Plasma levels of estradiol during vitellogenesis and early gestation in oviparous and viviparous *Lacerta* (*Zootoca*) *vivipara*

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Abstract. The evolution of viviparity in lizards and snakes is always associated with a reduction of the eggshell membrane. There is strong evidence indicating that estradiol is the primary factor involved in seasonal development of the uterine glands in preparation for eggshelling. However, the hypothesis that the thinner eggshells of viviparous species could be the consequence of lower pre-ovulatory levels of circulating estradiol has not been tested. In a previous histological study we showed that the pre-ovulatory growth of the uterine shell glands is significantly more pronounced in oviparous than in viviparous females of the lizard *Lacerta* (*Zootoca*) *vivipara.* During the current study we assayed plasma levels of estradiol before and during vitellogenesis and during early gestation. We did not find any significant difference of estradiol concentrations between oviparous and viviparous females. In both reproductive forms the plasma estradiol concentration was significantly higher during late vitellogenesis than during early gestation. Future research should address whether variation in the growth of the uterine shell glands could be predominantly mediated by modification affecting estrogen receptors of the uterus rather than by concentration of the circulating hormone.

Keywords: estradiol, live-bearing, lizards, oviparity, reproductive mode.

The parchment-like eggshell observed in most oviparous species of squamates is composed of a thick layer of proteinaceaous fibers, the eggshell membrane, overlain by a thin (sometimes absent) calcite crust (Schleich and Kastle, 1988). Although some viviparous species of squamates still have an eggshell membrane enveloping the embryo during development, this structure is always much thinner than the eggshell membrane of oviparous species (Stewart, 1985; Heulin, 1990; Guillette, 1993; Qualls, 1996). Understanding the evolution of viviparity in squamates requires study of the factors that influence eggshell thickness. The eggshell membrane of oviparous and viviparous species is secreted by uterine glands immediately after ovulation (Guillette, 1993; Palmer et al., 1993; Heulin et al., 2005). Differentiation and growth of the uterine shell glands, which occurs during the period of vitellogenesis preceding ovulation, appears to be regulated by estradiol secreted by the ovary (Girling, 2002). Reduced development of uterine shell glands in viviparous forms of squamates is thought to be the consequence either of lower pre-ovulatory levels of circulating estradiol and/or of a lower sensitivity (lesser number or blocking of the estrogen receptors) of the uterine target tissue (Guillette, 1993).

Testing this hypothesis requires comparison of closely related oviparous and viviparous taxa in order to minimize the confounding effect of phylogenetic differences. The lizard *Lacerta* (*Zootoca*) *vivipara*, which is one of the rare species of squamates that are reproductively bimodal (i.e. species with allopatric oviparous and viviparous populations), is an ideal model for such a comparative study. Viviparous females of *L.* (*Z.*) *vivipara* give birth to fully formed offspring (stage 40 of Dufaure and Hubert, 1961) enveloped in a thin (6-10 μm) eggshell membrane, whereas oviparous females oviposit eggs containing embryos of stage 30 to 35 encased in a thicker (40-65 μm) eggshell membrane (Panigel, 1956; Heulin, 1990; Heulin

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et al., 1991, 2002; Stewart et al., 2004). A recent histological investigation revealed that the uterine shell glands are significantly thicker in oviparous than in viviparous females during vitellogenesis and that, in both forms, the secretion of the eggshell membrane and the regression of uterine shell glands occur very rapidly after ovulation (Heulin et al., 2005). In the present paper we report plasma estradiol levels measured on the blood samples collected from females autopsied in our histological study (i.e. Heulin et al., 2005) to test whether the lesser pre-ovulatory development of uterine shell glands in viviparous females is correlated with a lower level of circulating estradiol.

The data presented here were obtained from lizards that were caught in September 1998 in the oviparous population of Louvie (43°06′N, 0°23′W, Alt. 370 m) in south-western France and in the viviparous populations of Paimpont (48°N, 2°W, Alt 150 m) in north-western France. The lizards hibernated (4 months at 4°C) in our lab. The females were allowed to copulate with males for 2 or 3 days during the third week following hibernation. During the activity period (before and after hibernation) the lizards were reared separately in plastic terraria., in a room where large windows provided natural photoperiod. Each terrarium (30 × 20 × 20 cm) was equipped with a shelter, dishes of food and water, and a 40W bulb that provided heat for 6 h/day. The rearing conditions described above allow normal vitellogenesis and the onset of ovulation occurs about one month after the end of hibernation (Gavaud, 1983; Heulin, unpubl. obs.). In our study, we sacrificed some females during the two days following their removal from hibernation (N = 6 oviparous and N = 6 viviparous). The other females (27 oviparous, 29 viviparous) were sacrificed 20 to 30 days after the end of hibernation during the period of peak development of the uterine shell glands (in pre-ovulatory vitellogenic females) and the subsequent decrease in size of these glands following eggshell formation (in females with recently ovulated eggs). This timing allowed us to get a majority of samples (tissues and blood) for the period during which there are the most pronounced differences in growth and activity of shell glands between oviparous and viviparous females (Heulin et al., 2005).

Each female was sampled once (when sacrificed). The females were chilled to 3°C for 20 min before decapitation. Blood samples were collected from the carotid arteries into heparinised tubes immediately after decapitation. The blood sample was centrifuged at 1200 tpm for 10 min and the resultant plasma was stored at −70°C until assayed for estradiol. Standard solutions of non-radioactive estradiol-17β were prepared in phosphate buffer from 10 μg/ml stock solutions reconstituted from powdered steroids (Steraloid, Wilton, N.H., USA) dissolved in redistilled methanol. Radioactive estradiol-17β were purchased from Amersham-France (Les Ulis, France) ([2,4,6,7,16,17-^{3}H]Œstradiol; sp act 5.14 TBq/mmol, 139 Ci/mmol). Estradiol-17β, was extracted from plasma (0.03 to 0.1 ml) with 2 ml dichloromethane (Recovery > 80%). Steroid concentrations in the blood plasma were measured using radioimmunoassay according to Terqui et al. (1973). Bound and free fractions separations were made by the dextran-charcoal method. The antiserum, anti-estradiol-6-O-carboxymethoxyme-BSA (final dilution 1/240 000) cross-reacted with 6-keto-estradiol 17β (12.3%), but with neither estradiol-17α nor estrone (<1%) (Dray et al., 1971). Oviparous and viviparous plasma samples were assayed in a random order in 3 assays. Each sample was assayed in duplicate. We also ran quality control standards of known concentration in each assay. Sensitivity was 0.5 pg for the standard curve and <0.1 ng/ml for plasma. The inter-assay and intra-assay coefficients of variation were 15% and 13%, respectively.

All averages are given ± standard deviation. We analyzed the data using a two-factors analysis of variance (reproductive mode X stages of reproductive cycle), followed by pairwise (Student-*t* tests) comparisons of means. The Minitab 11.11 program was used for all statistics.

A detailed account of reproductive condition, including embryonic stage, eggshell thickness and the histology and morphometrics of the uterus of the females used in the current study has already been presented in Heulin et al. (2005). The categories of females used in our table 1 correspond to those described in Heulin et al. (2005): NV, non-vitellogenic with translucent follicles of less than 4 mm^3; VT1, vitellogenic with yellow follicles of 5 to 65 mm^3; VT2, vitellogenic with follicles of 65 to 125 mm^3; OE, with oviductal eggs containing stage 3 or 4 embryos of Dufaure and Hubert (1961).

The oviparous and viviparous data sets are presented in table 1. A two-way analysis of variance revealed significant variation of the plasma estradiol concentration between reproductive stages ($F = 4.96$, $P < 0.01$), no significant variation between reproductive modes ($F = 3.06$, $P = 0.09$), and no interaction between reproductive mode and stages ($F = 0.28$, $P = 0.84$). Pairwise comparisons (Student-*t* tests) of reproductive stages revealed that the plasma estradiol concentration was higher during late vitellogenesis (category VT2) than during early embryonic development (category OE), both in oviparous (significant at $P = 0.0005$) and in viviparous (significant at $P = 0.0001$) females. In viviparous females the plasma estradiol concentration was significantly higher during, than before vitellogenesis (significant at $P = 0.03$ for the comparison of categories VT1 versus NV; significant at

Table 1. Plasma estradiol levels, thickness of the eggshell membrane and thickness of the uterine shell glands of oviparous and viviparous *Lacerta* (*Zootoca*) *vivipara* during the pre-ovulatory and post-ovulatory periods. All data except estradiol levels are from Heulin et al., 2005.

Category	Days post-emergence	N	Uterine, shell glands (μm)	Eggshell (μm)	Estradiol (ng/ml)
NV oviparous	1-2	6	34 ± 6		4.17 ± 2.74
NV viviparous	1-2	6	32 ± 5		2.62 ± 0.98
VT1 oviparous	20-30	14	69 ± 17*		4.66 ± 2.69
VT1 viviparous	20-25	11	50 ± 13		3.93 ± 1.24
VT2 oviparous	22-30	9	101 ± 17**		4.34 ± 1.23
VT2 viviparous	20-25	12	63 ± 8		3.82 ± 1.17
OE oviparous	22-29	4	60 ± 13	52.0 ± 6.4**	2.08 ± 0.38
OE viviparous	23-29	6	54 ± 12	4.2 ± 2.3	1.69 ± 0.13

Categories of females: NV, non-vitellogenic; VT1, vitellogenic with follicles of 5 to 65 mm^3; VT2, vitellogenic with follicles of 65 to 125 mm^3; OE, with oviductal eggs containing stage 3 or 4 embryos of Dufaure and Hubert (1961).
Days post-emergence: days after removal from hibernation.
*, ** respectively indicate significant differences at $P < 0.01$ or $P < 0.001$ between oviparous and viviparous values, Student t-test.

$P = 0.04$ for the comparison of VT2 versus NV). Other pairwise comparisons of reproductive stages were not significant. Whatever the reproductive stage (NV, VT1, VT2 or OE) the mean values of plasma estradiol concentrations were always slightly elevated in oviparous females compared to viviparous females (table 1), but these differences were not statistically significant (Student-t tests, $P > 0.05$ in all cases).

The highest plasma concentrations of estradiol in squamate reptiles occur during the period of follicular growth preceding ovulation (Girling, 2002). Estradiol, which is secreted by the follicles of the ovaries, induces vitellogenin synthesis by the liver and simultaneously stimulates the development of the uterus in preparation for eggshelling and/or for gravidity. There is considerable interspecific variation in pre-ovulatory levels of circulating estradiol among squamates and the range for oviparous species overlaps that for viviparous species. In oviparous species, the peak of plasma concentration of estradiol measured during the pre-ovulatory period ranges from 0.12 ng/ml to 4 ng/ml (Bonna-Gallo et al., 1980; Gorman et al., 1981; Joss, 1985; Moore and Crews, 1986; Carnevali et al., 1991; Diaz et al., 1994; Phillips and Millar, 1998; Rhen et al., 2000; Radder et al., 2001; Weiss et al., 2002; Woodley and Moore, 2002). In viviparous species, this peak of estradiol concentration generally ranges from 0.07 ng/ml to 6 ng/ml, (Gorman et al., 1981; Kleis-San Francisco and Callard, 1986; Ghiara et al., 1987; Whittier et al., 1987; Bonnet et al., 1994; Jones and Swain, 1996; Woodley and Moore, 1999; Edwards and Jones, 2001; Girling et al., 2002). In the absence of a phylogenetically based comparative analysis, a possible relationship between reproductive mode and concentration of estradiol is obscured by the high level of interspecific variation. The sole previous study of lacertid lizards reported a concentration of pre-ovulatory plasma estradiol (4 ng/ml) in a wild population of the oviparous *Podarcis sicula* (Carnevali et al., 1991) that is very close to the value we obtained for oviparous *Lacerta* (*Zootoca*) *vivipara*. Although our investigations show that the mean values of plasma estradiol concentrations were slightly elevated in oviparous females compared to viviparous females for all three of our pre-ovulatory categories, this tendency was not statistically significant. Although the lack of significant differences in circulating levels of estradiol might be due to the small samples, it strikingly contrasts with the significant difference in pre-ovulatory growth of the uterine shell glands

in oviparous females compared to viviparous females (Heulin et al., 2005, and table 1).

There is strong experimental evidence indicating that estradiol, secreted by the ovaries during follicular growth, is the main hormone triggering the pre-ovulatory development of uterine shell glands in Squamates (Panigel, 1956; Christiansen, 1973; Botte, 1974; Girling et al., 2000; Girling, 2002). However, other substances (androgens, gonadotropin, growth factors) might also be involved in the regulation of this development, at least in some species (see review in Girling, 2002). Such additional influences might explain why, despite a marked difference in the pre-ovulatory growth of the uterine shell glands, we only found weak (if any) differences in plasma estradiol concentration between oviparous and viviparous females of *Lacerta* (*Zootoca*) *vivipara*. In addition, the response of uterine shell glands to estradiol certainly results from the interaction between changing hormone and hormone-receptor levels. For example, the estrogen receptor concentration in nuclei of uterine cells significantly increases during the pre-ovulatory reproductive stage in the lacertid lizard *Podarcis sicula* (Paolucci et al., 1992). This is consistent with a major role for estradiol in uterine gland stimulation. Guillette (1993) hypothesized that the lesser development of uterine shell glands in viviparous forms of squamates could result either from a lesser level of circulating estradiol or modification (lesser number, blocking) of the estrogen receptors. As our data do not support the former alternative, our future research will focus on the comparison of estrogen receptors between oviparous and viviparous females of *Lacerta* (*Zootoca*) *vivipara.*

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