be ovulated. By winter quiescence, females had, on average, deposited half of the yolk needed for their reproductive effort the following year. In our study, females had preovulatory follicles of ovulatory size approximately 2–3 weeks prior to the typical starting date of nesting locally.

Hormone concentrations varied both among follicle classes within a female and across the nesting season. The best predictor of elevated progesterone levels was the ovulatory state of the follicle. Yolks from oviductal eggs contained considerably more progesterone than did preovulatory follicles of comparable size. This contrast is consistent with our previous finding that progesterone concentrations were highest in the external layer of yolks from shelled eggs, regardless of season (Bowden et al., 2001). We also found relatively low concentrations of progesterone in the interior portions of egg yolks. Again, this outcome is compatible with our present finding of a low concentration of progesterone in the preovulatory follicles.

The concentration of progesterone measured in yolks from ovulated follicles far exceeds that reported in plasma of female *C. picta* by Callard et al. ('78). Increasing levels of progesterone do occur prior to ovulation in plasma (Callard et al., '78; McPherson et al., '82), but progesterone also increases in the granulosa cells of the follicular epithelium just prior to ovulation (Lombardi, '98). Corpora lutea are also known to synthesize progesterone for some time after the follicle has been ovulated (Lombardi, '98), but due to the addition of both albumin and shell during the post-ovulatory period, the corpora lutea seem an unlikely source. Since elevated progesterone levels are not occurring in all follicles, this suggests that the most likely source of the elevated levels of progesterone in ovulated follicles are the granulosa cells.

Progesterone is a precursor for both testosterone and estradiol. Progesterone can be converted to androstenedione by cytochrome P450 17 α -hydroxylase/C17-20lyase, which can then be converted to testosterone by 17 β -hydroxysteroid dehydrogenase; testosterone can, in turn, be converted to estradiol by cytochrome P450 aromatase (Freking et al., 2000). Thus, the elevated levels of progesterone in the yolk could function as a reservoir for testosterone and estradiol production in the developing embryo,

potentially influencing embryonic sex determination by altering the ratio of estradiol to testosterone available to the embryo (Bowden et al., 2000). Aromatase transcript is present in the brains of embryonic diamondback terrapins (*Malaclemys terrapin*) at least as early as developmental stage 12 (Jeyasuria and Place, '98), well prior to the temperature sensitive period, but whether the embryo is able to convert progesterone to androgens remains to be demonstrated.

Testosterone concentrations were low in all follicles regardless of size. Again, this finding is consistent with our previous work on *C. picta*, as is the finding that the concentrations of estradiol were lower in the largest follicles than in smaller follicles. In an earlier study of multiclutching female temperate-zone turtles, plasma estradiol did not peak prior to the ovulation of the first clutch (McPherson et al., '82). Therefore, the yolk added to follicles in the spring should be lower in estradiol than the yolk added in the fall when estradiol concentrations are higher (McPherson et al., '82; Bowden et al., 2001), although we did not have a sample of enlarged follicles from late fall to verify this point. A similar pattern of estradiol distribution with follicle size was found in another population of *C. picta* (P.K. Elf, personal communication). The observed increase in estradiol in second clutch follicles also follows from the seasonal plasma profiles. The majority of the yolk in second clutch follicles appears to be added after the first clutch is ovulated, at the time of maximal estradiol in the plasma. Thus, more estradiol is present in second (or later) clutches.

The presence of elevated levels of estradiol in eggs from second clutches corroborates our previous findings that estradiol levels increase seasonally (Bowden et al., 2000, 2001) and is consistent with the idea that hormones may be a mechanism for maternal effects (Conley et al., '97; Rhen and Lang, '98; Roosenburg and Niewiarowski, '98). Whether by active sequestration or passive diffusion, the apparent seasonal variation in accumulation of hormones in yolk suggests that female reptiles have some potential to influence offspring sex and, thus, other phenotypic traits, and perhaps fitness (as in redwing blackbirds, above).

If the maternal levels and partitioning of sex-determining hormones are not inherited by offspring but are, rather, environmentally determined, then the effective heritability of the sex-determining mechanism may be reduced, constraining any response to selection (Wade, '98). If so, heterogeneity among clutches in maternally-

derived hormones could slow the rate of evolution of sex determining mechanisms.

Further studies are needed to elucidate the interactions between TSD and maternal effects such as endogenous hormone concentrations and nest site choice (Roosenburg, '96; Janzen and Morjan, 2001). Studies of this type may help clarify any role that endogenous hormones play in seasonal effects on sex determination in reptiles with TSD.

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