

RAPID COMMUNICATION

Yolk Testosterone Varies With Sex in Eggs of the Lizard, *Anolis carolinensis*

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ABSTRACT In the green anole (*Anolis carolinensis*), a lizard with genotypic sex determination, yolk testosterone (T) concentration is greater in male-producing than female-producing eggs at oviposition, but the source and potential effects were not clear from previous studies. If yolk T levels are also sex-specific before eggs are laid, a period during which embryonic steroidogenesis is unlikely, it would strongly suggest that the difference in yolk T is maternally derived. We collected yolk samples from eggs shelling within the oviducts of anesthetized females, and then allowed these females to lay the eggs naturally. Eggs were incubated to hatching to determine sex morphologically, and yolk T concentrations were analyzed by radioimmunoassay. As is the case just after they are laid, yolk T is higher in male than female oviductal eggs. To our knowledge, this is the earliest sex difference reported for any yolk steroid. We suggest that maternally derived yolk T levels could influence sex by differentially affecting male- and female-inducing sperm, because fertilization occurs after yolk deposition and ovulation, while the egg is in the oviduct. Our results, together with those of an increasing number of studies, suggest that a relationship between hormones and vertebrate sex determination may be more widespread than generally appreciated. *J. Exp. Zool.* 295A:206–210, 2003. © 2003 Wiley-Liss, Inc.

INTRODUCTION

Whether fertilization produces a male or female is often thought to occur by chance. However, in some insect, fish, amphibian, and reptile species, environmental conditions during development (e.g., temperature, population density) can bias offspring sex ratios (reviewed in Korpelainen, '90). In these cases, it is not the genetic material inherited by the offspring but rather particular environmental features that initiate whether males or females develop. Perhaps more surprisingly, accumulating data suggest that numerous epigenetic factors can influence offspring sex in species in which sex is determined at fertilization (i.e., species with genotypic sex determination). For example: (1) food availability appears strongly related to offspring sex ratios in Seychelles warblers (Komdeur et al., '97) and zebra finches (Kilner, '98); (2) in Mongolian gerbils and house mice, whether females develop in utero next to male or female littermates influences the sex ratios of offspring that they themselves produce as adults (Clark et al., '93; Vandenbergh and Huggett, '94); and (3) the timing of insemination with respect to ovulation influences offspring sex in numerous mammalian species (reviewed in

James, '96). In these examples, hormones may be proximately involved in the link between parental phenotype and offspring sex (e.g., Krackow, '95; James, '96).

If parental hormone levels can influence offspring sex, then it should be possible to detect hormone differences around the time of ovulation and fertilization and to relate such differences to the sex of the offspring produced. In peafowl, yolk concentrations of the sex steroids androstenedione, testosterone, dihydrotestosterone, and estradiol differ between male-producing and female-producing eggs sampled on day 10 of a 28-day incubation period (Petrie et al., 2001). The authors suggest that these steroid levels reflect differences in maternal input during yolking, which in turn influenced whether male- or female-inducing gametes were ovulated (females are the heterogametic sex in birds). However, since sampling occurred well after the eggs were laid, it is possible

Grant Sponsor: NIH NRSA HD08661 (MBL), NSF IBN-9733074 (JW).

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Received 2 April 2002; Accepted 21 October 2002

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.a.10225

that the embryos themselves were producing sexually dimorphic steroid levels that diffused into the yolk. Although unknown for peafowl, both chicken and Japanese quail embryos are steroidogenically active within the first week of incubation (reviewed in Ottinger et al., 2001).

A sex difference in yolk steroid levels is also present in the green anole (*Anolis carolinensis*), a lizard in which sex is determined genetically, with no influence of incubation temperature (Viets et al., '94). Specifically, male-producing eggs contain higher yolk testosterone (T) than female-producing eggs on the day of oviposition (Lovern et al., 2001; Lovern and Wade, 2001). Yolking follicles produce T (Lovern and Wade, 2001), but whether embryos are steroidogenic at this early stage is unknown. Compared to birds, lizards are well-developed at oviposition, although the gonads and adrenal cortices are still weeks away from morphological differentiation (Forbes, '56; Austin, '88). In the present study, we examined yolk T concentrations of oviductal eggs to see if the sex difference in yolk T found after the eggs are laid is present earlier in development. If yolk T levels are also sex-specific before eggs are laid, when it is even less likely that embryonic steroidogenesis occurs, it would suggest that the sex difference at oviposition is maternally derived. Furthermore, such an early sex difference in yolk T levels would suggest a mechanism by which mothers could influence genetic sex.

MATERIALS AND METHODS

Adult green anoles were purchased from a commercial supplier (Fluker Farms, Port Allen, LA) and housed in the laboratory under conditions to stimulate breeding (Lovern and Wade, 2001). Reproductively active female anoles lay single-egg clutches every 7–14 days alternately from the left and right ovaries (Smith et al., '73; Andrews, '85). We palpated females to determine if they had an oviductal egg, and if so, anesthetized them and made a small (5 mm) ventrolateral incision through the skin and muscle layers to expose the egg. Then, a 6–13 mg yolk sample (<10% total egg mass) was removed by inserting a sterile 25-gauge needle through the oviduct wall and developing egg. After sample removal, the incision was sutured with silk. Yolk samples were diluted in 0.5 ml dH₂O, vortexed, and frozen at –80° C until analysis. All sampled eggs were in the middle to late stages of shell formation, and hence post-fertilization (Conner and Crews, '80).

Following surgery, females were placed individually into 21-liter aquaria furnished with a peat moss substrate, artificial vegetation for climbing and hiding, a shallow water dish, and a small (0.5 liter) plastic nest box filled with moistened peat moss to encourage oviposition. Each day, females were fed vitamin-dusted crickets, cages were sprayed with water, and nest boxes were checked for eggs. We confirmed that oviposited eggs were the ones that were sampled by palpating females the day eggs were found on the appropriate side (left or right, depending on which oviduct contained the sampled, shelling egg). Eggs were placed individually into plastic cups containing a 1:1 (mass) mixture of vermiculite: dH₂O and sealed with plastic wrap and a rubber band. All eggs were incubated at 28° C to hatching (34 days) to determine sex by postanal scale dimorphism (only males have enlarged post-anal scales). In total, we sampled one egg from each of 37 different females, of which 14 hatched (38%; eight males and six females). Although it is not possible to know the exact fertilization-to-sampling intervals, females laid their eggs on average 3.5 days after sampling (range=1–11 days). Given this information and a typical interval between ovulation and oviposition of 4–8 days (e.g., Crews, '80), we estimate that eggs in our study were generally sampled 1–5 days after fertilization. This estimate assumes that fertilization occurs on the same day as ovulation; if the interval between ovulation and fertilization is longer, then yolk sampling may have occurred even closer to fertilization.

Yolk T concentrations were analyzed by radioimmunoassay following extraction and chromatographic purification as previously described (Lovern and Wade, 2001). Samples were run in duplicate in a single assay; the standard curve was run in triplicate. The intra-assay coefficient of variation was 3%. Nonparametric, Kruskal-Wallis tests were used because the data were not normally distributed, and all tests were two-tailed with $\alpha=0.05$.

RESULTS AND DISCUSSION

Male-producing eggs had significantly higher yolk T concentrations than did female-producing eggs ($H=5.1$, $P=0.024$; Fig. 1). The five highest yolk T concentrations came from male-producing eggs, the five lowest came from female-producing eggs, and the four intermediate yolk T concentrations came from a combination of both sexes. This result documents that the sex difference in yolk T

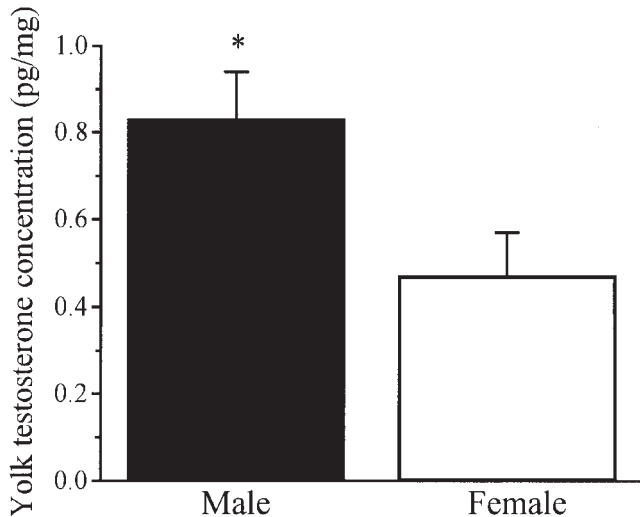


Fig. 1. Mean+SE yolk testosterone (T) concentration in male-producing (N=8) and female-producing (N=6) oviductal eggs of *Anolis carolinensis*. * $P < 0.05$.

present at oviposition (Lovern et al., 2001; Lovern and Wade, 2001) is also present earlier in development, only days after fertilization. To our knowledge, this is the earliest sex difference reported for any yolk steroid. We suggest that maternally derived yolk T levels present at fertilization could influence sex by differentially affecting male- or female-inducing sperm (the mode of genetic sex determination has not been confirmed for *A. carolinensis* specifically, but it is most likely male heterogamety based on karyotypes of other species in the genus; Gorman, '73). Such an effect of T is possible because fertilization occurs—and sex is determined—after yolk deposition and ovulation.

In a potentially related finding, female anoles tend to produce successive eggs alternately from the left and right ovary (Smith et al., '73) and, in *A. carolinensis*, they also alternate the sex of their offspring with successive eggs more frequently than expected by chance (Lovern and Passek, 2002). Although it is not the case that each ovary produces just one sex (Lovern and Passek, 2002), under normal conditions females may have a lateral bias in the production of males and females. If such an asymmetry exists in anoles, it would be similar to a phenomenon found in gerbils, in which females more often develop in the left uterine horn and males more often in the right (Clark and Galef, '90). Clark et al. ('94) confirmed that the ovaries play a role in the lateralization seen in gerbils, and they speculated that differences in ovarian hormone secretions

could affect the fertilization process. Indeed, asymmetries in ovarian sex steroid production have been reported for humans (e.g., Fukuda et al., 2000). In the present study, we addressed whether yolk T concentrations differed depending on whether eggs originated from left or right ovaries, but found no such effect ($H=0.1$, $P=0.76$). However, we may have missed a subtle effect, if one exists, for at least two reasons. First, our sample sizes were small (N=14; 4 left, 10 right) compared to those in Clark and Galef ('90) and Fukuda et al. (2000), whose sample sizes numbered in the hundreds. Second, we measured yolk T from one egg from each female. Thus, our comparison of the T concentrations of eggs originating from left and right ovaries was among rather than within females. Given the variability in yolk T deposition that exists among females (Lovern and Wade, 2001), a within-female examination would be more appropriate for assessing potential lateral differences in yolk T concentrations.

Accumulating research indicates that parental hormone levels can affect offspring sex in diverse taxa with different modes of sex determination; the degree of influence ranges from absolute control to subtle modulation. In oviparous reptiles with temperature-dependent sex determination, in which incubation temperature triggers the development of male or female gonads, yolk steroids can directly override the effects of temperature on sex, and they covary with clutch sex ratios in at least one turtle species (Wibbels et al., '94; Bowden et al., 2000). Similarly in birds, yolk steroids may cause nonrandom ovulation of the sex-determining gamete (Petrie et al., 2001), and hormone manipulations after fertilization can sex-reverse genetic females into gonadal males (Elbrecht and Smith, '92; Wade, 2001). In mammals, including humans, levels of parental hormones and substances that compete with or modify those levels (e.g., endocrine disruptors, fertility drugs) around the time of spermatogenesis and conception may bias offspring sex (e.g., Krackow, '95; James, '96; Mocarelli et al., 2000; Karmaus et al., 2002). We suggest that this relationship between hormones and sex determination may be more widespread than generally appreciated.

It is tempting to try to compare relationships between hormone levels and offspring sex across taxa, especially when patterns appear superficially similar (as described above). However, it is important to remember that if parental hormones

broadly influence sex determination, the specific hormones and mechanisms involved are likely diverse, given species differences in reproductive mode (oviparity, viviparity) and in which sex, if either, contributes the sex-determining gamete. Among the possibilities, steroids may influence the ratio of male- and female-producing ova or sperm available for fertilization, or they may affect the female reproductive tract in such a way as to make it more likely for one sex to be produced than the other (e.g., by altering vaginal pH or mucosity; Krackow, '95).

In *A. carolinensis*, T accumulates in yolk during vitellogenesis, and in parallel is highest in concentration in plasma around the time of ovulation (Lovern and Wade, 2001). Importantly, both yolk T in follicles and plasma T just prior to ovulation are quite variable across individual samples from females (Lovern and Wade, 2001). This variability could facilitate modulation of sex ratios and if so, it may function in an adaptive manner, or it may be an indirect consequence of another role for the hormone. For example, the increased concentration of and variability in yolk T might simply result from the pre-ovulatory rise in ovarian production that is assumed to facilitate female receptive behavior (exogenous T has that effect; Mason and Adkins, '76; Winkler and Wade, '98). Thus, it remains to be determined—for *A. carolinensis* and more generally—whether the hypothesized hormonal influence on offspring sex occurs via parental control, or if such an influence is instead a byproduct, or constraint, of the nearly ubiquitous hormonal regulation of reproduction.

ACKNOWLEDGMENTS

We thank Marc Breedlove for helpful comments on the manuscript, and Erin O'Bryant, Greta Rosen, and Claudia Ruiz for help with animal care.

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