

## Calcium provision to oviparous and viviparous embryos of the reproductively bimodal lizard *Lacerta (Zootoca) vivipara*

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### SUMMARY

Embryos of oviparous squamate reptiles typically obtain calcium from both yolk and eggshell but differ from other oviparous amniotes (turtles, birds and crocodylians) because they are heavily dependent on calcium-rich yolk. Eggs of viviparous squamates lack calcareous eggshells, and embryos receive calcium solely from yolk or from both yolk and placenta. The pattern of calcium mobilization by amniote embryos has been predicted to influence the evolution of viviparity if embryos are dependent on calcium from the eggshell and calcium placentotrophy evolves subsequent to viviparity. We studied the pattern of maternal provision and embryonic utilization of calcium of an oviparous and a viviparous population of the reproductively bimodal lizard *Lacerta vivipara* to test the hypotheses: (1) oviparous embryos are not dependent on eggshell calcium and (2) calcium content of viviparous hatchlings does not differ from oviparous hatchlings. Our findings do not support either of these hypotheses because oviparous females oviposited eggs with heavily calcified shells and calcium-poor yolk, and embryonic mobilization of shell calcium was greater than for other oviparous squamates. The calcium content of yolk from viviparous females did not differ from oviparous yolk, but viviparous eggs lacked calcareous eggshells. Uterine secretion by viviparous females compensated for the low calcium content of yolk, and placental calcium transfer was among the highest recorded for squamates. The pattern of calcium provision in these two populations suggests that dependence on uterine calcium, either stored temporarily in an eggshell or transferred directly across a placenta, did not constrain the evolution of reproductive mode in this lineage.

Key words: lecithotrophy, placentotrophy, viviparity, calcium.

### INTRODUCTION

Embryos of oviparous squamate reptiles depend on calcium-rich yolk as the primary source of calcium for development, but calcium extracted from the eggshell contributes an important secondary source (Packard, 1994; Stewart et al., 2009). Viviparity has evolved independently in multiple lineages of squamates (Blackburn, 1982; Blackburn, 1985a; Blackburn, 2006; Shine, 1985) and the eggs of viviparous species lack a calcareous eggshell (Heulin, 1990; Blackburn, 1993; Qualls, 1996; Heulin et al., 2002). Loss of a thick, calcified eggshell is a predicted requisite to the evolution of viviparity because such a structure would impede respiratory exchange between the uterus and the embryo (Packard et al., 1977; Guillette, 1982; Guillette, 1992; Andrews and Mathies, 2000). The prevalence of viviparity among squamate reptiles has been attributed to their pattern of embryonic calcium provision based on the premise that oviparous embryos are not dependent on calcium from the eggshell (Packard et al., 1977). In the absence of eggshell calcium, most viviparous embryos take up calcium from placental transport, in addition to yolk reserves (Thompson et al., 2000). If the evolution of viviparity precedes the evolution of calcium placentotrophy, embryonic dependence on eggshell calcium for development would be a constraint on the evolution of viviparity, whereas heavy reliance on calcium-rich yolk would be an exaptation for the evolution of viviparity (Packard et al., 1977). One prediction from this model is that females from lineages with viviparous species should produce calcium-rich yolk, and embryos should not be dependent on eggshell calcium.

*Lacerta (Zootoca) vivipara* Jacquin 1787 is one of three lizard species that exhibits interpopulational variation in reproductive mode (Heulin et al., 1993; Fairbairn et al., 1998; Smith et al., 2001; Surget-Groba et al., 2001; Surget-Groba et al., 2006). Females from oviparous populations oviposit eggs with thick eggshells composed of a mat of fibrous protein overlain by a layer of calcium carbonate (Heulin, 1990; Heulin et al., 2002; Heulin et al., 2005). Eggs from viviparous populations lack an outer layer of calcium (Heulin, 1990) and the protein layer is thinner (Heulin, 1990; Heulin et al., 2005). We measured the distribution of calcium in egg contents of a developmental series of two populations (oviparous and viviparous) of *L. vivipara* to address two questions: (1) are oviparous embryos dependent on calcium from the eggshell and (2) are viviparous hatchlings calcium deficient compared with oviparous hatchlings?

### MATERIALS AND METHODS

Oviparous females were collected in September 2006 ( $N=24$ ) and September 2007 ( $N=21$ ) from Louvie, France (43°06'N, 0°23'W; elevation 370 m). Viviparous females were collected in September 2006 ( $N=22$ ) and September 2007 ( $N=15$ ) from Paimpont, France (48°N, 2°W; elevation 150 m). Animals were transported to Station Biologique de Paimpont, where they were maintained under hibernation conditions, as described previously (Heulin et al., 2005), until the following spring. Females were removed from hibernation, paired with males, which had been treated similarly, from the same populations and maintained in terraria prior to air shipment to East Tennessee State University in March 2007 and 2008. Animals were housed individually in glass terraria with nest

boxes filled with moist sphagnum moss. Room lights provided 14h:10h photophase:scotophase, and 40 W incandescent cage lights provided additional heat for 8 h per day. Water was provided *ad libitum*, and crickets dusted with phosphorus-free calcium with vitamin D (Rep-Cal) were offered twice per week. Oviposited eggs were incubated at 25°C in vermiculite:water (2:1), estimated to provide a water potential of  $-120\pm 40$  kPa as measured by thermocouple psychrometry using a Wescor C52 chamber and Wescor HR33 T microvoltmeter (Logan, UT, USA).

Oviparous eggs were sampled at periodic intervals throughout incubation, and viviparous eggs were removed from females that had been killed with an overdose of sodium pentobarbital. Hatchlings of both reproductive modes were killed by an overdose of sodium pentobarbital. Embryos were assigned to developmental stages based on the staging system of Dufaure and Hubert (Dufaure and Hubert, 1961). We defined recently ovulated eggs as those containing embryos up to the gastrula stage. Eggs were separated into yolk, embryo and eggshell components, placed in tared glass vials, weighed and frozen at  $-10^\circ\text{C}$ . These samples were lyophilized to constant mass in a Labconco Freezone 4.5 freeze dryer (Kansas City, MO, USA).

Preparation of the samples for calcium analysis followed the method of Shadrix et al. (Shadrix et al., 1994). Briefly, all samples were digested in borosilicate glass test tubes containing 3 ml concentrated nitric acid for 3 h at 125°C. Digests were cooled to room temperature for 1 h, and 1 ml of 30% hydrogen peroxide was added. The temperature was returned to 125°C and maintained for approximately 15 h. The digests were evaporated gently to near dryness on a hot plate, diluted in 1:1 hydrochloric acid and brought to a final volume of 2.5% hydrochloric acid with distilled water. Lanthanum chloride (1:10) was added to each sample prior to analysis for calcium. Calcium content was estimated using a Varian 220 FS atomic absorption spectrophotometer (Palo Alto, CA, USA) calibrated against samples of known calcium concentration.

We tested for differences in female size and in clutch size between populations with two-factor analysis of variance, with year of sample as a random factor, and used linear regression to reveal the relationship between female size and clutch size for each population. Differences between populations in dry mass of recently ovulated eggs were analyzed with three-factor analysis of variance, with reproductive mode as a fixed effect and female and year as random effects, and we tested for the possible influence of female size by entering female snout-vent length (SVL) as a covariate. Two-factor analysis of variance, with female as a random factor, was used to test for differences between recently ovulated eggs and hatchlings for each population. We tested for differences in dry mass and calcium mass for egg components (yolk, embryo, eggshell) among embryonic stages for each population with two-way analysis of variance, with embryonic

stage as a fixed effect and female (clutch) as a random effect. We compared dry mass and calcium mass among embryonic stages between populations using three-factor analysis of variance, with embryonic stage and reproductive mode as fixed effects and female as a random effect. Multiple comparisons among least-squares means were performed using the Scheffe procedure. All statistical tests were performed using SAS Institute programs (Cary, NC, USA).

## RESULTS

Viviparous females ( $N=37$ ) were significantly larger ( $56.9\pm 0.4$  mm SVL) than oviparous females ( $N=45$ ) ( $51.7\pm 0.4$  mm SVL) ( $F=72$ , d.f.=1, 80,  $P<0.0001$ ). Clutch size did not differ between viviparous ( $4.7\pm 0.3$ ) and oviparous ( $4.5\pm 0.3$ ) females ( $F=0.76$ , d.f.=1, 79,  $P=0.39$ ). Clutch size was positively correlated with female SVL for both viviparous (clutch size= $-4.4+0.16$  SVL,  $P=0.02$ ) and oviparous (clutch size= $-6.1+0.20$  SVL,  $P=0.002$ ) populations. There were no significant differences between sampling years (2007, 2008) for female size or clutch size.

Dry mass of recently ovulated eggs of oviparous and viviparous females did not differ significantly ( $F=0.03$ , d.f.=1, 11,  $P=0.87$ ) (Tables 1 and 2). There was no significant difference between sampling years for dry mass of recently ovulated eggs nor was there a significant effect of female size. The ontogenetic patterns of yolk depletion and embryonic acquisition of dry mass were similar for the two populations (Tables 1–4; Fig. 1). Dry mass of hatchlings of each population was significantly less than dry mass of recently ovulated eggs (oviparous,  $F=11.7$ , d.f.=1, 16,  $P<0.004$ ; viviparous,  $F=39$ , d.f.=1, 13,  $P<0.0001$ ) (Tables 1 and 2; Fig. 1).

Oviparous females oviposited clutches containing stage 31 ( $N=3$ ), stage 32 ( $N=3$ ) or stage 33 ( $N=4$ ) embryos. Eggs of oviparous females contained 1.40 mg of calcium at oviposition, 86% of which (1.21 mg) was deposited in the eggshell (Table 1). The relative distribution of calcium in egg compartments (yolk, embryo, eggshell) did not vary until after embryonic stage 36 (Table 1; Fig. 2). Between stages 36 and hatching, yolk and eggshell calcium levels dropped and embryonic calcium levels increased dramatically (Table 1; Fig. 2). Yolk of recently ovulated eggs accounted for 19% of hatchling calcium content (0.894 mg), 0.004 mg of which was contained in internal residual yolk (Table 1).

The mass of calcium in yolk of viviparous eggs at embryonic stage 32, the median stage at which oviparous females oviposited, did not differ from oviposited eggs of oviparous females (Tables 1, 2, 5; Fig. 2). However, because the eggshell contained significantly less calcium ( $F=233$ , d.f.=1, 12,  $P<0.0001$ ) (Tables 1 and 2), total calcium in stage 32 eggs of viviparous females ( $0.24\pm 0.07$  mg) was significantly less than in oviposited oviparous eggs ( $1.41\pm 0.04$  mg,  $F=218$ , d.f.=1, 12,  $P<0.0001$ ). The ontogenetic pattern of depletion of calcium from yolk

Table 1. Ontogeny of dry mass and calcium mass in eggs of oviparous *Lacerta (Zootoca) vivipara*

Embryonic stage	N	Yolk		Embryo		Eggshell	
		Dry mass (mg)	Calcium mass (mg)	Dry mass (mg)	Calcium mass (mg)	Dry mass (mg)	Calcium mass (mg)
Oviductal	8	54.4 $\pm$ 1.9 <sup>a</sup>	0.17 $\pm$ 0.01 <sup>a</sup>			4.3 $\pm$ 0.4 <sup>a</sup>	0.19 $\pm$ 0.08 <sup>a</sup>
Stage 31,32,33*	10	50.4 $\pm$ 1.7 <sup>a</sup>	0.17 $\pm$ 0.01 <sup>a</sup>	3.8 $\pm$ 1.2 <sup>a</sup>	0.02 $\pm$ 0.02 <sup>a</sup>	8.3 $\pm$ 0.3 <sup>b</sup>	1.21 $\pm$ 0.06 <sup>b</sup>
Stage 36	4	45.7 $\pm$ 2.7 <sup>a</sup>	0.17 $\pm$ 0.01 <sup>a</sup>	5.9 $\pm$ 2.4 <sup>a,b</sup>	0.02 $\pm$ 0.05 <sup>a</sup>	8.7 $\pm$ 0.6 <sup>b</sup>	1.22 $\pm$ 0.1 <sup>b</sup>
Stage 37	5	32.1 $\pm$ 2.4 <sup>b</sup>	0.10 $\pm$ 0.01 <sup>b</sup>	14.0 $\pm$ 2.1 <sup>b</sup>	0.14 $\pm$ 0.04 <sup>a</sup>	7.6 $\pm$ 0.5 <sup>b</sup>	1.10 $\pm$ 0.09 <sup>b</sup>
Stage 40	8	18.8 $\pm$ 1.9 <sup>c</sup>	0.03 $\pm$ 0.01 <sup>c</sup>	29.3 $\pm$ 1.7 <sup>c</sup>	0.42 $\pm$ 0.03 <sup>b</sup>	8.4 $\pm$ 0.3 <sup>b</sup>	1.00 $\pm$ 0.06 <sup>b</sup>
Hatchling	10	0.62 $\pm$ 1.7 <sup>d</sup>	0.004 $\pm$ 0.01 <sup>c</sup>	46.2 $\pm$ 1.2 <sup>d</sup>	0.89 $\pm$ 0.02 <sup>c</sup>	7.4 $\pm$ 0.4 <sup>b</sup>	0.72 $\pm$ 0.06 <sup>c</sup>

Sample sizes are number of females (clutches).

Values reported as least squares means  $\pm$  s.e.m.

\*Oviposited eggs.

<sup>a-d</sup>Comparisons within columns, values with the same letter denotation do not differ significantly ( $P\leq 0.05$ ).

Table 2. Ontogeny of dry mass (mg) and calcium mass (mg) in eggs of viviparous *Lacerta (Zootoca) vivipara*

Embryonic stage	N	Yolk		N	Embryo		N	Eggshell	
		Dry mass (mg)	Calcium mass (mg)		Dry mass (mg)	Calcium mass (mg)		Dry mass (mg)	Calcium mass (mg)
Oviductal	6	55.4±3.4 <sup>a</sup>	0.17±0.01 <sup>a</sup>						
Stage 30	5	57.6±3.7 <sup>a</sup>	0.14±0.01 <sup>a</sup>	5	1.5±2.4 <sup>a</sup>	0.004±0.05 <sup>a</sup>	5	0.8±0.2 <sup>a</sup>	0.08±0.03 <sup>a</sup>
Stage 32	4	54.6±4.2 <sup>a</sup>	0.16±0.02 <sup>a</sup>	4	2.4±2.7 <sup>a</sup>	0.006±0.05 <sup>a</sup>	4	1.0±0.3 <sup>a</sup>	0.08±0.04 <sup>a</sup>
Stage 34	4	63.1±4.1 <sup>a</sup>	0.16±0.02 <sup>a</sup>	4	3.7±2.7 <sup>a</sup>	0.008±0.05 <sup>a</sup>	4	0.8±0.3 <sup>a</sup>	0.13±0.04 <sup>a,b</sup>
Stage 36	4	50.5±4.2 <sup>a</sup>	0.12±0.02 <sup>a</sup>	4	7.6±2.7 <sup>a</sup>	0.03±0.05 <sup>a</sup>	4	1.1±0.3 <sup>a</sup>	0.24±0.04 <sup>a,b</sup>
Stage 40	6	18.0±3.4 <sup>b</sup>	0.03±0.01 <sup>b</sup>	6	32.4±1.8 <sup>b</sup>	0.46±0.04 <sup>b</sup>	6	1.6±0.2 <sup>a</sup>	0.24±0.03 <sup>b</sup>
Hatchling				9	40.2±1.6 <sup>c</sup>	0.65±0.03 <sup>c</sup>			

Sample sizes are number of females (clutches).

Values reported as least squares means ± s.e.m.

\*Values include eggshell.

<sup>a-d</sup>Comparisons within columns, values with the same letter denotation do not differ significantly ( $P \leq 0.05$ ).

for viviparous eggs was similar to that for oviparous eggs (Fig. 2; Tables 1, 2, 5), but there was a significant difference in the pattern of embryonic uptake of calcium (Fig. 2; Table 6). Embryonic calcium content did not differ between populations for stages 32, 36 or 40, but viviparous hatchlings contained significantly less calcium than oviparous hatchlings (Tables 1, 2, 6; Fig. 2). Viviparous hatchlings did not contain residual yolk with calcium. There was also a difference in the ontogenetic pattern of eggshell calcium flux (Tables 1 and 2). Eggshells of viviparous stage 40 embryos contained significantly more calcium than stage 32 eggs (Table 2) whereas eggshells of oviparous eggs contained less calcium at hatching than eggs at oviposition (Table 1).

## DISCUSSION

Previous research on patterns of calcium provision to embryonic oviparous amniotes identified differences among archosaurian, chelonian and squamate reptiles in the distribution of calcium in oviposited eggs and in the contribution of these sources to embryonic nutrition (Packard and Packard, 1984; Packard, 1994; Packard and Clark, 1996). The defining characteristics for oviparous squamates that emerged from this work are the production of calcium-rich yolk as a primary source of calcium for embryonic development and calcium-poor eggshells.

The pattern of calcium provision to oviposited eggs of *Lacerta vivipara* from Louvie is novel among oviparous squamates because these females oviposit eggs with calcium-rich eggshells and calcium-poor yolk. Indeed, the yolk contains the lowest concentration of calcium recorded for squamates, while the amount of calcium in the eggshell exceeds that of most oviparous squamates (Packard, 1994; Stewart et al., 2009). Further, the eggshell supplies most of the calcium to developing embryos; 81% of the calcium mass of hatchlings is extracted from the eggshell. In contrast to these distinctions, the ontogenetic pattern of calcium uptake by embryos is similar to all other oviparous lizards. Eggs are oviposited at the

Table 3. Three-way analysis of variance for the relationship between embryonic stage (as in Fig. 1) and embryonic dry mass for oviparous (Louvie) and viviparous (Paimpont) populations of *Lacerta vivipara*

	Num DF	Den DF	F	P
Reproductive mode	1	45.3	0.16	0.69
Embryonic stage	3	58.4	212	<0.0001
Mode × stage	3	58.4	3.14	0.03

Female (clutch) was entered as a random effect. Num DF, numerator degrees of freedom; Den DF, denominator degrees of freedom.

Table 4. Three-way analysis of variance for the relationship between embryonic stage (as in Fig. 1) and yolk dry mass for oviparous (Louvie) and viviparous (Paimpont) populations of *Lacerta vivipara*

	Num DF	Den DF	F	P
Reproductive mode	1	42.1	1.18	0.28
Embryonic stage	3	42	77	<0.0001
Mode × stage	3	42	0.38	0.77

Female (clutch) was entered as a random effect. Num DF, numerator degrees of freedom; Den DF, denominator degrees of freedom.

embryonic limb bud stage when embryonic calcium content is low and embryonic calcium levels increase dramatically during the latest embryonic stages (Fig. 2; Table 1).

Viviparous squamates also typically have calcium-rich yolk (Stewart and Ecaj, 2009) but, unlike their oviparous counterparts, they lack calcareous eggshells (Heulin, 1990; Qualls, 1996; Blackburn, 1998). However, all viviparous squamates have chorioallantoic placentae, and calcium placentotrophy is nearly universal among these species. Although placental transfer of calcium occurs in predominantly lecithotrophic viviparous species, i.e. those that receive most nutrients from yolk, the greatest dependence on calcium placentotrophy is among species that receive most other nutrients from placental transfer. Like most viviparous lizards, *Lacerta vivipara* from Paimpont are predominantly lecithotrophic (Table 2) with a neonate to ovulated egg dry mass ratio (0.71) comparable to both oviparous and many viviparous lizards (Thompson et al., 2000). However, the relative

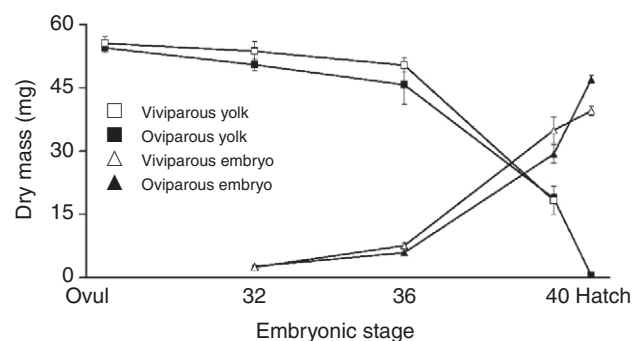


Fig. 1. Ontogeny of dry mass distribution in eggs of oviparous and viviparous *Lacerta vivipara*. Ovul, recently ovulated eggs; hatch, hatchlings/neonates.

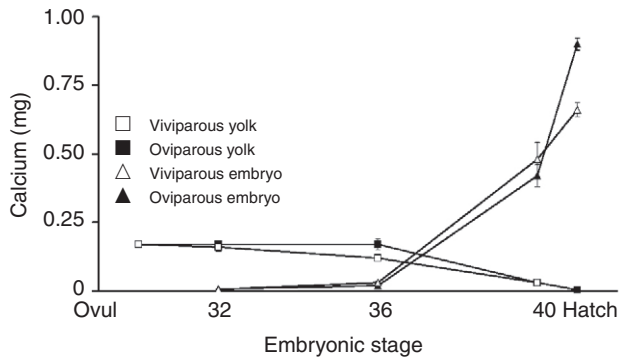


Fig. 2. Ontogeny of calcium distribution in embryos and yolks of oviparous and viviparous *Lacerta vivipara*. Ovul, recently ovulated eggs; hatch, hatchlings/neonates.

contribution of placental transfer to neonatal calcium content in *L. vivipara* is unusually high for predominantly lecithotrophic viviparous lizards and, indeed, is comparable to some highly placentotrophic species (Thompson et al., 2000; Stewart et al., 2009). Hatchlings obtain 76% of their calcium from placental transfer.

One of the distinguishing attributes of the pattern of embryonic calcium mobilization of oviparous squamates is that yolk does not gain calcium during development because extraction from the eggshell does not exceed embryonic uptake (Packard, 1994). Yolk calcium is gradually depleted during embryonic differentiation but both yolk and eggshell calcium are heavily exploited during the embryonic growth phase. Yolk calcium depletion of both reproductive modes of *L. vivipara* is consistent with this pattern. Total calcium in yolks and embryos remains the same for both oviparous and viviparous eggs between oviposition (embryonic stage 32 for viviparous females) and stage 36, indicating that non-yolk calcium sources are inconsequential during this developmental interval. Additionally, yolk calcium content of both populations remains stable, i.e. undergoes neither loss nor gain. However, yolk plus embryonic calcium in stage 40 eggs of both reproductive modes exceeds that of embryonic stage 36 eggs, and the total quantity of calcium in stage 40 embryos exceeds that in yolks (Fig. 2). Thus, during this developmental interval, embryos of both reproductive modes are taking up calcium from both yolk and non-yolk (i.e. eggshell or placenta) sources. Oviparous females secrete 1.21 mg of calcium that is trapped in the protein matrix of the eggshell prior to oviposition, and embryos extract a substantial amount of this calcium in the later stages (subsequent to embryonic stage 36) of development (Table 1). By contrast, eggshells of stage 32 eggs of viviparous females contain only 0.08 mg of calcium, whereas eggshells of embryonic stage 40 contain higher amounts of calcium (Table 2). Thus, most calcium secretion by the uterus of viviparous females occurs later in embryonic development than for oviparous females. In addition, this calcium does not form crystals on the protein matrix of the eggshell but is recovered by the embryo soon after it is secreted. Placental transport during this developmental interval is equivalent to recovery from the eggshell by oviparous embryos, because stage 40 embryos of the two populations do not differ in calcium content.

The absence of a layer of crystallized calcium on the eggshell in viviparous eggs results in a considerable reduction in the amount of calcium per clutch that viviparous females contribute compared with oviparous females because most of the calcium deposited in the eggshell of oviparous eggs is not utilized by embryos. Thus, one of the advantages to viviparity with calcium placentotrophy is that the nutritional cost in calcium to females is less and, furthermore, calcium secretion may be distributed over a longer time period.

Table 5. Three-way analysis of variance for the relationship between embryonic stage (as in Fig. 2) and yolk calcium mass for oviparous (Louvie) and viviparous (Paimpont) populations of *Lacerta vivipara*

	Num DF	Den DF	F	P
Reproductive mode	1	42.4	3.19	0.28
Embryonic stage	3	42.3	58.7	<0.0001
Mode × stage	3	42.3	1.5	0.23

Female (clutch) was entered as a random effect. Num DF, numerator degrees of freedom; Den DF, denominator degrees of freedom.

Table 6. Three-way analysis of variance for the relationship between embryonic stage (as in Fig. 2) and embryonic calcium mass for oviparous (Louvie) and viviparous (Paimpont) populations of *Lacerta vivipara*

	Num DF	Den DF	F	P
Reproductive mode	1	46.8	2.36	0.13
Embryonic stage	3	59.7	186	<0.0001
Mode × stage	3	59.7	7.44	0.0003

Female (clutch) was entered as a random effect. Num DF, numerator degrees of freedom; Den DF, denominator degrees of freedom.

The potential influence of the pattern of embryonic calcium mobilization on the evolution of viviparity was initially expressed by Packard et al. (Packard et al., 1977), who reasoned that if eggshells are a requisite source of calcium for developing embryos, loss of the calcareous layer of the eggshell, as occurs in viviparous squamates, would impose a constraint on the evolution of viviparity. This argument was based on observations that in exclusively oviparous amniote clades, i.e. birds, crocodylians and turtles, embryos are heavily dependent on eggshell calcium whereas lizards and snakes include a high proportion of viviparous species and these embryos depend primarily on calcium from yolk. Thus, the calcium-rich yolk and calcium-poor eggshells of oviparous squamates may be an exaptation for the evolution of viviparity. The model assumes that the evolution of viviparity precedes the evolution of placentotrophy. An important prediction of the model is that clades with viviparous species should have calcium-rich yolk, and embryos should not be dependent on eggshell calcium.

Oviparous females from Louvie and viviparous females from Paimpont each ovulate eggs containing a relatively low amount of calcium. As a result, oviparous embryos obtain most of their calcium from the eggshell, while viviparous embryos are substantially placentotrophic. Although the contribution of yolk calcium to embryonic nutrition is similar in the two populations, the two patterns of uterine calcium provision are so distinct that they nearly form the extremes of variation known for squamates. This degree of intraspecific variation suggests that embryonic reliance on non-yolk calcium does not constrain the evolution of reproductive mode.

Although the patterns of uterine calcium provision of these two populations are inconsistent with the Packard et al. model (Packard et al., 1977), our comparison reveals that an important assumption of the model is supported because oviparous *L. vivipara* receive a substantial amount of calcium from the eggshell and the absence of the calcareous eggshell in viviparous eggs has a negative impact on embryonic calcium nutrition. Calcium content of yolk of recently ovulated eggs of the viviparous Paimpont population does not differ from the oviparous Louvie population yet oviparous hatchlings contain more calcium than viviparous hatchlings because oviparous embryos mobilize more calcium during embryonic stage 40 (Fig. 2;



Tables 1 and 2). Placental transport in this species delivers less calcium than recovery from the eggshell. Whatever the mechanism that results in this difference, a possible outcome could be retarded skeletal ossification in viviparous hatchlings, as predicted by Maisano (Maisano, 2001).

A recent study of 142 populations of *L. vivipara* identified 48 haplotypes and yielded a robust phylogenetic hypothesis based on molecular genetic data (cytochrome *b* and 16S ribosomal RNA) (Surget-Groba et al., 2006). This study revealed that viviparous populations are not monophyletic and that either viviparity evolved on three occasions within the species or that there was a single origin of viviparity followed by a reversal back to oviparity. The viviparous population from Paimpont is a member of a large viviparous clade, with a geographic range extending throughout Europe and Asia, that diverged from an oviparous lineage geographically distributed in Italy, Austria and Slovenia. The hypothesis that oviparity is the ancestral condition for the species and that viviparity is derived in the Paimpont population is strongly supported (Surget-Groba et al., 2006), but the oviparous population from Louvie is embedded within the viviparous clade and thus is potentially a reversal from viviparity to oviparity. The basal oviparous lineage of *L. vivipara* contains a population with thicker eggshells than the Louvie population, and these eggshells have an outer layer of calcium (Heulin et al., 2002).

Broader sampling of populations of *L. vivipara* will be required to determine the relationship between reproductive mode and pattern of embryonic calcium provision and utilization. In addition, the pattern of embryonic calcium mobilization is unknown for other Lacertidae. However, data from populations at Louvie and Paimpont offer little support for the hypothesis (Packard et al., 1977) that dependence on uterine calcium secretion constrains the evolution of reproductive mode in this lineage.

In contrast to the gradualistic model (Packard et al., 1977), Blackburn (Blackburn, 1985b; Blackburn, 1992; Blackburn, 1995; Blackburn, 2006) proposed that viviparity and placentotrophy evolve concurrently and that incipient placentotrophy, placental transfer of nutrients supplemental to yolk, is a necessary correlate of viviparity. Oviparous lizards from Louvie differ from viviparous lizards from Paimpont in (1) length of time that the eggs are in the oviduct, (2) size of uterine shell glands in preparation for eggshell secretion (Heulin et al., 2005) and (3) timing and quantity of uterine calcium secretion. Instead of impeding the evolution of viviparity, embryonic dependence on uterine calcium secretion, coupled with plasticity in the mechanisms of calcium secretion by the uterus and of embryonic uptake of calcium, may facilitate the transition to placentotrophy as predicted by the Blackburn (Blackburn, 2006) model.

*Lacerta vivipara* provides an unusual opportunity to study the relationship between the evolution of viviparity and the evolution of placentotrophy. There is a well-resolved phylogeny for the species to guide further sampling of populations for comparative analyses and there are multiple transitions between reproductive modes within the lineage. Future studies should focus also on unraveling the mechanisms of calcium secretion and embryonic uptake and the possible interaction between the regulation of oviposition, eggshell formation and uterine calcium secretion.

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