General and Comparative Endocrinology 186 (2013) 67–71

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Yolk-albumen testosterone in a lizard with temperature-dependent sex determination: Relation with development

Victoria Huang [a], Rachel M. Bowden [b], David Crews [a,*]

[a] Department of Ecology, Evolution and Behavior, University of Texas at Austin, TX 78712, USA
[b] School of Biological Sciences, Illinois State University, Normal, IL, USA

ARTICLE INFO

Article history:
Received 2 October 2012
Revised 12 February 2013
Accepted 16 February 2013
Available online 4 March 2013

Keywords:
Yolk
Temperature-dependent sex determination
Testosterone
Reptile
Maternal effects

ABSTRACT

The leopard gecko (*Eublepharis macularius*) exhibits temperature-dependent sex determination as well as temperature-influenced polymorphisms. Research suggests that in oviparous reptiles with temperature-dependent sex determination, steroid hormones in the yolk might influence sex determination and sexual differentiation. From captive leopard geckos that were all from the same incubation temperature regime, we gathered freshly laid eggs, incubated them at one of two female-biased incubation temperatures (26 or 34 °C), and measured testosterone content in the yolk-albumen at early or late development. No differences in the concentration of testosterone were detected in eggs from different incubation temperatures. We report testosterone concentrations in the yolk-albumen were higher in eggs of late development than early development at 26 °C incubation temperatures, a finding opposite that reported in other TSD reptiles studied to date.

1. Introduction

Yolk steroid hormones contribute to phenotypic plasticity by modulating development. The function of yolk steroid hormones has been described as providing information from the environment via maternal effects. In birds, environmental factors correlated with yolk steroid hormone fluctuations include proximity to nesting conspecifics, maternal condition, and egg or clutch laying order (Gilbert et al., 2005; Reed and Vleck, 2001; Groothuis et al., 2005). Yolk steroid hormone differences, natural and manipulated, can contribute to differences in juvenile social rank, begging behavior, and growth rate, as well as changed secondary sexual characteristics and behavior in adults (Adkins-Regan et al., 1995; Bonisoli-Alquati et al., 2011; Partecke and Schwabl, 2008; Schwabl, 1993, 1996; Strasser and Schwabl, 2004). In various lizard species with genotypic sex determination, sex can be correlated or uncorrelated to yolk steroid hormone concentrations (Lovern et al., 2001; Radder et al., 2007). In an Australian montane skink species (*Bassiana duperreyi*), yolk dihydrotestosterone concentrations are higher in smaller eggs, and smaller eggs are more likely to produce males (Radder et al., 2009).

In reptile species with temperature-dependent sex determination (hereafter TSD), gonadal sex is determined by incubation temperature during a thermosensitive period in development. Gene expression patterns in the undifferentiated gonad is initiated by incubation temperature at the beginning of the thermosensitive period, and is sex specific but varies across taxa (Matsumoto and Crews, 2012; Shoemaker et al., 2007). After this window, the development of testes or ovaries is not affected by exogenous hormones or temperature changes (Bull, 1987; Bull et al., 1988). The influence of exogenous steroid hormones on gonadal sex has been studied in various reptile species, mainly turtles. In painted turtles (*Chrysemys picta*), at a 28 °C incubation temperature, the ratio of testosterone:estradiol is correlated with the sex ratio of clutches and season (Bowden et al., 2000). It has been noted in most oviparous species regardless of the mode of sex determination that yolk steroid hormones declined during development (Bowden et al., 2002; Conley et al., 1997; Elf et al., 2002), and the mechanism for yolk estradiol metabolism has recently been described (Paitz et al., 2012). Jeyasuria and Place (1998) proposed that testosterone acts as a substrate for the enzymes aromatase or 5a-reductase that furthers sexual differentiation in the brain, although the source, from local production in the brain, gonads, or yolk, was not specified. Aromatase activity has been found in the whole brain during the thermosensitive period of TSD reptiles, although its function has yet to be determined (Endo et al., 2008; Willingham et al., 2000).

In the leopard gecko (*Eublepharis macularius*), incubation temperature determines gonadal sex; low and high incubation temperatures (26 and 34 °C respectively) produce either only females or female-biased sex ratios, (100 and 95% females, respectively), while an intermediate temperature (32.5 °C) generates a male-biased sex ratio (approximately 75% males) (Viets et al., 1993). The embryo is at developmental stage 28 at oviposition, experiences a thermosensitive period from stages 32–37, and

* Corresponding author. Fax: +1 512 471 6078.
E-mail address: crews@mail.utexas.edu (D. Crews).

0016-6480/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.ygcen.2013.02.019

hatches at stage 42 (Bull, 1987; Wise et al., 2009). Incubation time is temperature-dependent; embryos incubated at lower temperatures spend more time at each stage than those incubated at high temperatures (Bull, 1987; Endo et al., 2008; Tousignant and Crews, 1994; Valleley et al., 2001).

Incubation temperature also contributes significantly to adult leopard gecko intrasexual polymorphisms; males and females from each incubation temperature exhibit significant within-sex variation hereafter called temperature morphs (Sakata and Crews, 2004). Animals of both sexes differ between male- vs. female-biased incubation temperatures, as well as between two female-biased incubation temperatures. Although the 26 °C and 34 °C incubation temperatures produce exclusively or predominantly females, temperature morphs from the 34 °C incubation temperature are more aggressive, reach sexual maturity later, and have higher levels of circulating corticosterone relative to females from the 26 °C incubation temperature, but they do not differ in baseline circulating sex steroid hormones (Flores et al., 1994; Tousignant et al., 1995). The two temperature morphs also differ in the metabolic capacity of hypothalamic regions associated with male-typical behavior (Coomber et al., 1997).

Studies demonstrate that steroid hormones activate sociosexual behaviors in leopard geckos (Sakata and Crews, 2004), and relatively little is known about the levels of steroid hormones in the egg (Elf, 2004; Rhen et al., 2006). Females treated with estradiol *in ovo* did not display significant growth differences at either a female-biased or male-biased incubation temperature (Tousignant and Crews, 1995), though it is known that estradiol can reverse the sex in these species (Bull et al., 1988). Like some other oviparous species, maternal condition does influence yolk steroid hormone concentration; in this species dihydrotestosterone concentration has an inverse relation to female mass depending on the laying season (Rhen et al., 2006).

It is important to note that there is no distinct separation between the yolk and albumen in leopard gecko eggs, therefore the extra-embryonic material is best described as yolk-albumen (YA). The leopard gecko system provides a unique opportunity to look at YA testosterone concentration across development within a sex, at two female-biased incubation temperatures with an 8 °C difference. Because of the large difference in incubation temperatures that both produce putative females, any hormone difference detected would be due to incubation temperature and less likely sex differences.

In this experiment, we measured testosterone concentrations in the YA of eggs incubated at 26 or 34 °C collected in either early or late in development to answer (1) how YA testosterone fluctuates during embryonic development, and (2) if the YA steroid hormone concentration across development is temperature-dependent. We predicted that YA testosterone levels would decrease during development, similar to most amniotes. Finally, we wanted to examine how the hydric state of the YA varied between high and low incubation temperatures across development, as different YA water content may provide a varying microenvironment *in ovo* (Reed and Vleck, 2001). In other TSD species substrate moisture can influence sex determination at low incubation temperatures (Gutzke and Paukstis, 1983). Despite the water exchange-conducive, parchment-like structure of leopard gecko eggshells, we predicted no difference in YA water content between incubation temperatures during early development due to the same initial hydric environment *ex ovo* (Werner, 1972).

2. Methods

2.1. Egg collection

Eggs and samples were collected in accordance of Institutional Animal Care and Use Committee (IACUC) protocol AUP-2008-00135. Eggs were collected from Tremper Leopard Gecko (Boerne, TX, USA), a leopard gecko breeder in July 2009. Within each breeding group, eggs could not be attributed to individual geckos, but all egg-laying females were of the same incubation temperature (26 °C for 2/3 of their development time). Eggs collected and frozen within 4 days of oviposition were considered "oviposition" with embryonic stages to be determined. The eggs were incubated for 7–30 days in a commercial incubator (Precision Instruments, OH, USA) at either 26 or 34 °C. After incubation, eggs were frozen at −20 °C until YA collection. Eggs frozen after seven days incubating at 34 °C and 14 days at 26 °C were categorized as "Early", and after 17 days incubating at 34 °C and 30 days at 26 °C were categorized as "Late". The days of incubation that correspond with early and later developmental stages including the thermosensitive period were estimated from (Bull, 1987) and Endo et al. (2008). Frozen YA was separated from embryos, which were staged according to Wise et al. (2009).

2.2. Radioimmunoassay

The yolk-albumen fraction collected from eggs was used to measure testosterone concentrations using a competitive-binding radioimmunoassay (RIA) with testosterone-specific antibody (Wien Laboratories T3003, Flanders, NJ, USA). The samples were divided into two assays, with each assay containing an equivalent number of samples from each treatment group. The RIA protocol followed that of Schwabl (1993). For each egg, 50 mg YA was weighed and homogenized in 1 ml water. To calculate recoveries, 2000 cpm of tritiated testosterone (Perkins Elmer NET553, Boston, MA USA) was added per sample.

After equilibrating over night, samples were extracted with 6 ml diethyl ether/petroleum ether (70:30, v:v), dried with nitrogen and reconstituted in 1 ml ethanol in −20 °C overnight. To pellet neutral lipids, samples were spun at 2000 rpm for 5 min, supernatants were dried with nitrogen at 37 °C. Samples were then resuspended in 500 ml 10% ethyl acetate in isooctane (2,2,4-trimethylpentane) before being transferred to Celite columns consisting of water phase and a propylene glycol/ethylene glycol (1:1, v:v) phase. After increasing concentrations of ethyl acetate in isooctane, testosterone samples were collected with 4.5 ml of 20% ethyl acetate in isooctane. The average recovery rate of testosterone for the samples was 51%. The intra-assay coefficients of variation were 26.6% and 13% respectively. The interassay coefficient of variation was 26.6%.

2.3. Yolk-albumen dehydration

Because YA water content can vary among eggs, with the possibility of dried YA mass increasing during development (Reed and Vleck, 2001), dried weights of the samples were measured. To determine how YA water content was related to embryonic development or incubation temperature, YA was aliquoted in a preweighed 1.5 ml Eppendorf centrifuge tubes (Eppendorf, Hamburg, Germany), and dried overnight at 45 °C. The YA aliquot and tube were collectively weighed before and after drying. Water content was calculated as the difference between the before and after drying YA weight. The dry YA weight was the difference between the after drying weight and the tube weight. The dry YA weight divided by the aliquot weight gave the proportion of YA as dry weight. This fraction multiplied by 100 gave the percent dry weight. As these aliquots were not taken at the same time as the radioimmunoassay aliquots, some samples for RIA were not available for dehydration.

2.4. Statistics

R: A Language and Environment for Statistical Computing was used for data analyses (R Foundation for Statistical Computing, Vienna, Austria 2012). A Pearson product correlation test was performed on samples of all developmental stages to detect correlation between developmental stage and YA testosterone concentration. Considering YA taken at the thermosensitive period, two factors were considered as factors influential to YA testosterone concentration: development (Early vs. Late) and incubation temperature (26 °C vs. 34 °C). The combination of factors generated four treatment groups: Early26, Early34, Late26 and Late34.

The combinations made four groups and each was normally distributed (Shapiro–Wilk $p > 0.05$). However, sample sizes were taken to account and the non-parametric analysis was used. Kruskal–Wallis with *post hoc* pairwise Wilcoxon rank-sum test with Benjamini-Hochberg corrections was used to detect the effects of development and incubation temperature on YA testosterone and water content (Benjamini and Hochberg, 1995). To detect an influence of development on dry YA weight, a Pearson product correlation test was performed.

3. Results

3.1. Yolk-albumen testosterone concentration

Analysis of our radioimmunoassay data detected a significant difference in YA testosterone concentration between Early and Late development but no difference between low and high female-biased incubation temperatures (Fig. 1). One egg frozen in the "oviposition" category had a developmental stage in the thermosensitive period, and was excluded from analysis of thermosensitive period because it did not undergo an incubation treatment. Some eggs frozen at the "Late" interval (see 2.1) had embryos that had developed beyond the thermosensitive period; these eggs were excluded when considering effects of thermosensitive period.

Including YA testosterone measurements from eggs at all recorded developmental stages (stage 28–41), there was a significant positive correlation between yolk-albumen testosterone concentration and developmental stage ($df = 51$, $p = 4.2e^{-7}$ $r^2 = 0.40$). During the thermosensitive period, YA testosterone concentrations differed among the four treatment groups (Kruskal–Wallis $X^2 = 14.801$, $p = 0.002$). The ranges of stages in Early and Late development were 33 to 35 and 36 to 37, respectively. A *post hoc* pairwise Wilcoxon rank-sum test with Benjamini-Hochberg corrections indicated that the Early vs. Late development difference in YA testosterone concentration was detected at 26 °C but not at 34 °C incubation temperature ($p = 0.003$, and $p = 0.056$, respectively).

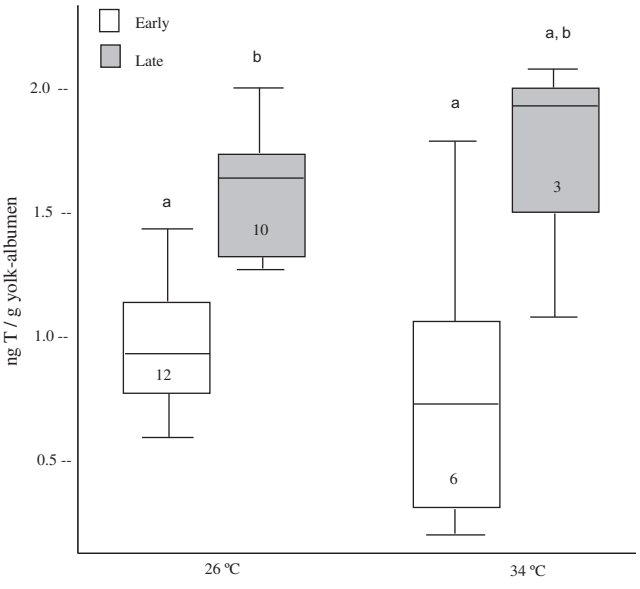

Fig. 1. In the leopard gecko, testosterone concentration in the yolk-albumen is higher in Late vs. Early thermosensitive period at 26 °C incubation temperature. Medians of four treatment groups ±1.5 interquartile ranges are represented; numbers in boxes indicate sample size. Groups not sharing the same letters indicate significant difference ($p < 0.05$).

3.2. Dried yolk-albumen weight

There was a significant positive correlation between the dry YA weight and developmental stage ($df = 50$, $p = 3.4e^{-4}$, $r^2 = 0.23$), but there was no significant difference between 26 or 34 °C incubation temperature at either early or late thermosensitive period (Kruskal–Wallis $X^2 = 0.8487$, $df = 3$, $p = 0.8378$). Nor was there significant correlation during the thermosensitive period between the dry YA weight and YA testosterone concentration at either 26 or 34 °C ($p = 0.085$, $p = 0.16$, respectively).

4. Discussion

In order to describe changes in testosterone across development in the leopard gecko, we measured egg YA testosterone concentrations Early and Late during the thermosensitive period. We had predicted that, according to most other studies of oviparous animals with genetic and temperature-dependent sex determination, testosterone concentration in the YA would decrease during embryonic development, possibly as a consequence of steroid metabolism. However, we detected the opposite, where concentration of testosterone in the YA was greater in eggs at the Late temperature-sensitive period than at the Early temperature-sensitive period, specifically at the 26 °C incubation temperature. There was a similar trend in YA testosterone concentration at 34 °C but the difference between Early vs. Late was not significant. Our data suggest that females of this Eublepharid species with temperature-dependent sex determination experience an increase in the concentration of testosterone in YA during development. In addition, although we detected a loss of YA water content across development, the YA water content remained similar during the thermosensitive period, and did not differ between incubation temperatures.

Testosterone concentration in the YA of eggs did not differ between 26 and 34 °C incubation temperatures during development. These findings complemented other studies of steroid hormone exposure, such as hormone synthesis and sensitivity during development. In leopard gecko embryos from 26 and 34 °C incubation temperatures, steroidogenic enzyme p450scc and androgen receptor expressions are detected in both the brain and gonad-adrenal-mesonephric complex at all stages of the thermosensitive period, but aromatase expression patterns across development are different (Endo et al., 2008). Steroidogenic enzyme aromatase expression is not detected in the gonad-adrenal-mesonephric complex of embryos at 26 °C incubation temperature until the late thermosensitive period, unlike embryos of 34 °C incubation temperature. However, in other TSD species gonadal activity separate from the adrenal and mesonephros have shown a different gene expression timeline (Ramsey and Crews, 2007). Alternative explanations are that behavioral differences in 26 and 34 °C incubation temperature females do not originate during the thermosensitive period, or that the behavioral differences are not organized by testosterone in the YA.

The increase over time in testosterone concentrations in YA suggests an increase in gonad steroidogenesis occurring in Early vs. Late temperature-sensitive period, across five developmental stages. Although YA water content decreased during development, the decrease was not detected during the temperature- sensitive period at either 26 or 34 °C. It is worth noting that the aliquots were taken from the same stock sample but not taken at the same time, but each egg YA was homogenized before collecting the stock sample. Another possible shortcoming is that in Late thermosensitive period samples, the YA becomes vascularized and hormones in circulating blood vessels could potentially contribute to an increase in testosterone concentration. However with frozen samples, it is easy to identify and remove the vessels from YA, so there was minimal input in our samples from circulating steroids present in the vasculature.

In the context of other species, the pattern of increasing YA testosterone is in the minority. In contrast to species with decreasing yolk steroid hormone, it is suggested that developing leopard gecko embryos may be generating testosterone during the thermosensitive period, in addition to metabolizing YA testosterone. A few studies in species with genetic sex determination have reported an increase in steroid hormones in later development, possibly attributing the increase to gonad and adrenal steroidogenesis or decrease in absolute yolk volume (Elf and Fivizzani, 2002; Lovern and Wade, 2001). In other TSD species, estrogen metabolites move between the yolk and embryo, suggesting that other steroid hormones, if conjugated and polar, can also move between yolk and embryo during development (Paitz et al., 2012). Neither the absolute YA volume nor gonadal steroidogenesis for these leopard gecko samples were quantified, so it is possible that both factors can contribute to the measured increase in testosterone concentration across development. Recently, the yolk testosterone level of another gecko species with TSD (*Gekko japonicus*) has been described across development among different incubation temperatures, with similar results; the two female-biased incubation temperatures did not differ in yolk testosterone levels, but both experienced an increase in testosterone from 1/3 to 2/3 of their incubation time (Ding et al., 2012). It was suggested that the increased yolk testosterone concentration followed a proposed mechanism in the GSD green anole (*Anolis carolinensis*), that nonpolar steroids generated by the embryo enter the lipophilic yolk (Lovern and Wade, 2003), but steroidal movement is also possible in a conjugated state (Paitz et al., 2012). Further work is needed to determine whether the observed increase in YA testosterone is the product of embryonic steroidogenesis, and to compare the mechanisms between opposing changes in YA steroid hormones.

In summary, the most important finding was that the YA testosterone concentration increased during development in eggs, specifically at the female-biased incubation temperature of 26 °C. This is an uncommon examination of within-sex variation of YA hormones in a species with TSD where a developmentally dependent increase was found, that there was no incubation temperature difference in YA testosterone concentration, even considering a decrease in YA water content during development. Because it is possible to separate sex and temperature effects in this system, future studies focused on determining the source of YA testosterone may be particularly interesting.

Acknowledgments

Research supported by UT EEB and NSF GRFP to VH and NSF IOS 0750938 to DC. Thanks to Ron Tremper, Yuiko Matsumoto, Sarah Simmonds, Christine Tran, Sandrine Clairardin, and Kelsey Coto for help in gecko egg and yolk collection, embryo staging, and radioimmunoassay.

References

Adkins-Regan, E., Ottinger, M.A., Park, J., 1995. Maternal transfer of estradiol to egg yolks alters sexual differentiation of avian offspring. J. Exp. Zool. 271, 466–470.
Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. B Meth. 57, 289–300.
Bonisoli-Alquati, A., Matteo, A., Ambrosini, R., Rubolini, D., Romano, M., Caprioli, M., et al., 2011. Effects of egg testosterone on female mate choice and male sexual behavior in the pheasant. Horm. Behav. 59, 75–82.
Bowden, R.M., Ewert, M.A., Nelson, C.E., 2000. Environmental sex determination in a reptile varies seasonally and with yolk hormones. Proc. R. Soc. Lond. B 267, 1745–1749.
Bowden, R.M., Ewert, M.A., Nelson, C.E., 2002. Hormone levels in yolk decline throughout development in the red-eared slider turtle (*Trachemys scripta elegans*) Gen. Comp. Endocrinol. 129, 171–177.
Bull, J.J., 1987. Temperature-sensitive periods of sex determination in a lizard: similarities with turtles and crocodilians. J. Exp. Zool. 241, 143–148.
Bull, J.J., Gutzke, W.H.N., Crews, D., 1988. Sex reversal by estradiol in three reptilian orders. Gen. Comp. Endocrinol. 70, 425–428.
Conley, A.J., Elf, P., Corbin, C.J., Dubowsky, S., Fivizzani, A., Lang, J.W., 1997. Steroids decline during sexual differentiation in the alligator. Gen. Comp. Endocrinol. 107, 191–200.
Coomber, P., Crews, D., Gonzalez-Lima, F., 1997. Independent effects of incubation temperature and gonadal sex on the volume and metabolic capacity of brain nuclei in the leopard gecko (*Eublepharis macularius*), a lizard with temperature-dependent sex determination. J. Comp. Neurol. 380, 409–421.
Ding, G.-H., Yang, J., Wang, J., Ji, X., 2012. Offspring sex in a TSD gecko correlates with an interaction between incubation temperature and yolk steroid hormones. Naturwissenschaften 99, 999–1006.
Elf, P., Lang, J., Fivizzani, A., 2002. Dynamics of yolk steroid hormones during development in a reptile with temperature-dependent sex determination. Gen. Comp. Endocrinol. 127, 34–39.
Elf, P.K., Fivizzani, A.J., 2002. Changes in sex steroid levels in yolks of the leghorn chicken, *Gallus domesticus*, during embryonic development. J. Exp. Zool. 293, 594–600.
Elf, P.K., 2004. Yolk steroid hormones and their possible roles in TSD species. In: Valenzuela, N., Lance, V. (Eds.), Temperature-dependent Sex Determination in Vertebrates. Smithsonian, Washington DC, pp. 111–118.
Endo, D., Kanaho, Y.-I., Park, M.K., 2008. Expression of sex steroid hormone-related genes in the embryo of the leopard gecko. Gen. Comp. Endocrinol. 155, 70–78.
Flores, D., Tousignant, A., Crews, D., 1994. Incubation temperature affects the behavior of adult leopard geckos (*Eublepharis macularius*). Physiol. Behav. 55, 1067–1072.
Gilbert, L., Rutstein, A.N., Hazon, N., Graves, J.A., 2005. Sex-biased investment in yolk androgens depends on female quality and laying order in zebra finches (*Taeniopygia guttata*). Naturwissenschaften 92, 178–181.
Gutzke, W.H., Paukstis, G.L., 1983. Influence of the hydric environment on sexual differentiation of turtles. J. Exp. Zool. 226, 467–469.
Jeyasuria, P., Place, A.R., 1998. Embryonic brain-gonadal axis in temperature-dependent sex determination of reptiles: a role for P450 aromatase (CYP19). J. Exp. Zool. 281, 428–449.
Lovern, M.B., McNabb, F.M.A., Jenssen, T.A., 2001. Developmental effects of testosterone on behavior in male and female green anoles (*Anolis carolinensis*). Horm. Behav. 39, 131–143.
Lovern, M.B., Wade, J., 2001. Maternal plasma and egg yolk testosterone concentrations during embryonic development in green anoles (*Anolis carolinensis*). Gen. Comp. Endocrinol. 124, 226–235.
Lovern, M.B., Wade, J., 2003. Sex steroids in green anoles (*Anolis carolinensis*): uncoupled maternal plasma and yolking follicle concentrations, potential embryonic steroidogenesis, and evolutionary implications. Gen. Comp. Endocrinol. 34, 109–115.
Matsumoto, Y., Crews, D., 2012. Molecular mechanisms of temperature-dependent sex determination in the context of ecological developmental biology. Mol. Cell. Endocrinol. 354, 103–110.
Paitz, R.T., Sawa, A.R., Bowden, R.M., 2012. Characterizing the metabolism and movement of yolk estradiol during embryonic development in the red-eared slider (*Trachemys scripta*). Gen. Comp. Endocrinol. 176, 507–512.
Partecke, J., Schwabl, H., 2008. Organizational effects of maternal testosterone on reproductive behavior of adult house sparrows. Dev. Neurobiol. 68, 1538–1548.
Radder, R., Ali, S., Shine, R., 2007. Offspring sex is not related to maternal allocation of yolk steroids in the lizard *Bassiana duperreyi* (Scincidae). Physiol. Biochem. Zool. 80, 220–227.
Radder, R.S., Pike, D.A., Quinn, A.E., Shine, R., 2009. Offspring sex in a lizard depends on egg size. Curr. Biol. 19, 1102–1105.
Ramsey, M., Crews, D., 2007. Adrenal–kidney–gonad complex measurements may not predict gonad-specific changes in gene expression patterns during temperature-dependent sex determination in the red-eared slider turtle (*Trachemys scripta elegans*). J. Exp. Zool. 307A, 463–470.
Reed, W., Vleck, C., 2001. Functional significance of variation in egg-yolk androgens in the American coot. Oecologia 128, 164–171.
Rhen, T., Crews, D., Fivizzani, A., Elf, P., 2006. Reproductive tradeoffs and yolk steroids in female leopard geckos, *Eublepharis macularius*. J. Evol. Biol. 19, 1819–1829.
Sakata, J.T., Crews, D., 2004. Developmental sculpting of social phenotype and plasticity. Neurosci. Biobehav. Rev. 28, 95–112.

Schwabl, H., 1993. Yolk is a source of maternal testosterone for developing birds. PNAS 90, 11446–11450.
Schwabl, H., 1996. Maternal testosterone in the avian egg enhances postnatal growth. Comp. Biochem. Phys. A 114, 271–276.
Shoemaker, C., Ramsey, M., Queen, J., Crews, D., 2007. Expression of *Sox9*, *Mis*, and *Dmrt1* in the gonad of a species with temperature-dependent sex determination. Dev. Dyn. 236, 1055–1063.
Strasser, R., Schwabl, H., 2004. Yolk testosterone organizes behavior and male plumage coloration in house sparrows (*Passer domesticus*). Behav. Ecol. Sociobiol. 56, 491–497.
Groothuis, T.G.G., Muller, W., von Engelhardt, N., Carere, C., Eising, C., 2005. Maternal hormones as a tool to adjust offspring phenotype in avian species. Neurosci. Biobehav. Rev. 29, 329–352.
Tousignant, A., Crews, D., 1994. Effect of exogenous estradiol applied at different embryonic stages on sex determination, growth, and mortality in the leopard gecko (*Eublepharis macularius*). J. Exp. Zool. 268, 17–21.
Tousignant, A., Crews, D., 1995. Incubation temperature and gonadal sex affect growth and physiology in the leopard gecko (*Eublepharis macularius*), a lizard with temperature-dependent sex determination. J. Morphol. 224, 159–170.
Tousignant, A., Viets, B., Flores, D., Crews, D., 1995. Ontogenetic and social factors affect the endocrinology and timing of reproduction in the female leopard gecko (*Eublepharis macularius*). Horm. Behav. 29, 141–153.
Valleley, E.M.A., Cartwright, E.J., Croft, N.J., Markham, A.F., Coletta, P.L., 2001. Characterisation and expression of Sox9 in the leopard gecko, *Eublepharis macularius*. J. Exp. Zool. 291, 85–91.
Viets, B.E., Tousignant, A., Ewert, M.A., Nelson, C.E., Crews, D., 1993. Temperature-dependent sex determination in the leopard gecko, *Eublepharis macularius*. J. Exp. Zool. 265, 679–683.
Werner, Y.L., 1972. Observations on eggs of eublepharid lizards, with comments on the evolution of the Gekkonoidea. Zool. Meded. 47, 221–224.
Willingham, E., Baldwin, R., Skipper, J.K., Crews, D., 2000. Aromatase activity during embryogenesis in the brain and adrenal–kidney–gonad of the red-eared slider turtle, a species with temperature-dependent sex determination. Gen. Comp. Endocrinol. 119, 202–207.
Wise, P.A.D., Vickaryous, M.K., Russell, A.P., 2009. An embryonic staging table for in ovo development of *Eublepharis macularius*, the leopard gecko. Anat. Rec. 292, 1198–1212.