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

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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

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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.