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Embryonic mobilization of calcium in a viviparous reptile: Evidence for a novel pattern of placental calcium secretion

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ABSTRACT

Yolk reserves supply the majority of embryonic nutrition in squamate reptiles, including calcium. Embryos of oviparous squamates exploit the eggshell for supplemental calcium, while embryos of viviparous species may receive additional calcium via the placenta. Developmental uptake of calcium in oviparous snakes increases during the interval of greatest embryonic growth (stage 35 to parturition). However, the pattern of embryonic calcium acquisition is unknown for viviparous snakes. Furthermore, while the uterus of oviparous species transports calcium early in embryonic development during mineralization of the eggshell, the timing of uterine calcium secretion in viviparous snakes is unknown. We studied a viviparous snake, *Virginia striatula*, to determine the ontogenetic pattern of yolk and embryonic calcium content. The pattern of embryonic calcium uptake of *V. striatula* is similar to that of oviparous snakes but the sources of calcium differ. In contrast to oviparous species, embryos of *V. striatula* acquire half of total neonatal calcium via placental provision, of which 71% is mobilized between stage 35 and parturition. Furthermore, we report for the first time in a viviparous squamate an increase in yolk calcium content during early stages of embryonic development, indicating that uterine secretion of calcium occurs in *V. striatula* coincident with shelling in oviparous squamates. Thus, uterine calcium secretion in this viviparous species may either occur continuously or in two phases, coincident with the timing of shelling in oviparous species and again during the last stages of development. Whereas, the pattern of embryonic calcium acquisition in *V. striatula* is plesiomorphic for squamates, the pattern of uterine calcium secretion includes both retention of a plesiomorphic trait and the evolution of a novel trait.

1. Introduction

Yolk is the primary source of calcium during embryogenesis for most squamate reptiles (Packard, 1994; Stewart and Ecay, 2010). Embryos acquire additional calcium by extraction from the eggshell in oviparous species (Packard, 1994) or by placental transfer in most viviparous species (Thompson et al., 2000; Stewart and Ecay, 2010). Calcium is deposited in yolk during follicular vitellogenesis prior to ovulation and fertilization, whereas the uterine epithelium is the source of calcium for eggshells and for placental transfer, which occur at discrete times during development (Stewart et al., 2009). Eggshell calcium is deposited after fertilization, but prior to oviposition in oviparous snakes, and is stored in the eggshell until it can be utilized by embryos. Embryos of oviparous species mobilize 14–36% of their calcium from eggshells (Packard et al., 1984; Packard and Packard, 1988; Ji et al., 1997a,b, 1999; Stewart et al., 2004). Mobilization of yolk nutrients, including calcium, increases in the later stages of development, synchronous with greatest increases in embryonic mass. The extraction of eggshell calcium does not commence until the latest developmental stages when embryonic growth is highest (Packard et al., 1984; Packard and Packard, 1988; Stewart et al., 2004).

Embryos of viviparous snakes obtain a comparable proportion of calcium from placental transfer (Stewart and Castillo, 1984; Stewart, 1989; Stewart et al., 1990) as oviparous snakes obtain from eggshells. However, the timing of placental calcium secretion in viviparous snakes is unknown. Herbert et al. (2006) suggest that the timing of uterine calcium secretion in viviparous lizards with simple placentae is conserved to occur early in embryonic development prior to the phase of greatest embryonic growth. The conclusions of Herbert et al. (2006) are based on the developmental expression of a uterine plasma membrane calcium ATPase and not on direct measures of embryonic calcium uptake. If correct, early embryonic transport requires that calcium be stored within the eggshell-devoid egg until the embryo is sufficiently mature to utilize the additional calcium. Yolk may serve as a storage vessel for uterine-secreted calcium during early embryogenesis of viviparous squamates (Herbert et al., 2006). However, apart from observations in birds, there are no reports of

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increases in yolk calcium mass following vitellogenesis in reptiles (Packard, 1994). We studied the ontogenetic pattern of yolk and embryonic calcium content in the viviparous snake *Virginia striatula* to test the hypothesis that the timing of uterine calcium secretion coincides with the interval of highest embryonic growth and thus is temporally distinct from eggshell formation in oviparous snakes.

2. Materials and methods

Gravid female *V. striatula* ($n=30$) were collected from localities in Mark Twain National Forest (Taney County, Missouri, USA) during May 2008. Snakes were transported to East Tennessee State University where they were housed in aquaria (51 L × 26.5 W × 32 H cm) in groups of up to three individuals. The aquaria were equipped with 25 W incandescent bulbs, set on 14:10 photophase: scotophase, which elevated ambient temperature (21–24 °C) on one end of the enclosure allowing the snakes to move freely from one end of the enclosure to the other to thermoregulate. A refuge was placed under the light bulb in each aquarium, which consisted of a Petri dish with a bottom layer of topsoil and an upper layer of sphagnum moss. A plastic container with a hole cut in the center to allow the snakes to enter and exit covered the soil and sphagnum moss. The sphagnum moss was kept moist at all times, cages were misted on a daily basis and water was provided *ad libitum* in a small dish. Earthworms were provided as a food source.

Females were killed by a lethal injection of sodium pentobarbital at periodic intervals to sample yolks, embryos, and neonates in a series of developmental stages. Embryos were assigned to stages using Zehr's (1962) staging system (refer to Table 1 for stages and sample sizes). Wet mass was determined for yolks, embryos, and neonates which were stored in individual tared glass vials at −10 °C.

Harvested yolks, embryos, and neonates were lyophilized to a stable mass using a Labconco Freezone 4.5. Specimens were processed according to a modified protocol of Shadrix et al. (1994). Samples were digested in 3 mL concentrated nitric acid contained in borosilicate glass test tubes for 3 h at 125 °C. Digests were cooled to room temperature for 1 h. One mL of 30% hydrogen peroxide was added to each digest and allowed to react for 1 h. The samples were gradually returned to 125 °C and maintained for approximately 15 h. Following digestion, digests were evaporated gently to near dryness on a hot plate, diluted in 1:1 HCl, and brought to a final volume of 2.5% HCl with distilled water. Lanthanum chloride (1:10) was added to each sample prior to calcium analysis. Calcium content was estimated using a Varian 220 FS atomic absorption spectrophotometer calibrated against samples of known calcium concentration.

The relationship between female size and clutch size was analyzed using a linear regression analysis. Ontogenetic differences in dry mass and calcium mass of yolks and embryos were analyzed by two-factor analysis of variance with embryonic stage as a fixed factor and female (clutch) as a random factor. Comparisons among least squares means were analyzed using Scheffe's multiple comparisons test. Differences in relative yolk calcium content among five embryonic stages of oviductal eggs were tested with three-factor analysis of variance with embryonic stage as a fixed factor, female (clutch) as a random factor and egg dry mass as a covariate followed by Scheffe's multiple comparisons test. All statistics were generated with SAS 9.1 statistical software.

3. Results

Clutch size (mean, 5.21 ± 0.22; range, 2–9) was positively correlated with size of females (mean, 192.9 ± 2.16 mm snout-vent; range, 157–232) (Clutch size = −7.8 + 0.07 fsvl; $F=24.26$, $P<0.0001$).

Following ovulation, embryos gain little dry mass by stage 31 (Table 1). By stage 35, embryos undergo a roughly four-fold increase in dry mass. Stage 37 embryos contain 180% more dry mass than that of stage 35 embryos, while neonates contain 31% more dry mass than stage 37 embryos. Additionally, neonatal dry mass was 29% lower than dry mass of ovulated eggs (Table 1). Our earliest embryos, stage 31, contained little dry mass and we were unable to accurately estimate calcium content for these samples (Table 1).The ontogenetic pattern of embryonic calcium content was similar to dry mass with significant differences between stages 35 and 37 and between neonates and stage 37 embryos (Table 1). Calcium content of stage 37 embryos was equal to or exceeded total calcium in yolk at any embryonic stage and neonates contained 56% more calcium than stage 37 embryos. Neonates contained significantly more calcium (least squares means, 3.39 ± 0.13) than was available in ovulated yolk (least squares means, 1.67 ± 0.19) ($F=56.53$, $df=7.14$, $P<0.0001$) (Table 1).

Dry mass of yolk in ovulated eggs did not differ significantly from yolk of stage 31 embryos. At stages 35 and 37, yolk dry mass was reduced to 60% and 26% respectively of yolk dry mass in ovulated eggs (Table 1). Yolk calcium mass of stage 31 and stage 35 embryos did not differ from calcium mass in ovulated eggs. Calcium mass of yolk of stage 37 embryos was less than half the mass of calcium in yolk of stage 35 embryos and residual yolk from neonates contained little calcium (Table 1). Total yolk calcium adjusted for dry mass increased significantly following ovulation and remained relatively stable between stage 31 and parturition (Tables 1 and 2).

4. Discussion

Embryos of *V. striatula* gain dry mass and deplete yolk nutrients in a pattern that is similar to oviparous snakes (Table 1) (Packard et al., 1984; Packard and Packard, 1988; Stewart et al., 2004). The small dry mass of stage 31 embryos, associated with a gradual loss in yolk dry mass between ovulated eggs and embryonic stage 31, indicates that embryos grow slowly prior to this stage. Embryonic growth and yolk depletion remains gradual between embryonic stages 31 and 35. An increase in yolk consumption after stage 35 parallels an increase in embryonic growth that continues until parturition. The ratio of neonate dry mass to dry mass of recently ovulated eggs (0.71) (Table 1) is comparable to another population of *V. striatula* (Stewart, 1989) and to other viviparous (Stewart and Castillo, 1984; Stewart, 1989; Stewart et al., 1990) and oviparous species of snakes (Packard

Table 1
Ontogeny of wet mass (mg), dry mass (mg), and calcium (mg) content for yolk and embryos of *Virginia striatula*. Values represent least squares means ± s.e.m. Sample sizes are numbers of females (clutches). Embryonic staging from Zehr (1962).

	Yolk					Embryo			
Embryonic stage	*N*	Wet mass	Dry mass	Calcium	Calcium adj yolk dry mass*	*N*	Wet mass	Dry mass	Calcium
Ovulated	3	229.7 ± 22.8^{a}	126.1 ± 8.6^{a}	1.67 ± 0.21^{a}	0.25 ± 0.15^{a}				
Stage 31	4	207.3 ± 20.3^{a}	$101.8 \pm 7.8^{a,b}$	2.10 ± 0.19^{a}	1.18 ± 0.12^{b}	4	75.4 ± 17.7^{a}	6.5 ± 6.5^{a}	
Stage 35	4	182.4 ± 19.9^{a}	76.1 ± 7.6^{b}	1.78 ± 0.19^{a}	1.41 ± 0.08^{b}	4	231.0 ± 16.1^{b}	24.4 ± 6.5^{a}	0.61 ± 0.27^{a}
Stage 37	9	91.6 ± 13.3^{b}	32.6 ± 5.1^{c}	0.62 ± 0.12^{b}	1.15 ± 0.07^{b}	9	389.3 ± 10.9^{c}	68.7 ± 4.3^{b}	2.17 ± 0.18^{b}
Neonate	5	5.4 ± 17.7^{c}	2.5 ± 6.7^{d}	0.01 ± 0.17^{b}	1.16 ± 0.12^{b}	6	391.0 ± 12.5^{c}	89.9 ± 5.3^{c}	3.39 ± 0.22^{c}

a,b,c,d indicate significant differences between stages, $P \leq 0.05$.
*Yolk calcium mass with yolk dry mass as covariate.