

# Concentrations of Steroid Hormones in Layers and Biopsies of Chelonian Egg Yolks

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The actions of circulating hormones, although relatively well understood for adults, are largely unknown for their developing embryos. Transfer of maternal hormones to the egg is known to occur in oviparous species, and recently the presence of hormonally heterogeneous yolk layers has been described in two avian species. To investigate the possibility of a similar phenomenon occurring in chelonian species, egg yolk layers were analyzed in the painted turtle (*Chrysemys picta marginata*) and the red-eared slider turtle (*Trachemys scripta elegans*), two species that exhibit temperature-dependent sex determination. There was a similar pattern of hormonally heterogeneous yolk layers in both species: concentrations of progesterone and testosterone were significantly higher in the external yolk layer while concentrations of  $17\beta$ -estradiol were significantly higher in the intermediate and internal layers. This pattern of hormone deposition concurs with previously published studies of plasma hormone profiles from females of temperate-zone turtle species. Yolks of freshly laid eggs were also sampled using a biopsy technique to examine the concordance of early yolk hormone concentrations and offspring sex. No relationship was found between yolk hormone concentrations and individual offspring sex. Previous work showing that maternally derived yolk estradiol concentrations are correlated with female-biased sex ratios was, however, replicated. These findings suggest that off-

spring sex is influenced, in part, by the maternal hormone environment. © 2001 Academic Press

**Key Words:** egg; yolk; progesterone; testosterone; estradiol; biopsy; painted turtle; red-eared slider; maternal effects; temperature-dependent sex determination.

The presence of hormonally heterogeneous yolk layers has been described recently in freshly laid eggs of two bird species and has been attributed to temporal patterns of steroidogenesis in the female (Lipar *et al.*, 1999). In birds, follicles accumulate most of their yolk in a few days and experimental evidence illustrates that elevating plasma steroid concentrations in the female results in elevated levels of steroids in her yolk (Adkins-Regan *et al.*, 1995). To investigate whether hormonally heterogeneous yolk layers occur in other oviparous species, we examined yolks from the eggs of two emydid turtles and compared our results to published chelonian seasonal plasma profiles.

Follicle enlargement due to yolk accumulation takes much longer in turtles than in birds, perhaps as much as 10 months in the painted turtle (*Chrysemys picta*; Congdon and Tinkle, 1982). Throughout this prolonged period of yolk deposition, maternally derived steroid hormones could be deposited in the yolk as has been demonstrated in birds (Schwabl, 1993; Adkins-Regan *et al.*, 1995). In turtles, and in contrast to birds, all follicles for a given clutch are yolked simultaneously (Congdon and Tinkle, 1982) and therefore all would be exposed to the same maternal hormone environment. Recent work demonstrates that hor-

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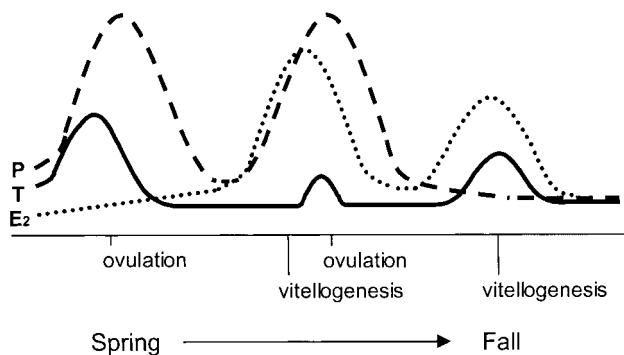


FIG. 1. Schematic representation of seasonal cycling of plasma hormone titers in a multiclutching temperate-zone turtle species. The figure is based on data from Callard *et al.* (1978) and McPherson *et al.* (1982). P = progesterone, T = testosterone, and  $E_2$  = estradiol.

mones are present in reptilian yolks at oviposition (Conley *et al.*, 1997; Janzen *et al.*, 1998; Bowden *et al.*, 2000). Turtle embryos are approximately at gastrulation when oviposited (reviewed in Ewert, 1985). At this early developmental stage it is unlikely that the embryo would be able to synthesize hormones, and therefore any hormones present in the yolk are most likely of maternal origin.

In adult female *C. picta*, plasma concentrations of progesterone, testosterone, and estradiol do change seasonally (Callard *et al.*, 1978). Levels of all three hormones peak in the spring, just prior to ovulation, and levels of testosterone and estradiol peak again in the fall. Although painted turtles can lay from one to three clutches per season (Snow, 1980; Iverson and Smith, 1993), hormone profiles have only been described for females that have laid a single clutch (Callard *et al.*, 1978). Circulating hormone profiles also have been described for females of other chelonians that lay multiple clutches per nesting season (*Chelonia mydas*; Licht *et al.*, 1979, 1980; *Sternotherus odoratus*; McPherson *et al.*, 1982). In *S. odoratus*, these hormone profiles are similar to those reported for *C. picta*, but with oscillations in hormone titers corresponding to individual nesting events (Fig. 1). Progesterone levels peak just prior to or during ovulation for each clutch and show a clutch-independent peak in the fall (no fall progesterone peak was noted for *C. picta*; Callard *et al.*, 1978). Testosterone levels are highest during the ovulation of the first clutch, with much smaller peaks occurring during subsequent ovulations, and then increase again in the fall. Both progesterone and testos-

terone are known antagonists of estradiol (Ho *et al.*, 1981). With early peaks of progesterone and testosterone, estradiol activity is suppressed, but as levels of these two hormones decrease there is a rapid increase of estradiol and vitellogenic activity (Fig. 1). As progesterone and testosterone levels rise, estradiol is again suppressed and the second clutch is ovulated. Estradiol levels increase again in the fall and do not peak again until shortly before ovulation of the second clutch in the following season. Estradiol levels are also elevated prior to the ovulation of the third clutch in *S. odoratus*, which lays three clutches per season (McPherson *et al.*, 1982).

In 1998 progesterone, testosterone, and  $17\beta$ -estradiol levels were measured in three layers of yolk from eggs of female painted turtles (*C. picta marginata*) and red-eared sliders (*Trachemys scripta elegans*) to assess both the amount and the predictability of interlayer heterogeneity for hormone concentrations. In *C. picta*, seasonal variation in yolk hormone concentrations was also assessed. Presumably, any observed variation would reflect hormonal profiles of the first and second clutches as most females in this locality lay more than one clutch per season (MAE, unpublished data). If plasma hormones in *C. picta* and *T. scripta* cycle as in *S. odoratus* (Fig. 1), then both progesterone and testosterone should follow a similar layering pattern, but estradiol should follow a different layering pattern and this pattern should vary seasonally.

We have previously found an association between maternally derived yolk hormone titers and clutch sex ratios using homogenized yolk samples of *C. picta*, a turtle with temperature-dependent sex determination, TSD (Bowden *et al.*, 2000). To further explore this association, small yolk biopsies were extracted from eggs of both species in 1999 and the concentrations of testosterone and  $17\beta$ -estradiol were determined. Although our previous methods did not allow for the comparison of initial yolk hormone concentration and the sex of the individual developing from the same egg, the biopsy method would make this direct comparison possible. This study also allowed for validation of the reliability of the biopsy technique, given the possibility of variation in hormone concentration among yolk layers. Based on the study of plasma levels across multiple clutches in *S. odoratus* (McPherson *et al.*, 1982) and on our previous analysis of whole yolk homogenate levels of hormones (Bowden *et al.*,

2000), we predicted that clutches laid early in the nesting season would have higher yolk testosterone and lower yolk estradiol levels than clutches laid later in the nesting season. Variation in maternally derived yolk steroid concentrations could provide a means for maternal influence of sex determination in species with TSD.

## METHODS

Gravid female *C. picta* were collected from several locations near Bloomington, Indiana, between 26 May and 4 July 1998 ( $n = 28$ ) and between 2 June and 17 July 1999 ( $n = 26$ ). Gravid female *T. scripta* were collected from Reelfoot Lake, Tennessee, from 8 to 11 June 1998 ( $n = 10$ ) and from 7 to 9 June 1999 ( $n = 15$ ). All females were brought into the lab and the state of the maturation of the eggs was assessed with careful palpation. Most females were captured while they were apparently searching for a nesting site or in the process of building the nest. These females were induced to oviposit with oxytocin within 48 h (Ewert and Legler, 1978). If a nonnesting female was captured at an earlier stage of gravidity, she was held in the lab until the presence of fully shelled, mature eggs was noted and was then injected. Once injected, completion of oviposition takes 2–6 h (Ewert and Legler, 1978). Females typically spent less than 72 h in the lab before being returned to their collection site.

Each year the clutches of *C. picta* were divided for analysis into early season and late season clutches. In 1998, clutches collected on or before 6 June were considered early; those collected on or after 16 June were considered late. No clutches were collected from 6 to 16 June 1998. In 1999, clutches collected on or before 15 June were considered early; those collected on or after 22 June were considered late. No clutches were collected from 15 to 22 June 1999.

### Yolk Layers

In 1998, two or three eggs from each clutch were selected randomly for hormone analysis and were frozen at  $-20^{\circ}$ . One frozen egg per clutch was used for layer analysis, one or two others were used for whole yolk analysis, and the remainder were incubated for

sex determination. Whole yolks were separated from shell and albumin while frozen. Yolk layer samples were taken as described in Lipar *et al.* (1999). In brief, we cut a medial section across the long axis of the yolk; two sides of the resulting disk were cut off leaving only a narrow medial strip; the yolk layers were then cut from this strip. To separate the layers, the middle of the section was found, and a cut was made on each side of the center, attempting to maintain the integrity of the innermost core of yolk (which was usually distinct in coloration). A cut was then made on each side of the innermost core; the other two layers (intermediate and external) were obtained by cutting one of the remaining sections in half. The use of the term layer, in this context, is not meant to imply biologically discrete segments, but rather that the yolk was separated into rough thirds for the purpose of the analysis. Samples varied from 14 to 80 mg; however, sample mass does not contribute to error in the measurement of hormone concentrations as the concentration was determined as nanograms per gram of yolk (Lipar *et al.*, 1999).

### Yolk Biopsy

In 1999, two or three eggs from each clutch were selected randomly for biopsy, and the rest were incubated for sex determination. Prior to performing the biopsies on the eggs, we first rinsed the egg under a stream of deionized water. Each egg was dried and marked with a unique clutch-egg identification number. A small area of the egg shell was swabbed with betadine solution. The egg was illuminated with a fiberoptic light, and a sterile butterfly infusion set (Abbott Laboratories) was used to extract 10 mg of yolk from each egg. Yolk samples were suspended in 500  $\mu$ l of distilled water and frozen at  $-20^{\circ}$  until hormone assays were performed. The puncture site was sealed with several drops of Nexaband glue (Veterinary Products Laboratories). Once the glue had dried the egg was placed into a clean incubation box that contained 1:1 vermiculite to water by weight ( $\sim -170$  kPa) and the box was placed into an incubator at  $28^{\circ}$  (the temperature that yields approximately a 1:1 sex ratio). All biopsied eggs were checked for survival by candling on a weekly basis up to the temperature-sensitive period. Sex of hatchlings was determined by macroscopic examination of gonads and Müllerian

ducts (Schwarzkopf and Brooks, 1985; Ewert and Nelson, 1991; Janzen, 1994).

### Radioimmunoassay

A competitive-binding steroid radioimmunoassay (RIA) was used to measure levels of progesterone, testosterone, and 17 $\beta$ -estradiol in eggs of both species. Three assays were run: one that contained both painted turtle samples and red-eared slider samples and two that contained only painted turtle samples.

We followed the RIA procedure as laid out in Wingfield and Farner (1975): 2000 cpm of tritiated progesterone, testosterone, and 17 $\beta$ -estradiol (New England Nuclear) was added to each of our samples to serve as a tracer. Samples were then vortexed and allowed to equilibrate overnight at 4°. The hormones were then extracted from the samples using petroleum and diethyl ethers and reconstituted in 90% ethanol (Schwabl, 1993). Samples were again allowed to sit overnight and were 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 a 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 (progesterone = 2%, testosterone = 20%, and estradiol = 40%). All 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, antibody for estradiol from Arnel).

Yolk layer samples were run in duplicate and hormone concentrations were compared to a standard curve that ranged from 3.91 to 1000 pg for progesterone and from 1.95 to 500 pg for testosterone and estradiol. Recovery values, averaged across the assays, were 35.0% for progesterone, 55.7% for testosterone, and 56.5% for estradiol. The intra-assay variation, calculated as the coefficient of variation of the standards, for progesterone was 17.63, 11.54, and 29.08%, with an interassay variation of 11.15%. The intra-assay variation for testosterone was 17.98, 16.63, and 8.51%, with an interassay vari-

ation of 9.86%, and the intra-assay variation for estradiol was 9.6, 11.27, and 14.9%, with an interassay variation of 5.69%. *C. picta* samples were run in each of the three assays, but only two samples were run in the last assay. The values obtained for the last assay did not differ from the previous two assays. *T. scripta* samples were run only in the first assay.

Yolk biopsy samples were also run in duplicate and compared to standard curves that ranged from 1.95 to 500 pg for testosterone and estradiol. Recovery values averaged 57.2% for testosterone and 50.6% for estradiol. All biopsy samples for both *C. picta* and *T. scripta* were run in a single assay with an intra-assay variation of 12.36% for testosterone and 9.91% for estradiol.

### Statistics

We used a repeated measures analysis of variance to look for differences among yolk layers for progesterone, testosterone, and 17 $\beta$ -estradiol. For *C. picta*, season was included as an additional variable in the repeated measures analysis of variance. Post hoc paired *t* tests were used to identify any significant effects of either egg layer or season whenever ANOVAs were significant. We performed one-way analysis of variance tests on seasonal variation and differences among clutches in hormone levels for the biopsy data. Linear regression analysis was used to examine associations between hormones, clutch sex ratios, and oviposition date in the biopsy data. All hormone data were square root transformed prior to statistical analyses to meet assumptions of parametric tests (Sokal and Rohlf, 1995). Untransformed data are presented in the figures.

## RESULTS

### Yolk Layers

In *C. picta*, mean progesterone values ( $\pm 1$  SE) in the exterior, intermediate, and interior layers were 13.95  $\pm$  2.17, 3.65  $\pm$  0.77, and 3.34  $\pm$  0.99 ng/g of yolk, respectively, and decreased significantly from the exterior to the interior of the yolk ( $F_{2,25} = 24.13$ ,  $P = 0.0001$ , Fig. 2). For progesterone concentrations, the exterior layer differed significantly from both the in-

intermediate and the interior layers ( $P < 0.01$ ), but the intermediate and interior layers did not differ ( $P = 0.420$ ). There was no detectable effect of season ( $F_{2,25} = 0.274$ ,  $P = 0.605$ ).

Testosterone in *C. picta* was, like progesterone, more concentrated in the exterior layer, but at much lower levels; mean testosterone values were  $0.35 \pm 0.04$ ,  $0.21 \pm 0.04$ , and  $0.19 \pm 0.03$  ng/g of yolk for the exterior, intermediate, and interior layers, respectively. Concentrations decreased significantly from the exterior to the interior of the yolk ( $F_{2,25} = 8.44$ ,  $P = 0.0007$ , Fig. 2). For testosterone concentration, the exterior layer differed significantly from both the intermediate and the interior layers ( $P < 0.01$ ), but the intermediate and interior layers did not differ ( $P = 0.587$ ). There was again no detectable effect of season ( $F_{2,25} = 0.141$ ,  $P = 0.711$ ).

In contrast, mean estradiol concentrations in *C. picta* were  $1.37 \pm 0.31$ ,  $1.90 \pm 0.49$ , and  $2.20 \pm 0.58$  ng/g of yolk for the exterior, intermediate, and interior layers, respectively. There was a significant effect of season on estradiol concentrations ( $F_{1,24} = 22.573$ ,  $P = 0.0001$ ). There also was a significant interaction between egg layer and season ( $F_{2,24} = 4.470$ ,  $P = 0.017$ ). When only early season eggs were tested, there were no statistically significant differences among layers ( $F_{2,19} = 1.260$ ,  $P = 0.295$ ). However, in the late season eggs there were significant differences between layers ( $F_{2,5} = 12.749$ ,  $P = 0.0018$ ). Concentrations in the exterior layer of late season eggs were significantly less than those from both the intermediate ( $P = 0.0189$ ) and the interior layers ( $P = 0.0135$ ); those in the intermediate and interior layers again did not differ significantly ( $P = 0.561$ ).

In *T. scripta*, mean progesterone values were  $13.95 \pm 4.10$ ,  $3.06 \pm 0.79$ , and  $2.92 \pm 0.72$  ng/g of yolk for the exterior, intermediate, and interior layers, respectively, and decreased significantly from the exterior to the interior of the yolk ( $F_{2,9} = 8.916$ ,  $P = 0.002$ , Fig. 3). Testosterone again followed a pattern similar to progesterone, but with much lower concentrations;  $0.36 \pm 0.08$ ,  $0.15 \pm 0.03$ , and  $0.15 \pm 0.03$  ng/g of yolk for the exterior, intermediate, and interior layers, respectively. Concentrations decreased significantly from the exterior to the interior of the yolk ( $F_{2,9} = 7.110$ ,  $P = 0.0053$ , Fig. 3). Mean estradiol concentrations were  $8.074 \pm 1.626$ ,  $14.925 \pm 3.628$ , and  $14.620 \pm 4.904$  ng/g of yolk for the exterior, intermediate, and interior

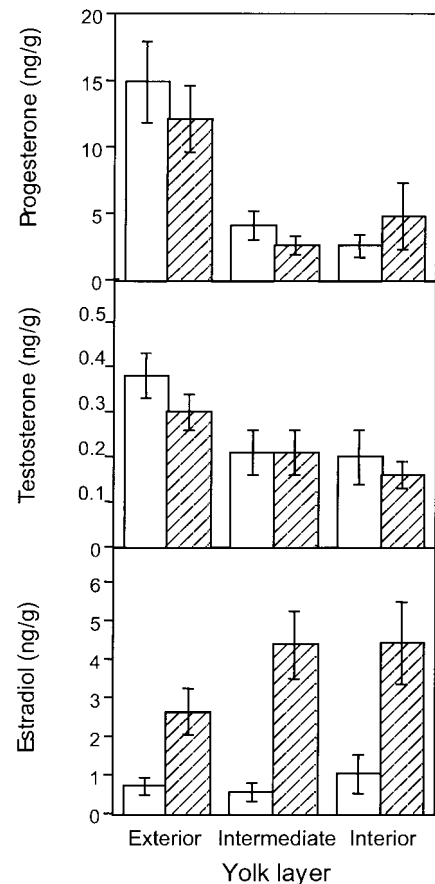


FIG. 2. Mean ( $\pm$ SEM) concentrations of progesterone, testosterone, and  $17\beta$ -estradiol in the exterior, intermediate, and interior layers of yolk of *Chrysemys picta marginata*. Open bars represent early clutches; hatched bars represent late clutches.

layers, respectively, and increased significantly from the exterior layer to the two interior layers of the yolk ( $F_{2,9} = 7.601$ ,  $P = 0.004$ , Fig. 3) as in *C. picta*. Since all clutches of *T. scripta* were collected within a 3-day period, seasonal comparisons were not possible.

### Yolk Biopsies

We biopsied 58 eggs from 26 *C. picta* clutches and 44 eggs from 15 *T. scripta* clutches; survival rates were 29.31 and 45.45%, respectively. Survival of *C. picta* eggs was 42.86% in 15 early season clutches but was only 8.70% in 11 late season clutches.

In *C. picta*, mean estradiol was  $1.237 \pm 0.421$  ng/g and testosterone was  $1.625 \pm 0.107$  ng/g. There was a significant correlation in hormone titers among eggs from the

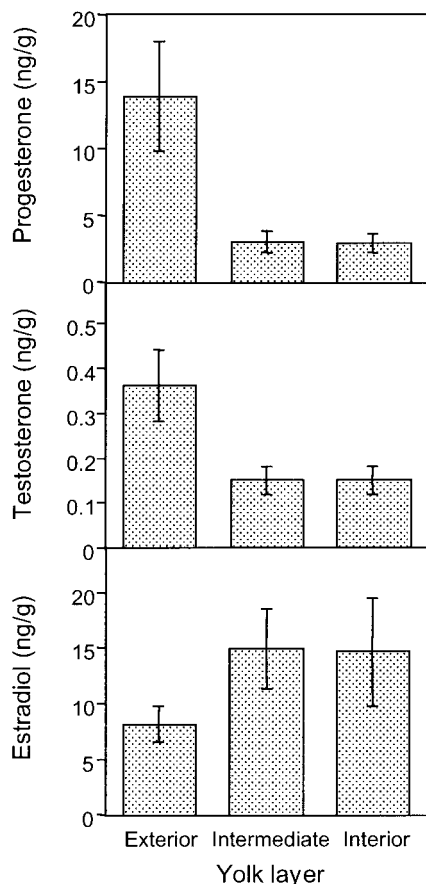


FIG. 3. Mean ( $\pm$ SEM) concentrations of progesterone, testosterone, and  $17\beta$ -estradiol in the exterior, intermediate, and interior layers of yolk of *Trachemys scripta elegans*.

same clutch for estradiol ( $F_{1,24} = 29.760$ ,  $P = 0.0001$ ,  $r^2 = 0.554$ ), but not for testosterone ( $F_{1,24} < 0.0001$ ,  $P = 0.998$ ,  $r^2 < 0.0001$ ). We also found significant among-clutch heterogeneity for estradiol ( $F_{25,26} = 6.986$ ,  $P = 0.0001$ ), but not for testosterone ( $F_{25,26} = 0.998$ ,  $P = 0.501$ ). Estradiol concentrations varied seasonally ( $F_{1,24} = 7.700$ ,  $P = 0.0105$ ), but testosterone concentrations did not ( $F_{1,24} = 1.478$ ,  $P = 0.236$ ). Early clutches had a mean estradiol value of  $0.353 \pm 0.151$  ng/g of estradiol and late clutches averaged  $2.442 \pm 0.914$  ng/g (Fig. 4). Clutch sex ratios also varied seasonally ( $F_{1,24} = 8.936$ ,  $P = 0.0064$ ), with early clutches being male-biased and late clutches being female-biased. The proportion of female-biased clutches increased with increasing estradiol concentrations ( $F_{1,24} = 7.547$ ,  $P = 0.0112$ ). We found no significant association between yolk estradiol concentrations and the sex of an individual ( $F_{1,13} = 0.327$ ,  $P = 0.577$ ) in the small sample of embryos that survived to term.

For *T. scripta*, testosterone had a mean of  $0.674 \pm 0.178$  ng/g and mean estradiol was  $2.815 \pm 0.703$  ng/g. Hormone titers were not correlated among eggs from the same clutch for either testosterone ( $F_{1,13} = 0.038$ ,  $P = 0.849$ ,  $r^2 = 0.003$ ) or estradiol ( $F_{1,13} = 0.062$ ,  $P = 0.807$ ,  $r^2 = 0.005$ ). We failed to find any among-clutch heterogeneity for either testosterone ( $F_{10,12} = 1.127$ ,  $P = 0.416$ ) or estradiol ( $F_{10,12} = 1.334$ ,  $P = 0.314$ ). There was no detectable association of yolk estradiol concentrations with either clutch sex ratio ( $F_{1,14} = 0.694$ ,  $P = 0.419$ ) or the sex of an individual ( $F_{1,19} = 0.034$ ,  $P = 0.8554$ ). These samples were collected over only a 3-day period, so seasonal comparisons were not possible.

## DISCUSSION

### Yolk Layers and Maternal Hormone Titers

In birds and crocodilians, there appears to be an association between the maternal hormonal milieu and deposition of hormones in yolk (Adkins-Regan *et al.*, 1995; Schwabl, 1996; Conley *et al.*, 1997; Lipar *et al.*, 1999). A similar association is suggested by an apparent concordance between the differences in hormone layers in the yolk found here and the pattern of seasonal variation in maternal hormone levels previously reported by Callard *et al.* (1978) and McPherson *et al.* (1982). In turtles, yolk is deposited in concentric layers over an extended period throughout which maternal

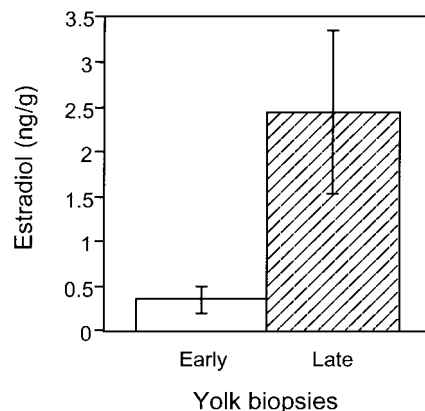


FIG. 4. Seasonal variation in mean estradiol levels ( $\pm$ SEM) from yolk biopsies of *Chrysemys picta marginata* eggs. Open bars represent early clutches; hatched bars represent late clutches.

hormone profiles constantly vary. If the levels in *C. picta* and *T. scripta* fluctuate as in *S. odoratus*, plasma progesterone levels should peak just prior to the ovulation of each clutch, and testosterone levels should be highest in the early spring. Plasma estradiol levels should be elevated in the fall, decrease during the winter (a period of gonadal quiescence), slowly recover to fall levels in the late spring/early summer, and then peak just prior to the ovulation of the second clutch.

The hormonal contrasts among layers in the egg yolk of *C. picta* and *T. scripta* concurred with the seasonal pattern in *S. odoratus*. Progesterone was highest in the external layer of yolk, a finding that is compatible with this layer being deposited shortly before or during ovulation, when progesterone was reported to peak. Testosterone was also highest in the external layer, which would correspond with its peak coincident with progesterone just before or during ovulation. Testosterone concentrations were higher, but not significantly so, in early clutches which agrees with the early spring plasma peak previously described. Estradiol concentrations were higher in the inner portions of yolk and lower in the external portion of yolk. In painted turtles, we also found elevated levels of estradiol for clutches laid later in the nesting season (Fig. 2), which is compatible with the occurrence of a preovulatory estradiol peak for the second clutch as in *S. odoratus* (McPherson *et al.*, 1982).

The internal and the intermediate layers had similar hormone concentrations regardless of season. However, clutches ovulated early in the season (i.e., first clutches) had much lower concentrations of estradiol in each layer than clutches ovulated later (i.e., second clutches). This finding is attributable to the lack of an estradiol peak prior to the ovulation of the first clutch and the preovulatory estradiol peak that occurs for the second clutch. There were no apparent seasonal differences in yolk levels for either progesterone or testosterone.

In *C. picta* approximately 50% of a female's energetic investment in yolk occurs during the fall, and the remainder occurs during the spring (Congdon and Tinkle, 1982). In the fall, a female most likely adds yolk to all follicles that are to be ovulated the following year, but more yolk is added to those follicles which are to be ovulated first. Therefore, the early clutches we describe here would contain a larger por-

tion of fall yolk relative to late clutches, and any yolk added to these follicles in the spring would be low in estradiol. The late clutches would also be started in the fall, but a larger portion of their yolk would come after the ovulation of the first clutch and would therefore be exposed to the late spring estradiol peak. This could account for the seasonal differences we observed in estradiol.

If yolk is laid down in roughly concentric spheres, as is suggested for birds (Romanoff and Romanoff, 1949), each new layer added is slightly larger than the previous one. We estimate that our external layer represented approximately 78% of the volume of any given yolk, thus representing more than spring investment, at least in the early clutches. The technique we used could be refined in further studies by taking a narrower "external" layer which should increase the correspondence of this layer and spring deposition. Additionally, the heterogeneity among layers suggests that if a single sample is to be used to represent a given egg, homogenization of the yolk will yield the most accurate results.

We previously assayed hormone concentrations in samples of homogenized yolks of *C. picta* (Bowden *et al.*, 2000) from a sample that included the same eggs analyzed here for layers. Homogenized concentrations were compared with individual layer concentrations (discussed here) for the same egg. The values for the homogenized yolks best compare to the external yolk layer for all three hormones, as expected from the relative contribution of the external layer to the volume of the homogenate.

We also detected differences in hormone concentrations between samples collected in 1998 (yolk layer data) and 1999 (yolk biopsy data). This variation could well be attributable to year-to-year fluctuations in hormones, as noted for yolk steroids by Conley *et al.* (1997) and for male and female plasma steroids by Shelby *et al.* (2000).

### ***Yolk Biopsies and Seasonal Variation***

When the yolk biopsies were taken, we were unable to control the placement of the needle within the yolk due, in part, to the mobility of the yolk within the egg. We were therefore unable to control the region from which the yolk was withdrawn, which may account for the lack of a significant association of the biopsy

hormone data with the eventual sex of the hatchling from the same egg. However, the seasonal differences in estradiol concentrations were so large that the biopsy technique reliably detected these differences.

The seasonal variation in both biopsy estradiol levels and clutch sex ratio (at 28°) for the *C. picta* clutches from 1999 and the significant association between biopsy estradiol levels and clutch sex ratio corroborate our previous findings (Bowden *et al.*, 2000). There was no significant association of clutch estradiol and clutch sex ratio or of estradiol concentrations among eggs within a clutch in *T. scripta*. However, the clutches were collected over just a 3-day period, approximately in the middle of the nesting season. The strongest correlation between sex and hormone concentrations occurs for clutches laid during the later part of the nesting season in *C. picta* (Bowden *et al.*, 2000). A parallel relationship between sex and hormones could exist in *T. scripta*, but, if so, a more seasonally prolonged sampling array would be required to detect the pattern.

We failed to find an association between sex and hormone concentrations in yolk biopsies for either species. As previously mentioned, biopsied eggs from early in the season had a higher survival rate than those biopsied later in the season, a period which had previously yielded a significant relationship between sex and estradiol concentrations. Perhaps contamination of the glue used to seal off the puncture site resulted in the late season mortality in *C. picta*. Further attempts at any such procedure should focus on maintaining a more sterile environment to limit potential sources of contamination.

The association of maternally derived estradiol and sex ratio is particularly interesting because it is evident that estradiol plays an important role in reptilian sex determination (Crews, 1996; Pieau, 1996). In addition, there is significant interclutch variation in estradiol concentrations both within a short sampling period and seasonally for *C. picta*. Thus, individual females could vary their estradiol investment across clutches. These differences in maternal contribution could account for at least part of the similarity in gender among clutch-mates that is usually attributed to heritability (Bull *et al.*, 1982; Janzen, 1992; Rhen and Lang, 1998).

As development proceeds, much of the yolk is reserved for posthatching development. It has been es-

timated for *C. picta* that only 38% of the total lipids in an egg are utilized for primary development (Congdon and Tinkle, 1982). Although many details of yolk utilization remain unclear, it is known that yolk becomes vascularized as development proceeds and that the vascularization results in an extensive network of vessels throughout the entire yolk. This network would imply that yolk is not simply used from the exterior to the interior, but, at least following the earliest stages of development, the embryo could access many or all yolk regions. Additionally, as development proceeds, the structural integrity of the yolk appears to break down such that the yolk layers become homogenized (Lipar *et al.*, 1999, for bird eggs; pers. obv., R. Bowden, for turtle eggs). This increase in apparent homogeneity occurs during the first third of development but gonadal differentiation does not occur until the middle third of development in TSD species (Crews, 1996). The timing suggests that differences among layers in hormone concentrations may only influence initial development and that the egg's total hormone supply might become accessible for embryonic metabolism early in development.

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## REFERENCES

- Adkins-Regan, E., Ottinger, M. A., and Park, J. (1995). Maternal transfer of estradiol to egg yolks alters sexual differentiation of avian offspring. *J. Exp. Zool.* **271**, 466–470.
- Bowden, R. M., Ewert, M. A., and Nelson, C. E. (2000). Environmental sex determination in a reptile varies seasonally and with yolk hormones. *Proc. R. Soc. Lond. B* **267**: 1745–1749.
- Bull, J. J., Vogt, R. J., and Bulmer, M. G. (1982). Heritability of sex ratio in turtles with environmental sex determination. *Evolution* **36**, 333–341.

- Callard, I. P., Lance, V., Salhanick, A. R., and Barad, D. (1978). The annual ovarian cycle of *Chrysemys picta*: Correlated changes in plasma steroids and parameters of vitellogenesis. *Gen. Comp. Endocrinol.* **35**, 245–257.
- Congdon, J. D., and Tinkle, D. W. (1982). Reproductive energetics of the painted turtle (*Chrysemys picta*). *Herpetologica* **38**, 228–237.
- Conley, A. J., Elf, P., Corbin, C. J., Dubowsky, S., Fivizzani, A., and Lang, J. W. (1997). Yolk steroids decline during sexual differentiation in the alligator. *Gen. Comp. Endocrinol.* **107**, 191–200.
- Crews, D. (1996). Temperature-dependent sex determination: The interplay of steroid hormones and temperature. *Zool. Sci.* **13**, 1–13.
- Ewert, M. A., and Legler, J. M. (1978). Hormonal induction of oviposition in turtles. *Herpetologica* **34**, 314–318.
- Ewert, M. A. (1985). Embryology of turtles. In "Biology of the Reptilia, Vol. 14" (C. Gans, F. Billett, and P. F. A. Maderson, Eds.), pp. 75–267. Wiley, New York.
- Ewert, M. A., and Nelson, C. E. (1991). Sex determination in turtles: Diverse patterns and some possible adaptive values. *Copeia* **1991**, 50–69.
- Ho, S. M., Danko, D., and Callard, I. P. (1981). Effect of exogenous estradiol-17 $\beta$  on plasma vitellogenin levels in male and female *Chrysemys* and its modulation by testosterone and progesterone. *Gen. Comp. Endocrinol.* **43**, 413–421.
- Iverson, J. B., and Smith, G. R. (1993). Reproductive ecology of the painted turtle (*Chrysemys picta*) in the Nebraska sandhills and across its range. *Copeia* **1993**, 1–21.
- Janzen, F. J. (1992). Heritable variation for sex ratio under environmental sex determination in the common snapping turtle (*Chelydra serpentina*). *Genetics* **131**, 155–161.
- Janzen, F. J. (1994). Vegetational cover predicts the sex ratio of hatchling turtles in natural nests. *Ecology* **75**, 1593–1599.
- Janzen, F. J., Wilson, M. E., Tucker, J. K., and Ford, S. P. (1998). Endogenous yolk steroid hormones in turtle with different sex-determining mechanisms. *Gen. Comp. Endocrinol.* **111**, 306–317.
- Licht, P., Owens, D., and Wood, F. (1979). Serum gonadotropins and steroids associated with breeding activities in the green sea turtle, *Chelonia mydas*. I. Captive animals. *Gen. Comp. Endocrinol.* **39**, 274–289.
- Licht, P., Rainey, W., and Clifton, K. (1980). Serum gonadotropins and steroids associated with breeding activities in the green sea turtle, *Chelonia mydas*. II. Mating and nesting in natural populations. *Gen. Comp. Endocrinol.* **40**, 116–122.
- Lipar, J. L., Ketterson, E. D., Nolan, V., Jr., and Casto, J. M. (1999). Egg yolk layers vary in the concentration of steroid hormones in two avian species. *Gen. Comp. Endocrinol.* **115**, 220–227.
- McPherson, R. J., Boots, L. R., MacGregor, R., III, and Marion, K. R. (1982). Plasma steroids associated with seasonal reproductive changes in a multiclutched turtle, *Sternotherus odoratus*. *Gen. Comp. Endocrinol.* **48**, 440–451.
- Pieau, C. (1996). Temperature variation and sex determination in reptiles. *Bio Essays* **18**, 19–26.
- Rhen, T., and Lang, J. W. (1994). Temperature-dependent sex determination in the snapping turtle: Manipulation of the embryonic sex steroid environment. *Gen. Comp. Endocrinol.* **96**, 243–254.
- Rhen, T., and Lang, J. W. (1998). Among-family variation for environmental sex determination in reptiles. *Evolution* **52**, 1514–1520.
- Romanoff, A. L., and Romanoff, A. J. (1949). "The Avian Egg." Wiley, New York.
- Schwabl, H. (1993). Yolk is a source of maternal testosterone for developing birds. *Proc. Natl. Acad. Sci. USA* **90**, 11446–11450.
- Schwabl, H. (1996). Environment modifies the testosterone levels of a female bird and its eggs. *J. Exp. Zool.* **276**, 157–163.
- Schwarzkopf, L., and Brooks, R. J. (1985). Sex determination in northern painted turtles: Effect of incubation at constant and fluctuating temperatures. *Can. J. Zool.* **63**, 2543–2547.
- Shelby, J. A., Mendonça, M. T., Horne, B. D., and Seigel, R. A. (2000). Seasonal variation in reproductive steroids of male and female yellow-blotched map turtles, *Graptemys flavimaculata*. *Gen. Comp. Endocrinol.* **119**, 43–51.
- Snow, J. E. (1980). Second clutch laying by painted turtles. *Copeia* **1980**, 534–536.
- Sokal, R. R., and Rohlf, F. J. (1995). "Biometry," 3rd ed. Freeman, New York.
- Wingfield, J. C., and Farner, D. S. (1975). The determination of five steroids in avian plasma by radioimmunoassay and competitive protein-binding. *Steroids* **26**, 311–327.