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Hormone levels in yolk decline throughout development in the red-eared slider turtle (*Trachemys scripta elegans*)

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Abstract

This study investigates the potential effects of maternally derived hormones present in the yolk of reptile eggs. Specifically, we ask when are these hormones utilized by developing red-eared slider turtles (*Trachemys scripta elegans*), a species with temperature-dependent sex determination (TSD). Eggs were incubated at 27 °C, a male-producing temperature, and at 31 °C, a female-producing temperature. Concentrations of progesterone, testosterone, and 17 β -estradiol were measured at four points during development: at oviposition, at the start of the temperature sensitive period (TSP), at the end of the TSP, and at hatching. No effects of incubation temperature on yolk hormone concentrations were detected. The highest concentrations of all three hormones were measured at oviposition. Hormone-specific patterns of decline occurred throughout development. Each hormone declined between oviposition and the early TSP. Although estradiol was present in detectable quantities at oviposition, it was virtually undetectable by the early TSP. Testosterone showed no further decline after the early TSP. Progesterone continued to decline between the early and post-TSP. These results demonstrate that maternally derived yolk hormones decline at different rates. Alternative explanations for the disappearance of these yolk hormones are presented.

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1. Introduction

In organisms with temperature-dependent sex determination (TSD), the incubation temperature of the eggs largely determines the sex of the resulting offspring. Organisms with TSD lack sex chromosomes and have offered only relatively indirect evidence for genetic involvement in sex determination (e.g., Rhen and Lang, 1998; Zaborski et al., 1988). However, one of these putative genetic influences (Bulmer and Bull, 1982), the similarity of sex among clutchmates incubated at the temperature which produces a population-wide 1:1 sex

ratio (the “pivotal” temperature), may be due to maternally derived hormones present in the yolk, rather than to genes per se (Bowden et al., 2000).

We have previously demonstrated that maternally derived yolk steroid hormone levels are correlated with clutch sex ratios at the pivotal temperature in an emydid turtle (Bowden et al., 2000). Moreover, work on the American alligator (*Alligator mississippiensis*) has demonstrated that hormones are present in the yolk early in development, and decline sharply during the middle third of development (Conley et al., 1997) suggesting that these hormones may be utilized during development. The purpose of the present study was to examine whether and when the steroid concentrations present in the yolk of turtle eggs at oviposition vary throughout development.

The possibility that endogenous yolk hormones could influence sex determination is supported by work with

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exogenously applied hormones. The ability of exogenously applied steroid hormones to override the sex determining effects of incubation temperature has been thoroughly documented in turtles. The exogenous application of estrogens during the middle third of development (the temperature sensitive period or TSP) to eggs incubating at male-producing temperatures produces females; the application of testosterone to eggs incubating at either male- or female-producing temperatures also results in females (Crews, 1996; Pieau, 1996). The induction of females by exogenous testosterone under both male- and female-temperature regimes may reflect the metabolism of testosterone to estradiol by aromatase (Crews and Bergeron, 1994).

The time course of utilization of yolk hormones may be crucial. Exogenous hormone application during the first third of development is less effective at overriding the effects of incubation temperature on sex determination than application during the TSP. In the red-eared slider turtle (*Trachemys scripta elegans*), the TSP starts at approximately developmental stage 15 (Wibbels et al., 1991a). In this species, the application of 10 µg of 17β-estradiol was capable of producing 100% females at a given male-producing temperature when applied as early as developmental stage 11, whereas 1 µg of 17β-estradiol produced no females when applied at developmental stage 14 under the same incubation conditions (Wibbels et al., 1991b). In contrast, within the TSP very low doses of estradiol are capable of causing sex reversal in embryonic *T. scripta*. For example, application of only 400 pg/egg of estradiol during the TSP to eggs incubated at a temperature that otherwise produces a male-biased sex ratio resulted in 14.4% more females in the estradiol-treated group than in the control group (Sheehan et al., 1999). In the same study, endogenous estradiol concentrations were estimated at 1.7 ng/egg using a modified Michaelis–Menten equation. We have previously measured endogenous estradiol concentrations in *T. scripta* egg yolks at oviposition (Bowden et al., 2001), and found estradiol concentrations in excess of the estimate by Sheehan et al. (1999).

There is also a synergistic interaction between the concentration of 17β-estradiol and incubation temperature. In eggs incubated at a moderately male-biased temperature, doses of 17β-estradiol as low as 0.10 µg resulted in approximately 70% females, whereas eggs incubated at an exclusively male-producing temperature yielded approximately 10% females when given the same dose (Wibbels et al., 1991b). At or near the pivotal temperature significantly more females resulted from as little as 1.0 ng of exogenously applied 17β-estradiol when compared to untreated controls (Wibbels and Crews, 1995). These studies indicate that both the timing of the hormone application and the distance from the pivotal temperature are important factors for deter-

mining how a given hormone level influences sex determination in reptiles with TSD.

Steroid hormones are important for the normal development and differentiation of primary and secondary sexual characters in vertebrates (vom Saal et al., 1992). Although gonadal sex is chromosomally determined in mammals, secondary sexual character development in males is reliant upon the presence of androgens: without androgens, female development occurs. In contrast, in birds and reptiles, estrogens appear to be responsible for ovary formation; in the absence of estrogens, testes develop (Lance, 1997; Pieau, 1996; Wade and Arnold, 1996; Wolfe and Wolfe, 1951).

In organisms with TSD, the demonstrated ability of sex steroids, both endogenous and exogenous, to override the sex determining effects of temperature would suggest that these hormones play an important role in the early organization of structures such as the gonad. In addition to 17β-estradiol, testosterone, progesterone, and androstenedione have all been detected in the yolks of reptile eggs at oviposition (Bowden et al., 2000, 2001, 2002; Conley et al., 1997; Janzen et al., 1998). Although 17β-estradiol is clearly the most thoroughly studied of these hormones, the potential for testosterone, progesterone, or androstenedione to influence sexual differentiation needs to be explored as all three hormones can be converted to 17β-estradiol via various enzymatic pathways. Further investigation into the timing and magnitude of steroid action and enzyme production is necessary to elucidate the mechanism(s) by which steroid hormones influence sexual differentiation. In this study, we assay for progesterone in addition to testosterone and 17β-estradiol because of its potential to be converted to testosterone and subsequently to 17β-estradiol, and because we have previously detected progesterone in freshly oviposited eggs at concentrations that far exceeded those of testosterone and 17β-estradiol (Bowden et al., 2001, 2002).

2. Materials and methods

Nine-gravid female *T. scripta* were collected from Claiborne and Ouachita Parishes, LA between 9 and 13 May 1998 as they emerged from aquatic areas to nest. Females were induced to oviposit with oxytocin injections in the laboratory (Ewert and Legler, 1978). They laid a mean of 9.14 eggs per clutch (range = 7 to 12). Freshly laid eggs were cleaned and marked with a unique clutch-egg identification number, and the eggs from each clutch were randomly divided into a no-incubation group and two temperature-treatment groups. The latter were then placed into incubation boxes that contained 1:1 vermiculite to water by weight (~170 kPa). Eggs were incubated at a constant temperature of either 27 °C (a 100% male-producing temperature) or 31 °C (a 100%

female-producing temperature). All eggs were periodically candled to check for survival and to determine approximate developmental stage (Beggs et al., 2000; Ewert, 1985).

We sampled eggs at four stages of development to determine yolk hormone concentrations. The eggs in the no-incubation group (first sampling period) were frozen at -20°C within 24 h of oviposition. Embryos from this group would be close to Yntema (1968) stage zero, a late gastrula stage, as known for the closely related emydid turtle *Chrysemys picta* (Mahmoud et al., 1973). The groups of eggs placed at either 27 or 31 $^{\circ}\text{C}$ were further divided into three sub-groups. One sub-set (second sampling period) was collected and frozen at the start of the TSP (as defined for *T. scripta* by Wibbels et al., 1991a) between developmental stages 15 and 17. Another sub-set (third sampling period) was collected and frozen at the end of the TSP between developmental stages 21 and 23. The last group (fourth sampling period) was collected and frozen at pipping, stage 26 when embryonic development was complete. To obtain yolk samples, the frozen yolk was slightly thawed and separated from its embryo and extraembryonic membranes. Yolk samples were collected and re-frozen for later hormone analysis. The thawed embryo was then patted dry and definitively staged under a dissecting microscope. We used several characteristics including shape and pigmentation status of the carapace, forelimb formation, status of the ocular sclera, and the position of the eyelids to assess developmental stage following Yntema (1968). We also macroscopically examined the gonads of the stage 26 embryos to confirm the effect of the incubation temperatures on sex determination.

Radioimmunoassay

We used a competitive-binding steroid radioimmunoassay (RIA) to measure levels of progesterone, testosterone, and 17β -estradiol in *T. scripta* yolk samples. To prepare yolk samples for the assay, each yolk was homogenized and a sample of approximately 50 mg yolk was collected and suspended in 500 μl distilled water. We followed the RIA procedure of Wingfield and Farner (1975), see also Bowden et al. (2000, 2001): 2000 cpm tritiated progesterone, testosterone, and 17β -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°C . The hormones were then extracted from the samples using petroleum and diethyl ethers and reconstituted in 90% ethanol (Schwabl, 1993). 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 isoctane in preparation for column chro-

matography. 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 (progesterone = 2%, testosterone = 20%, and 17β -estradiol = 40%). 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 1000 pg for progesterone and from 1.95 to 500 pg for testosterone, and 17β -estradiol. Recovery values averaged 43.2% for progesterone, 57.3% for testosterone, and 53.9% for estradiol. All samples were run in a single assay. The intra-assay variation, calculated as the coefficient of variation for the standards, was 23.4% for progesterone, 5.73% for testosterone, and 11.88% for 17β -estradiol.

2.1. Statistics

For statistical analyses, unless otherwise stated, any sample that had an undetectable value for a hormone was assigned a value of zero for that hormone only. We performed a Kruskal–Wallis analysis of variance on ranks to examine differences among sampling period and hormone concentration for both progesterone and 17β -estradiol due to the non-normal distribution of these data sets. Post hoc Mann–Whitney *U* tests were conducted to explore pairwise comparisons. A Kolmogorov–Smirnov test was run to compare progesterone concentrations at both incubation temperatures across the second and third sampling periods. We used a one-way analysis of variance to examine differences among sampling periods and testosterone concentration. Any significant main effects were evaluated using Fisher's Protected LSD.

3. Results

Progesterone concentrations declined significantly through development (Kruskal–Wallis test, $H = 50.571$, $P < 0.0001$, $df = 3$), and varied significantly among sampling periods (Mann–Whitney *U* test: $P_s < 0.01$; Fig. 1). Mean progesterone ($\pm\text{SE}$) was 9.013 ± 0.967 ng/g yolk at the time of oviposition (stage 0), declined to 0.450 ± 0.184 ng/g yolk at the beginning of the TSP (mean stage 16), and declined again to 0.019 ± 0.009 ng/g by the end of the TSP (mean stage

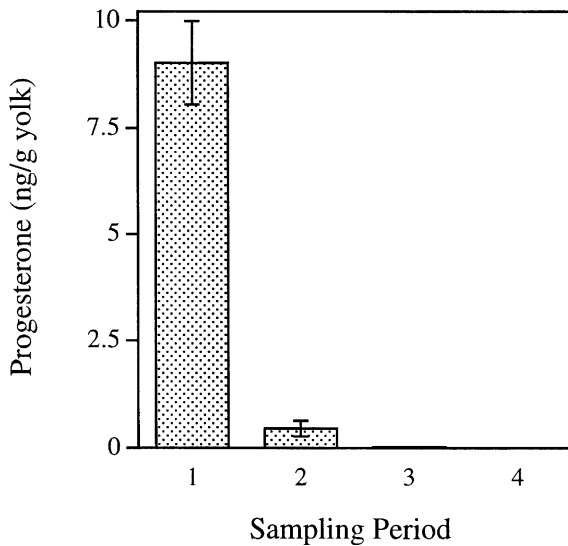


Fig. 1. Mean (\pm SEM) concentrations of progesterone in the yolk of *Trachemys scripta* for the four sampling periods. Sampling period 1 = oviposition, $n = 12$; sampling period 2 the start of the temperature sensitive period (early TSP), $n = 15$; sampling period 3 = the end of the TSP (post-TSP), $n = 23$; and sampling period 4 = hatching, $n = 20$.

22). Progesterone was usually undetectable in yolk from recently hatched individuals (stage 26): only 1 of 20 samples registered a detectable level. Although more progesterone was present at 31 °C (mean = 0.760 ± 0.385 ng/g yolk; $n = 6$) than at 27 °C (mean = 0.218 ± 0.111 ng/g yolk; $n = 8$) in the early TSP samples, the differences were not statistically significant (Kolmogorov–Smirnov test, $P = 0.137$). Levels of progesterone at both temperatures declined to similar, near zero, levels during the post-TSP periods (mean at 31 °C = 0.028 ± 0.014 ng/g yolk; $n = 8$; mean at 27 °C = 0.014 ± 0.012 ng/g yolk; $n = 14$).

Testosterone concentrations also changed significantly during development ($F_{3,64} = 3.792$, $P = 0.014$). Mean testosterone (\pm SE) was 0.725 ± 0.137 ng/g yolk at oviposition and declined to 0.355 ± 0.084 ng/g yolk at the beginning of the TSP. Post-TSP testosterone concentrations were 0.372 ± 0.061 ng/g yolk and 0.371 ± 0.066 ng/g yolk at pipping. Post hoc analyses show that sampling period 1 differed significantly from all other sampling periods ($P_s < 0.01$), but that there were no statistically detectable changes in yolk testosterone concentrations among subsequent sampling rounds ($P_s > 0.05$; Fig. 2). There was not a significant effect of incubation temperature on testosterone concentration ($F_{1,54} = 1.227$, $P = 0.273$).

Concentrations of 17 β -estradiol also changed significantly (Kruskal–Wallis test, $H = 44.999$, $P < 0.0001$, $df = 3$). Mean 17 β -estradiol (\pm SE) was 2.378 ± 0.419 ng/g yolk at oviposition. Estradiol was non-detectable for the remainder of development, except that one individual (from different clutches) in each period did have

detectable hormone levels (Fig. 3). Consequently, we were unable to test for any effect of incubation temperature on 17 β -estradiol levels.

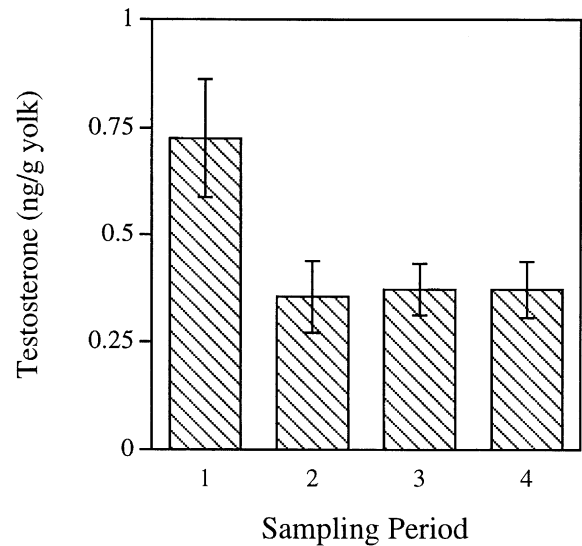


Fig. 2. Mean (\pm SEM) concentrations of testosterone in the yolk of *Trachemys scripta* for the four sampling periods. Sampling period 1 = oviposition, $n = 12$; sampling period 2 the start of the temperature sensitive period (early TSP), $n = 15$; sampling period 3 = the end of the TSP (post-TSP), $n = 23$; and sampling period 4 = hatching, $n = 20$.

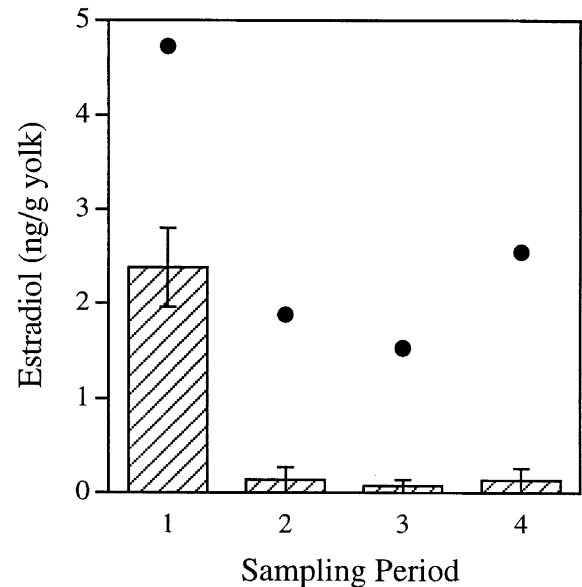


Fig. 3. Mean (\pm SEM) concentrations of estradiol in the yolk of *Trachemys scripta* for the four sampling periods. Circles represent the maximum concentration obtained for a given sampling period. Sampling period 1 = oviposition, $n = 12$; sampling period 2 the start of the temperature-sensitive period (early TSP), $n = 15$; sampling period 3 = the end of the TSP (post-TSP), $n = 23$; and sampling period 4 = hatching, $n = 20$. We performed a χ^2 -test on the number of detectable and undetectable samples due to the highly skewed distribution of the estradiol samples in the last three sampling periods. Sampling period 1 had 11 detectable samples and 1 undetectable sample while sampling periods 2–4 combined had 3 detectable samples and 55 undetectable samples ($\chi^2 = 74.35$; $P < 0.0001$).

4. Discussion

4.1. Patterns of yolk hormone decline

Although progesterone, testosterone, and 17β -estradiol were each present in the yolk at oviposition, they were not present at equivalent levels, nor did they appear to decline at equivalent rates. At oviposition, progesterone was the most abundant of these yolk steroids, followed by 17β -estradiol, and, then, testosterone; this pattern was previously demonstrated for all three hormones in the painted turtle (*C. picta*) and for testosterone and 17β -estradiol in *T. scripta* (Bowden et al., 2000, 2001, 2002). Estradiol was most rapidly depleted, usually reaching non-detectable levels prior to the start of the TSP. Progesterone concentrations decreased progressively with levels falling below the detection limits of the assay by the end of the TSP. Interestingly, testosterone concentrations decreased between oviposition and the start of the TSP, but then did not decline further. We suspect that these declines in yolk steroid levels were due to embryonic metabolism of the steroids, as was demonstrated in coho salmon, *Oncorhynchus kisutch* (Feist et al., 1990). Additionally, preliminary evidence suggests that steroids are not metabolized by the yolk itself, at least not in the alligator (Conley et al., 1997).

We did not detect any significant differences in yolk hormone decline between the two incubation temperatures. We report a differential drop in progesterone between sampling periods 2 and 3; we detected higher levels of progesterone in eggs incubating at 31 °C than at 27 °C at sampling period 2, but by sampling period 3 both had declined to similarly low levels. The timing of this decline is similar to that reported for estradiol in the alligator (Conley et al., 1997). We were unable to examine differences in estradiol across the TSP in *T. scripta* because the major decline in estradiol occurred prior to sampling period 2. Estradiol was largely below the detection limits of our assay for samples collected in periods 2–4.

4.2. A role for endogenous yolk hormones

The current study demonstrates that all three hormones were present in the yolk at oviposition and then declined, with 17β -estradiol declining most rapidly. Conley et al. (1997) suggested that yolk hormones could influence sex determination in species with TSD. We have previously demonstrated a correlation between the ratio of 17β -estradiol to testosterone in the yolk and sex ratios for clutches of *C. picta* (Bowden et al., 2000).

Previous experimental studies of the effects of 17β -estradiol on sex determination have focussed on the role of exogenously applied estradiol. Although these studies have clearly demonstrated the importance of estrogens in sex determination of species with TSD, they have not examined how the applied dosages compare to endoge-

nous hormone concentrations. Exogenous hormone application studies established that relatively minute quantities of hormone can be sufficient to cause a change in sex determination in species with TSD (Wibbels and Crews, 1995). Additionally, one study estimated that less than 0.2% of the exogenously applied steroid has actually been found to penetrate the eggshell (Willingham and Crews, 2000). Therefore, if 1.0 ng of 17β -estradiol is applied to the surface of the eggshell, only roughly 0.002 ng reaches the interior, yet this dosage was enough to cause significant sex reversal when administered at or near the pivotal temperature during the TSP (Wibbels and Crews, 1995). Together, these data illustrate the extraordinary hormonal sensitivity in the pathway that leads to sex determination in reptiles with TSD. These data also suggest that hormone concentrations much lower than those reported here for progesterone and testosterone at the start of the TSP would be sufficient to influence sexual differentiation provided that the appropriate steroidogenic enzymes (i.e., those necessary for conversion to 17β -estradiol) are active at this time.

Recent research on *T. scripta* has revealed that aromatase activity is present at the earliest stage examined (developmental stage 15), the start of the TSP, in the brain, and in the adrenal–kidney–gonad (AKG) complex of both putative males and putative females (Willingham et al., 2000). Other work on *T. scripta* demonstrated the presence of estrogen receptor mRNA (ER mRNA) production in both putative males and putative females at developmental stage 15, with apparent sex-specific differences in ER mRNA production occurring at this stage (Bergeron et al., 1998).

Further, aromatase transcription occurs as early as stage 12 (pre-TSP) in brains of embryonic diamondback terrapins (*Malaclemys terrapin*) at both male and female incubation temperatures (Jeyasuria and Place, 1998). While emphasizing the limited nature of the stage-by-stage data available for endogenous yolk hormone concentrations, aromatase, and ER mRNA activity, we offer three scenarios for how yolk hormones might function to determine sex in reptiles with TSD.

Maternally derived steroids present in yolk might organize the production of steroidogenic enzymes or affect steroid hormone receptor distribution or density. They could also affect the steroidogenic capacity of gonadal primordia, thus indirectly influencing sexual differentiation. Currently, little is known about an embryo's ability to respond to steroidogenic cues early in development. Exogenous steroid application studies conducted early in development (before stage 14) suggest that exogenous steroids applied at this time are not directly altering gonadal sex (Wibbels et al., 1991b); however, other endpoints for steroid activity have not been examined. Recent studies in both the diamondback terrapin and the olive ridley sea turtle (*Lepidochelys olivacea*) suggest that the brain might control gonadal

differentiation, and thus, the brain could be the site for early hormonal effects (Jeyasuria and Place, 1998; Salame-Mendez et al., 1998). In *T. scripta*, whole-embryo hormone analysis revealed that testosterone and estradiol were present at stage 14 (White and Thomas, 1992a), but secretion of these steroids from the AKG complex was not detectable until stage 21 (White and Thomas, 1992b). These data suggest an extragonadal source of testosterone and estradiol prior to the TSP.

Alternatively, maternally derived steroids mobilized from the yolk early in development might be stored, at least partially, by the embryo until it is competent to respond (i.e., during the temperature-sensitive period). As previously mentioned, aromatase activity has been measured in both the brain and AKG complex of embryonic red-eared slider turtles as early as developmental stage 15 (Willingham et al., 2000). The current study demonstrates that peak levels of hormones are present in the yolk much earlier, that is, at oviposition.

Finally, lacking more direct data, it remains possible that maternally derived steroids have no influence on sex determination or sex-determining pathways. The patterns of hormone removal described here might simply be artifacts of yolk utilization by the developing embryo. However, the apparently slow rate of yolk utilization during embryogenesis does not appear to match the rapid depletion of steroids from the yolk. Whereas all three hormones were depleted, the rate of depletion differs among hormones suggesting that the removal is not passive. Additionally, the correlative evidence in a closely related species that high ratios of estradiol to testosterone in recently oviposited clutches correspond to an increased proportion of female offspring (Bowden et al., 2000), suggests a role for hormonal influence on sex determination.

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