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RESEARCH ARTICLE

HATCHLING SEX IS NOT CORRELATED WITH THE MATERNAL ALLOCATION OF SEX STEROIDS AT OVIPOSITION IN THE LIZARD, *CALOTES VERSICOLOR*

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ABSTRACT

In reptiles exhibiting temperature-dependent sex determination (TSD), maternally derived yolk steroids may play a role in the sex determination. The present study explores the correlation, if any, existing between hatchling sex ratio and maternally derived yolk steroid hormone concentrations [17β-estradiol (E_2) and testosterone (T)] in the lizard, *Calotes versicolor,* which exhibits a unique FMFM (Female-Male-Female-Male) pattern of temperature-dependent sex determination. Ten clutches of *C. versicolor* eggs were collected and incubated at two male-producing (MPTs) (25.5±0.5°C & 34±0.5°C), two female-producing temperatures (FPTs) (23.5±0.5°C & 31.5±0.5°C) and at pivotal temperature (28.5±0.5°C). The yolk material was collected from eggs at oviposition (stage 27) as well as at hatching (stage 42) and homogenized. Subsequent to extraction of steroids from yolk, the concentrations of E_2 & T were measured by ELISA using specific antibodies for each hormone. Results reveal a noteworthy within season inter-clutch and negligible intraclutch variation in both the yolk steroid hormone (E_2 &T) concentrations among the 10 clutches. At oviposition, the steroid profile reveals that the level of T was much higher than that of E_2 in all the clutches examined. However, at hatching concentration of E_2 was greater than that of T. Eggs incubated at low FPT (23.5±0.5°C) and low MPT (25.5±0.5°C) had low steroid hormone concentrations when compared to the eggs incubated at high FPT (31.5±0.5°C) and MPT (34±0.5°C). Intermediate levels of yolk steroids were observed in eggs incubated at pivotal temperature (28.5±0.5°C). Hence, it is emphasized that high temperature has stimulatory effect on yolk steroid levels in this species. Therefore, based on these findings we conclude that the sex ratios are rather temperature dependent than on the maternal allocation of steroids at oviposition.

INTRODUCTION

In many vertebrate species, sex is determined at fertilization of zygotes by sex chromosome composition, known as genotypic sex determination (GSD). In some but not several other species of vertebrates such as fish and reptiles, sex determination does not merely hinge on the combination of sex chromosomes alone, but is influenced by environmental and social factors as well. Notably, the gonadal sex in most turtles, few lizards and in all crocodiles is determined via a temperature cue during temperature sensitive period (TSP) referred to as temperature-dependent sex determination (TSD) (Bull,'80, '87; Valenzuela and Lance, 2004; Ramsey and Crews, 2009; Nakamura, 2010). The involvement of sex steroid hormones (E_2 and T) or hormone precursors present in the embryonic gonads (Rew: Pieau *et al*., 1999, 2001; Pieau and Dorizzi, 2004; Ramsey and Crews, 2009) and brain (Jeyasuria and Place, 1998; Place *et al*., 2001) have been reported to influence sex determination in TSD reptiles. However, during the past decade maternally derived steroid hormones in the egg yolk of reptiles have been the focus of attention for their possible role in sex determination (Elf, 2003, 2004). Since the endocrine system develops relatively late in embryonic life, it is generally accepted that hormones of maternal origin direct much of the early development in oviparous species (Rew: Radder, 2007). The earlier reports on TSD reptiles also suggested that maternally derived yolk steroids are believed to be the primary hormone reservoir influencing a variety of aspects of development (Elf *et al*., 2002a) including offspring sex (Conley *et al*., '97; Bowden *et al*., 2000, 2002; Lovern and Wade, 2003; Elf, 2003, 2004; Kratochvil *et al*., 2008). However, more recent studies have cast noteworthy doubt on their role in sex determination, for instance, no correlation between the yolk steroid hormone concentration and sex ratio is noticed in the lizards, *Bassiana duperreyi* (Radder *et al*., 2007); *Amphibolurus muricatus* (Warner *et al*., 2007) and in snapping turtle, *Chelydra serpentina* (St Juliana *et al*., 2004). Hence, it is interesting to study the interplay between incubation temperature, yolk steroids and the offspring sex in the Indian oviparous lizard, *Calotes versicolor* which lacks sex chromosomes (Singh, 1974) and exhibits a potentially unique FMFM (Female-Male-Female-Male) pattern of TSD. The Incubation temperatures of 23.5±0.5 and 31.5±0.5°C produce 100% females and incubation temperatures of 25.5±0.5 and 34±0.5°C produce 100% males. Intermediate temperatures produce both the sexes with different % of males and females (Inamdar *et al*., 2012a). The Thermo-sensitive period (TSP) for gonadal sex differentiation occurs during the embryonic stages 30-33 which coincides with early stages of gonadal sex differentiation. In view of the above described scientific background and rationale the present investigation aims to focus on the following objectives.

- to explore the magnitude of inter- vs. intraclutch variation in the yolk steroid hormone levels in *Calotes versiolor*
- to understand correlations, if any, exist between maternal allocation of yolk steroid hormone concentrations at oviposition and offspring sex/ sex ratio,

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MATERIALS AND METHODS

Egg collection and incubation

Calotes versicolor, a polyautochronic multiclutched lizard, has an extended breeding phase (May - October) and retains its eggs in the oviduct for about 2 weeks. Gravid female lizards of *C. versicolor* possessing oviductal eggs were caught during the breeding season from the areas around Dharwad (15°17'N, 75°3'E), Karnataka, India. They were maintained in reptile dwelling 20x20x10′covered with mesh on all sides. Food (grasshoppers/silkworms/cockroaches) and water were supplied *ad libitum*. CPCSEA guidelines for care and use of animals in scientific research were followed and approved by Institutional Animal Ethical Committee (IAEC). In total, Ten (10) clutches of freshly laid eggs (N = 180) were collected from *C. versicolor* and incubated at two male-producing (100% MPTs) (25.5±0.5°C & 4±0.5°C), two female-producing (100% FPTs) (25.5±0.5°C & 31.5±0.5°C) temperatures and at pivotal temperature (28.5±0.5°C) which yields 1:1 sex ratio. Incubation temperatures were monitored and recorded twice daily using mercury thermometer. Other incubation details have already been described (Doddamani, 1994; 2006; Vani, *et al*., 2010; Inamdar *et al*., 2012a). Eggs were dissected at regular intervals to assess developmental stage as per the criteria adopted for *C. versicolor* (Muthukkaruppan *et al*., 1970). The eggs were incubated from oviposition till hatching (stage 42) stage at all 5 incubating temperatures. Determination of sexual phenotypes. The cloacal sexing (presence vs. absence of hemipenis) was performed prior to yolk collection in order to distinguish the sex of embryo (Inamdar *et al*., 2012b). Further, this sexing method was 100% congruent with gonadal histology. Reliability of sex was also verified by gonadal histology and the presence of secondary sexual characteristics.

Yolk steroid hormone extraction and analysis

Yolk T and E_2 concentrations of the initial (at oviposition- stage 27– prior to incubation) and final yolk samples (at hatching-stage 42) were extracted based on a previously established and validated protocol (Schwabl, 1993). At sacrifice, the whole yolk was collected, weighed and a sample of 1 gm each was suspended in 500 µl distilled water. All the samples were vortexed and allowed to equilibrate overnight at 4°C. The steroid hormones were then extracted from the samples using petroleum and diethyl ethers and reconstituted in 90% ethanol (Schwabl, 1993). The extraction of T and E_2 and other protocol have been already reported for this species (Vani *et al*., 2010). After extraction the steroid hormone (E_2 and T) concentrations were measured by ELISA using antibodies specific for each hormone (Equipar Diagnostics, Saronno, Italy). The E_2 and T concentrations were assayed in duplicate and compared to a standard curve with concentrations ranging from 2 to 16ng/ml for T and from 20 to 8000 pg/ml for E_2. Average inter- assay Coefficients of Variation (CVs) were 3.2% and 3.9%, and average intra-assay CVs were 5.4% and 6.2 % for E_2 and T respectively. Yolk steroid levels are expressed as ng/ml.

Data analysis

Data were expressed as the mean ± S.E. Yolk steroid levels at oviposition were log transformed to meet the assumptions of normality, and analyzed by one-way ANOVA, followed by Tukey's HSD post hoc test (Sokal and Rohlf, 1995) to evaluate the inter-clutch variation. Yolk E_2 and T levels female and male hatchlings were also analyzed by one-way ANOVA, followed by Tukey's HSD post hoc test. The possible sex differences in yolk steroid levels were analyzed by Unpaired *t* Test or independent sample *t*-Test. Actual values (untransformed data) of E_2 and T (ng/ml) are presented in graphs (Figs.1, 2 and 3). All statistical analysis was performed by SPSS statistical package for windows (version 16.0). Statistical significance level was accepted at P<O.05.

RESULTS

The variation in yolk steroid hormone levels of E_2 and T at oviposition (Stage 27)

In the present study a significant inter-clutch variation in the yolk E_2 (F=1.245, df=9, P<0.05) and T (F=152.098, df=9, P<0.05) concentrations was noticed among 10 clutches of *C. versicolor* revealing a clutch specific hormone levels at oviposition. The yolk E_2 concentration varied from minimum of 0.18 ng/ml to maximum of 2.10ng/ml. and yolk T from minimum of 0.38 ng/ml to 4.0 ng/ml. A noteworthy inter-clutch variation and negligible intraclutch variation was observed in yolk steroid concentrations among the clutches of the different females (Fig.1). Nevertheless, a much elevated mean initial level of T was observed than that of E_2 concentrations in all the clutches examined (Fig. 2).

Fig. 1 Maternal yolk steroid hormone profile (E2 and T) among the females of *C. versicolor* at oviposition

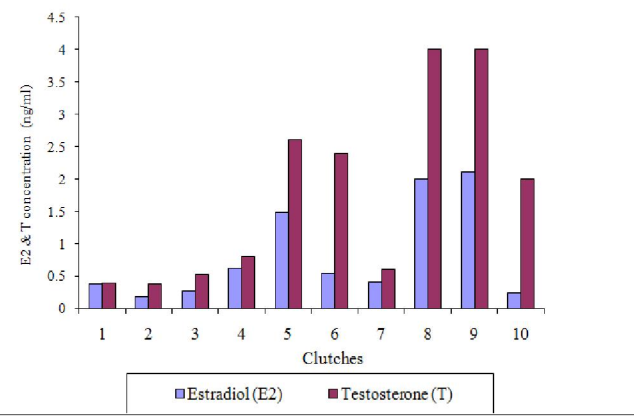
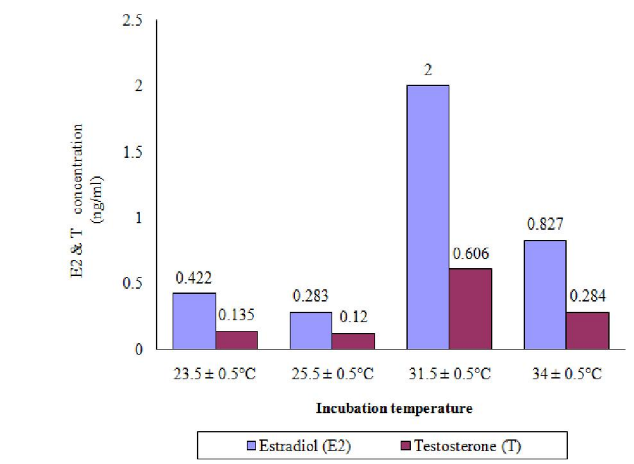


Fig. 2 Yolk steroid hormone profile (E_2 & T) at hatching. Eggs incubated at two MPTs (25.5 ± 0.5°C and 34 ± 0.5°C) and two FPTs (23.5 ± 0.5°C and 31.5 ±

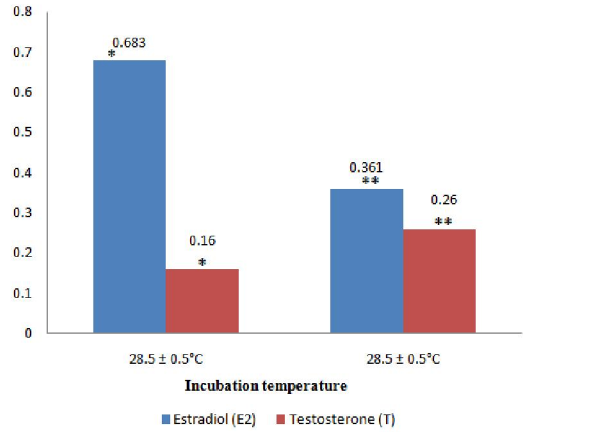


The yolk steroid hormone levels (E_2 and T) at hatching (Stage 42)

It was noted that yolk E_2 concentration increased greatly from oviposition till hatching whereas yolk T concentration declined significantly from oviposition stage to hatching in the both MPT and FPTs, as well as at pivotal temperatures (Figs.2 and 3). Significant difference in yolk E_2 and T concentrations were found in the eggs incubated at 2FPTs and pivotal temperatures (F=1.649, df=2, P<0.05 for E_2; F=1.148, df=2, P<0.05 for T) and in the eggs (male embryos) incubated at 2MPTs and pivotal temperatures (F=4.247, df=2, P<0.05 for E_2; F=71. 684, df=2, P<0.05 for T). Also at hatching, irrespective of the incubating temperature a significant sex difference in yolk steroid concentrations was observed (Unpaired *t*-Test. t= 2.781; F=33.966; df=28; P<0.05 for E_2; t= 1.295; F=39.447; df=28; P<0.05

for T) (Fig. 2). The Eggs incubated at high FPT (31.5±0.5°C) and at high MPT (34±0.5°C) had high yolk steroid concentrations when compared to the eggs incubated at low FPT (23.5±0.5°C) and at low MPT (25.5±0.5°C) (Fig. 2). Intermediate levels of yolk steroids were observed in eggs incubated at pivotal temperature (Fig. 3). The observed results revealed that high incubation temperature has stimulatory effect on both the hormones irrespective of the embryonic sex. Further it is interesting to note that at hatching yolk E_2 concentration was greater than that of T concentrations in all the clutches examined. Overall our results suggest that there is no significant correlation between steroid hormone concentration of embryos at oviposition and offspring sex in this species (Fig. 2).

Fig. 3 Yolk steroid hormone sprofile (E_2 & T) at hatching. Eggs incubated at pivotal temperatures(28.5 ± 0.5°C) * female embryos , ** male embryos



DISCUSSION

Maternal hormones in birds and reptilian eggs have recently received much attention, since they represent an intriguing pathway for maternal effects (Rew: Elf, 2003; 2004; Radder, 2007).

Inter-clutch variation in yolk steroid levels at oviposition

In the present study, the significant difference in initial levels of both yolk hormones (E_2 and T) noticed among all 10 clutches of different females. A high inter-clutch and negligible intraclutch variation observed in the E_2 and T concentrations reveal that the natural seasonal variations in yolk hormone levels of females might be responsible for these differences. An inter-clutch variation in concentrations of T and E_2 noted among different females of alligator, *Alligator mississippiensis* (Conley *et al*., '97; Elf *et al*., 2001); turtles, *Chelydra serpentina* and *Chrysemys picta* (Elf *et al*., 2002; Elf, 2003) supports our interpretations. Conversely, in some species of Eublepharidae family (Kratochvil *et al*., 2006; Rhen *et al*., 2006) a conspicuous low intra-clutch variation in yolk steroid concentration has also been detected. Further in *C. versicolor* the initial concentration of T was greater than that of E_2 levels in all the clutches examined is similar to that reported in lizard species, *Amphibolurus muricatus* (Warner *et al*., 2007); *Paroedura picta* (Kratochvil *et al*., 2006) and in turtle, (*Emydoidea blandingii*) (Elf, 2004). On the contrary, clutches of *Alligator mississippiensis* (Elf, 2003, 2004); *Chelydra serpentine*; *Chrysemys picta* (Elf *et al*., 2002; Elf, 2003; 2004); the lizards, *Eublepharis macularius* (Elf, 2004); *Bassiana duperreri* (Radder *et al*., 2007) and also in most of the turtles species studied to date (Janzen *et al*., 1998; Elf, 2003, 2004) exhibited greater E_2 concentration than T at the time of laying. Hence, we propose that clutch effect is common within species as well as among the species.

Sex difference in yolk steroid levels at hatching

Incubation temperature exhibits differential effect on yolk steroid levels as a consequence clutch effects persist even at hatching. Besides, eggs incubated at high FPT (31.5±0.5°C) and MPT (34±0.5°C) had highest level of yolk steroid concentrations when compared to the eggs incubated at low FPT (23.5±0.5°C) and MPT (25.5±0.5°C). Intermediate levels of yolk steroids are observed in eggs incubated at pivotal temperature (28.5±0.5°C). This indicates that high temperature has stimulatory effect on both the yolk hormone levels in turn suggesting that the incubation temperature determine the hormonal milieu. It is important to note that irrespective of initial allocation of maternal yolk steroids an elevated E_2 concentration and a decline in T level is noticed at hatching. The observed results indicate that there is not significant correlation between yolk steroid hormone concentration of embryos at oviposition and hatchling sex in this species. Otherwise only males would have hatched from the eggs with high T level at the time of oviposition which is not the case. Similarly more recent studies cast ample doubt on yolk steroids' role in sex determination, for instance, maternal allocation of steroids is not related to the offspring sex in the lizards, *Bassiana duperreyi* (Radder *et al*., 2007); *Amphibolurus muricatus* (Warner *et al*., 2007) and in snapping turtle, *Chelydra serpentina* (St Juliana *et al*., 2004). These studies emphasize that yolk steroids of maternal origin are not critical for sex determination in these species. Based on the yolk steroid hormone profile and gonadal histology we propose that hatchling sex is not correlated with the maternal allocation of steroids at oviposition. Hence it is concluded that in *C. versicolor* sex ratios are rather temperature-dependent than on the maternal allocation of steroids at oviposition.

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