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Yolk hormone levels in the synchronously developing eggs of *Paroedura picta*, a gecko with genetic sex determination

Lukáš Kratochvíl, Lukáš Kubička, and Eva Landová

Abstract: Sex steroid hormones of presumably maternal origin have been found in yolk of many oviparous vertebrates. Their effects on behavioural or physiological traits are well documented in birds, but until now are largely unknown in reptiles. The investigations of yolk steroids in reptiles have been focused mainly on species with temperature-dependent sex determination, where steroid levels are suggested to determine the sex of progeny. Here we report initial oestradiol (E2) and testosterone (T) levels in the yolk of the Madagascar ground gecko, *Paroedura picta* (Peters, 1854), a species with genetic sex determination. The yolk concentration was 0.39 ± 0.02 ng/g (mean ± SE) in E2, whereas the concentration of T was much higher (1.48 ± 0.06 ng/g, mean ± SE). Geckos usually lay two exceptionally large eggs per clutch; vitellogenesis and ovulation of both eggs proceed in phase. Individual two-egg clutches differed considerably in E2 and T levels. A clutch mean of E2 levels varied from 0.22 to 0.53 ng/g, whereas T levels varied from a clutch mean of 1.02 to 1.99 ng/g. Both eggs in a clutch possessed very similar levels of E2 and T. Initial yolk steroid levels thus presumably reflect maternal conditions during egg formation rather than differential allocation of hormones according to offspring sex.

Résumé : On trouve des hormones stéroïdes sexuelles d'origine maternelle présumée dans le jaune des oeufs de plusieurs vertébrés ovipares. Leurs effets sur les caractéristiques comportementales et physiologiques sont bien connus chez les oiseaux, mais jusqu'à maintenant peu étudiés chez les reptiles. Les études sur les stéroïdes du jaune chez les reptiles se sont surtout intéressées aux espèces à détermination sexuelle dépendante de la température, chez lesquelles on a proposé que les concentrations de stéroïdes déterminent le sexe des rejetons. Nous présentons ici les données sur les concentrations initiales d'estradiol (E2) et de testostérone (T) dans le jaune du gecko terrestre de Madagascar, *Paroedura picta* (Peters, 1854), une espèce à détermination sexuelle génétique. La concentration d'E2 dans le jaune est de 0,39 ± 0,02 ng/g (moyenne ± erreur type) et celle de T est beaucoup plus importante à 1,48 ± 0,06 ng/g (moyenne ± erreur type). Les geckos produisent généralement deux oeufs particulièrement gros par ponte; la vitellogenèse et l'ovulation des deux oeufs sont synchronisées. Les pontes individuelles de deux oeufs diffèrent considérablement par leurs concentrations de E2 et de T. Les concentrations moyennes de E2 des pontes varient de 0,22 à 0,53 ng/g et celles de T de 1,02 à 1,99 ng/g. Les deux oeufs d'une même ponte ont des concentrations très semblables d'E2 et de T. Les concentrations initiales de stéroïdes dans le jaune semblent donc représenter les conditions maternelles durant la formation des oeufs plutôt qu'une allocation différentielle des hormones en fonction du sexe du rejeton.

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Introduction

Steroid hormones are a common yolk component in many vertebrates (Schwabl 1993; Conley et al. 1997; Janzen et al. 1998). Although a number of recent papers documented their effects on behavioural or physiological traits in birds (usually a positive effect on individual fitness is reported, e.g., Schwabl 1993; Gorman and Williams 2005; but see Sockman and Schwabl 2000), their quantity and role in the formation of phenotype in reptiles are still largely unknown. The investigations of yolk steroids in reptiles have been focused mainly on species with temperature-dependent sex determination (TSD), where steroid levels are suggested to determine the sex of progeny (Bowden et al. 2000; Elf 2003; cf. St. Juliana et al. 2004). Much less investigated is the role of yolk steroids in reptiles with genetic sex determination (GSD).

In spite of the recent interest in yolk steroid hormones, we have not yet had any unequivocal proof of their origin. Two alternatives were proposed: steroid hormones were suggested to be deposited to yolk either locally from the cells of the follicular wall (Hackl et al. 2003) or from the female

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L. Kratochvíl[1] **and L. Kubička.** Faculty of Science, Department of Ecology, Charles University in Prague, Viničná 7, 128 44 Praha 2, Czech Republic.
E. Landová. Faculty of Science, Department of Zoology, Charles University in Prague, Viničná 7, 128 44 Praha 2, Czech Republic.

[1]Corresponding author (e-mail: lukkrat@email.cz).

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systemic circulation. The correlation between circulating female hormone levels and initial quantities of steroids in yolk of eggs formed at the same time in birds and turtles (e.g., Adkins-Regan et al. 1995; Janzen et al. 2002; Williams et al. 2005) was taken as an indication for the latter possibility. Williams et al. (2005) cited two papers (Painter et al. 2002; Lovern and Wade 2003*a*) as evidence that the maternal and yolk steroid levels are uncoupled in lizards. However, Painter et al. (2002) only found that female plasma and yolk steroid levels are quantitatively different. Instead, they reported slight but significant increases in yolk progesterone levels after massive experimental elevation of maternal progesterone levels in a viviparous lizard, *Sceloporus jarrovi* Cope in Yarrow, 1875. In an oviparous congener (*Sceloporus graciosus* Baird and Girard, 1852), the same authors did not find a correlation between maternal and embryonic or hatchlings' steroid levels, but they did not test the relationship between maternal and initial yolk hormone levels. Lovern and Wade (2003*a*) reported uncoupled maternal plasma and yolk oestradiol (E2) and testosterone (T) concentrations in eggs and developing follicles in the green anole (*Anolis carolinensis* Voigt, 1832), but they tested the correlation between maternal plasma and yolk steroid levels in eggs and follicles at different stages of development. As E2 and T concentrations in yolk strongly depend on the phase of follicle/egg development (Lovern and Wade 2001, 2003*a*; Elf et al. 2002), we should restrict a test of correlation between maternal and yolk levels to the same developmental stage. The paper by Lovern and Wade (2003*a*) thus could not be taken as an affirmation that maternal and yolk hormone levels in lizards do not correlate. Moreover, in their previous work (Lovern and Wade 2001), the same authors found a correlation between maternal and initial yolk steroid levels in the same species.

Recent evidence implies that yolk steroid levels during a sensitive period, that depend on the initial quantities of steroids allocated by females in the interaction with incubation temperature, determine the sex of progeny in TSD reptiles (Bowden et al. 2000; Elf 2003; cf. St. Juliana et al. 2004). The role of yolk hormones in sex determination or differentiation in species with GSD is more controversial. The deposition of steroids allocated to yolk was shown to be sexually dimorphic in birds (ZW animals) (Petrie et al. 2001), but subsequent papers made it clear that the sex differences observed reflect embryonic steroid production, not maternal allocation (see e.g., Pilz et al. 2005). Lovern and Wade (2001, 2003*b*) found that yolk T concentrations of freshly laid or even freshly fertilized eggs that give rise to males are nearly twice that of eggs that give rise to females in green anoles, a lizard with GSD of XY type.

Owing to their unique life history, geckos present an interesting group to investigate yolk steroid allocation. Females usually lay two exceptionally large eggs per clutch (e.g., Kratochvíl and Frynta, 2006*a*, 2006*b*); vitellogenesis and ovulation of both eggs proceed in phase (Rhen et al. 2000). If circulating female hormone levels or female physiological condition influence the quantities of steroids in the yolk of eggs formed at the same time, synchronously formed gecko eggs should have identical yolk hormonal composition. High interclutch variance, but very low intraclutch variance, in T and E2 concentrations were found in freshly laid eggs in the leopard gecko (*Eublepharis macularius* (Blyth, 1854)), a TSD gecko species (Elf 2004). However, it is not clear whether we should expect the same pattern in GSD gecko species as well, where yolk steroid levels could reflect different allocation to male vs. female eggs. In GSD geckos having equal primary sex ratio, the proportion of clutches yielding 2 males to 1 male and 1 female to 2 females is 1:2:1 (L. Kratochvíl, L. Kubička, E. Landová, unpublished data). If different allocation into male and female eggs exists, about half of the clutches in GSD geckos should have large intraclutch variance in steroid hormone levels.

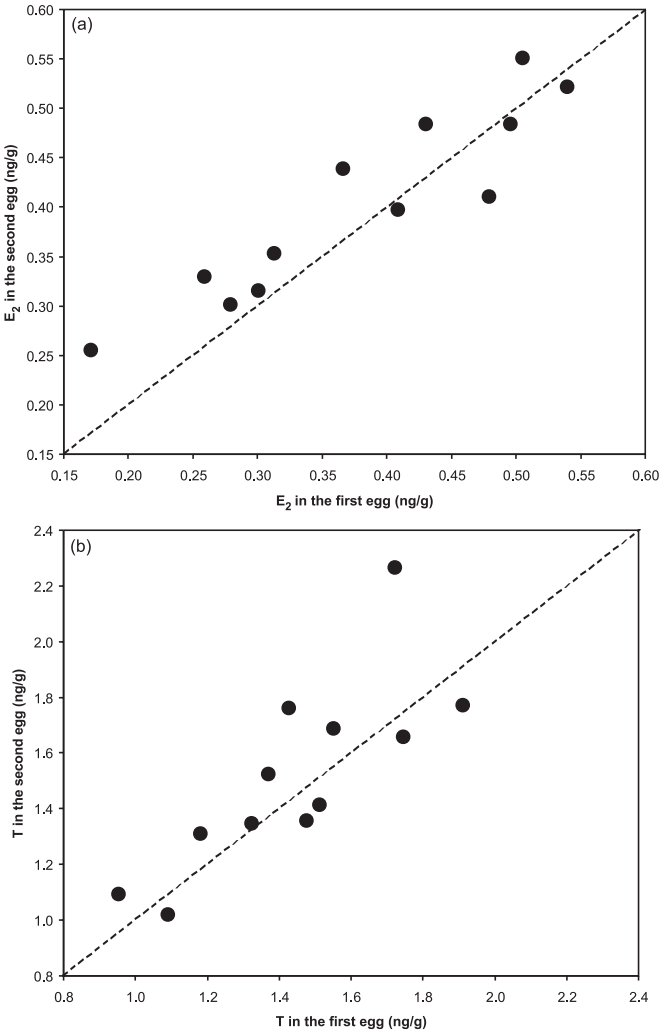
Here we report intra- and inter-clutch variance in initial E2 and T levels in yolk of the Madagascar ground gecko (*Paroedura picta* (Peters, 1854)), a species with GSD (Blumberg et al. 2002) that is relatively distantly related to the leopard gecko. We tested how particular clutches differ in allocated quantities of steroids and whether eggs in a given clutch share levels of these important hormones.

Material and methods

Experimental animals were kept in the laboratory breeding room under common conditions. Twelve experimental females were of the same age (18 months) and of similar size (mass 14.01 ± 0.59 g, mean ± SE; snout–vent length 80.1 ± 0.6 mm). Geckos were housed individually in glass terraria (30 cm × 30 cm × 20 cm) with sandy substrate, water dishes, shelters, and heating cables allowing active thermoregulation (gradient 26–45 °C) under 12 h light : 12 h dark cycle. Their diet consisted of vitamin-fed crickets. Gravid females were checked daily. Female *P. picta* laid hard-shelled eggs into the dry substrate. Freshly laid eggs (i.e., found a maximum of 12 h after laying) were cleaned of substrate remains and weighed.

To prepare samples for hormone concentration measurements, each egg was ruptured and the whole yolk was homogenized, weighed, and suspended in 500 μL of 0.154 mol/L of saline in a microtube, and stored at –20 °C until analyzed. E2 and T levels were measured in the Institute of Endocrinology, Praha, Czech Republic, using a competitive radioimmunoassay. Briefly, to measure T concentration, 200 μL of the homogenate was diluted with 400 μL of physiological solution. Samples were then extracted with diethyl ether (3 mL) and centrifuged (1500*g*, 5 min, 4 °C). The water phase of the extract was frozen in a solid carbon dioxide bath, decanted, and evaporated to dryness. To remove lipids, the dry residue was dissolved in the mixture of 80% aqueous methanol and *n*-hexane (1 mL each), shaken for 1 min in a vortex, centrifuged (1500*g*, 5 min, 4 °C), and the upper *n*-hexane phase was removed with a Pasteur pipette. The tubes that contained the dry residues of the ether extract were washed again with the 80% aqueous methanol and *n*-hexane mixture and the procedure was repeated. Lower methanolic phases were combined and evaporated again in a vacuum evaporator. The samples were reconstituted in 600 μL phosphate buffer. To reduce as much as possible the effect of losses during extraction and solvent partition, the standard solutions of both steroids in a physiological solution containing 0.1 g/100 mL of bovine serum albumin were processed in the same way and used to construct standard curves. The amount of

Fig. 1. Scatterplot of (*a*) oestradiol (E2) and (*b*) testosterone (T) yolk levels among 12 clutches of the Madagascar ground gecko (*Paroedura picta*). The Spearman rank correlations between the first and the second eggs in a clutch in E2 concentration and T concentration are highly significant (both $P < 0.0001$). The broken lines indicate a 1:1 relationship.

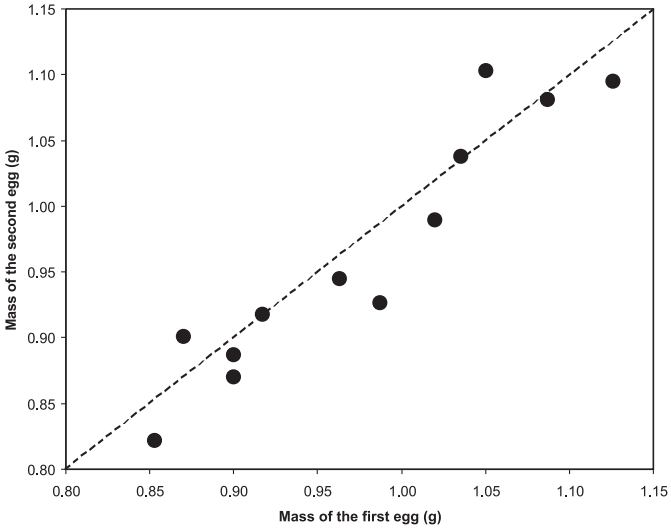


standards recovered, on average, was 81%. For the radioimmunoassays, a 100 μL aliquot of the radioligand (2616 cycles/min per 10 μL) and a 100 μL aliquot of specific antibody (dilution 1:25000) were added to each 100 μL sample or standard, respectively, and they were incubated at 4 °C for 16 h. After adsorption of unbound radioligand by dextra-coated charcoal, radioactivity was measured and T concentration in a sample was calculated using calibration curves.

E2 was assayed using a commercial estradiol RIA kit from Orion Diagnostica, Espoo, Finland, encompassing microtubes with immobilized antibody.

The hormone levels were measured blindly with respect to the present analysis. STATISTICA® version 6 (StatSoft Inc. 2001) was used for subsequent statistical analyses. First, the normal distribution of values of individual egg mass and of E2 and T concentrations was checked using Kolmogorov–Smirnov tests. The differences in yolk T vs. E2 concentrations and differences in egg mass among individual

Fig. 2. The Madagascar ground gecko exhibits large interclutch variation in egg mass; however, eggs within a clutch are of similar mass. The Spearman rank correlation between mass of the first and the second eggs in a clutch is highly significant ($P < 0.0001$). The broken line indicates a 1:1 relationship.



clutches were tested using generalized linear model (GLM) ANOVAs. Because of the small sample size, nonparametric Spearman's rank correlations between mass and E2 concentration, and between mass and T concentration, of the first and second eggs in a clutch were used to test for inter- vs. intra-clutch differences in these three variables. The same tests were used to check the relationship of mean egg mass, mean T concentration, and mean E2 concentration among clutches.

Results

Yolk steroid levels were analyzed in 24 eggs from 12 clutches, each clutch from a different female. Individual egg mass, E2 concentration, and T concentration did not significantly deviate from the normal distribution (nonsignificant Kolmogorov–Smirnov test results, $n = 24$). The egg mass was 0.98 ± 0.02 g (mean ± SE). All yolk samples had detectable E2 and T concentrations. The yolk concentration was 0.39 ± 0.02 ng/g in E2 and 1.48 ± 0.06 ng/g in T. Yolk T concentration was much higher than E2 concentration in all eggs (ANOVA; $F_{[2,46]} = 270.05$; $P < 0.00001$). Individual clutches significantly differ in egg mass, E2 concentration, and T concentration (GLM ANOVA, all $P < 0.001$). ANOVA models, including intercept and clutch identity, explain the majority of variability in egg mass (92%), E2 concentration (94%), and T concentration (87%). Spearman's rank correlations between mass and E2 concentration, and between mass and T concentration, of the first and the second eggs in a clutch were all highly significant (all $P < 0.0001$; Figs. 1, 2), again showing high interclutch but small intraclutch differences in all three variables. Spearman's rank correlation tests indicated no significant correlation between mean egg mass and mean T concentration, between mean egg mass and E2 concentration, and between mean T and mean E2 concentrations among clutches (all $P > 0.10$).

Discussion

Individual clutches of the Madagascar ground gecko differ considerably in E2 and T levels; there were approximately twofold differences in the mean concentrations of these steroids among particular clutches. Nevertheless, both eggs in a clutch possessed very similar concentrations of E2 and T (Fig. 1). We can conclude that the pattern of intra- vs. interclutch variance in the GSD gecko is technically the same as in previously studied TSD species (Elf 2004).

As our method of yolk sampling was destructive and the technique for molecular sexing of GSD geckos has not been developed yet, we have no information on the sex of embryos in the collected eggs. However, the proportion of double-egg clutches yielding 2 males to 1 male and 1 female to 2 females in the Madagascar ground geckos in our laboratory is approximately 1:2:1 (L. Kratochvíl, L. Kubička, E. Landová, unpublished data). Therefore, if there is a differential maternal allocation of steroids into male and female eggs in *P. picta* (as was reported in the green anoles; Lovern and Wade 2001, 2003*b*), about half of examined clutches should have large intraclutch variance in steroid hormone levels, which is obviously not the case (Fig. 1). It seems that the sex-specific allocation of yolk steroids in the Madagascar ground gecko does not exist or that the difference in hormonal levels between male vs. female eggs are negligible in comparison with interclutch variation.

Reproducing female geckos are apparently not able to differently allocate hormones into simultaneously yolked follicles. Nearly identical levels of initial yolk steroids in synchronously formed eggs of *P. picta* presumably reflect common maternal conditions during vitellogenesis, which further supports the parallel function of either ovary in geckos (Rhen et al. 2000). Nevertheless, individual clutches significantly differ in the hormone concentrations. Future research should explore the phenotypic (and fitness) consequences of different steroid concentrations for offspring.

An interesting question is whether differences in yolk E2 and T levels and their ratio across taxa reflect phylogenetic relationships or sex-determining modes (Janzen et al. 1998; Lovern and Wade 2003*a*). The information relevant to this issue is still scarce but seems to support the sex-determining mode. The initial yolk T to E2 concentration ratio tends to be >1 in birds and lizards with GSD, but <1 in a crocodile and turtles with TSD (Lovern and Wade 2003*a* and references therein; data listed in Elf 2004). Interestingly, the first data in geckos show the same trend. Yolks of freshly laid eggs possess higher concentration of E2 than T in the leopard gecko with TSD (Elf 2004), but the opposite is true in the Madagascar ground gecko (GSD). Initial yolk E2 levels between examined GSD and TSD geckos are comparable, but T levels are about 10 times lower in the TSD species. The taxonomic range of studied GSD and TSD geckos should be definitively broadened during subsequent research. For the resolution of the relationship between initial yolk steroid level and system of sex determination within an explicit phylogenetic framework, it will be particularly important to focus on closely related gecko species with different sex-determining modes. It will be also fascinating to uncover the potential functional differences and evolutionary shifts in steroid roles during offspring phenotypic differentiation in GSD vs. TSD lizards.

In summary, our study is only the first step in the comparative analysis of yolk steroid levels in lizards with different modes of sex determination. The examined TSD and GSD gecko species share similar yolk E2 concentrations; however, the level of T in the TSD species is much lower. The present paper reports for the first time the significant interclutch, but slight intraclutch, differences in initial yolk levels of E2 and T in a GSD species of gecko, the phenomenon previously described in a gecko with TSD (Elf 2004). As gecko eggs in a clutch develop in phase, these observations can be interpreted as support for the hypothesis that initial yolk steroid levels in synchronously developing eggs reflect common maternal conditions during egg formation. We believe that geckos laying clutches of similarly sized eggs with comparable initial hormone levels, but differing in the mode of sex determination, present an excellent model for studies of maternal effects, phenotype differentiation, and animal reproduction.

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