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Ex utero culture of viviparous embryos of the lizard, *Zootoca vivipara*, provides insights into calcium homeostasis during development

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ABSTRACT

The chorioallantoic membrane resides adjacent to either the inner surface of the egg shell or uterine epithelium in oviparous and viviparous reptiles, respectively. Chorionic cells face the shell or uterine epithelium and transport calcium to underlying embryonic capillaries. Calcium transport activity of the chorioallantois increases in the final stages of development coincident with rapid embryonic growth and skeletal ossification. We excised embryos from viviparous *Zootoca vivipara* females at a stage prior to significant calcium accumulation and incubated them *ex utero* with and without calcium to test the hypothesis that chorioallantois calcium transport activity depends on developmental stage and not calcium availability. We measured calcium uptake by monitoring incubation media calcium content and chorioallantois expression of calbindin-D_{28K}, a marker for transcellular calcium transport. The pattern of calcium flux to the media differed by incubation condition. Eggs in 0 mM calcium exhibited little variation in calcium gain or loss. For eggs in 2 mM calcium, calcium flux to the media was highly variable and was directed inward during the last 3 days of the experiment such that embryos gained calcium. Calbindin-D_{28K} expression increased under both incubation conditions but was significantly higher in embryos incubated with 2 mM calcium. We conclude that embryos respond to calcium availability, yet significant calcium accumulation is developmental stage dependent. These observations suggest the chorioallantois exhibits a degree of functional plasticity that facilitates response to metabolic or environmental fluctuations.

1. Introduction

Most vertebrate embryos accumulate calcium during development along a trajectory that parallels embryonic growth in mass and length. For reptiles and mammals, growth and calcium accumulation accelerate in later embryonic stages. Depending on mode of reproduction, the calcium acquired during development originates from maternally supplied yolk stores and either the calcified egg shell in oviparous reptiles or by placental transfer from uterine secretions in viviparous reptiles and mammals. Mammals are exclusively dependent on placental calcium transfer during prenatal development, whereas crocodilians and birds derive 80% or more of hatchling calcium from the eggshell (Packard, 1994). Species of squamate reptiles have converged on each of these divergent patterns of embryonic calcium nutrition (Stewart and Ecay, 2010). Oviparous squamates exhibit the greatest diversity in the relative contribution of yolk and eggshell calcium to embryonic development of any amniote lineage and, with >1500 viviparous species (Blackburn, 2006), pattern of embryonic calcium nutrition of viviparous species is equally diverse (Stewart and Ecay, 2010). Early speculation that development in squamate species is highly dependent on calcium-enriched yolk and seemingly independent of eggshell calcium (Simkiss, 1967) supported a hypothesis that transitions from oviparity to viviparity are not constrained by the loss of eggshell calcium that accompanies prolonged uterine egg retention culminating in live birth (Packard et al., 1977). However, subsequent descriptions of developmental calcium accumulation in oviparous and viviparous squamate species demonstrate that 1) eggshell calcium can account for up to 80% of hatchling calcium in species with geographic variation in mode of reproduction, i.e., species with both oviparous and viviparous populations (Stewart et al., 2009a), 2) calcium placentotrophy can precede complete loss of eggshell calcium (Linville et al., 2010), and 3) placental calcium transport can compensate fully for loss of eggshell calcium (Ramírez-Pinilla et al., 2011; Stewart and Thompson, 1993). These observations suggest that eggshell thinning with loss of calcium and calcium placentotrophy are not consecutive but coincident evolutionary events and further suggest that mechanisms defending embryonic calcium accumulation during development confer an adaptive advantage to hatchlings and neonates. A consequent hypothesis is that the plasticity of uterine and embryonic calcium transport mechanisms, which can maximize

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calcium accumulation during prolonged uterine egg retention, is an important facilitator of the many transitions from oviparity to viviparity in squamates.

Embryonic accumulation of calcium from the eggshell or uterine secretions is mediated by the chorioallantoic membrane (Packard and Clark, 1996). This tissue, which lies adjacent to the inner surface of the eggshell or the uterine epithelium depending on mode of parity, expresses protein markers for transcellular calcium absorption found in renal and intestinal epithelia (Hoenderop et al., 2005). These include the intracellular calcium binding protein calbindin-D_{28K} and a plasma membrane calcium-transporting ATPase (PMCA). Expression of calbindin-D_{28K} and PMCA in the chorioallantois increases coincident with calcium transport to the embryo in late stages of development suggesting that transcellular calcium movement is an important mechanism for embryonic calcium accumulation (Stewart and Ecay, 2010). Existence of a paracellular calcium transport pathway is also possible but remains to be investigated.

Zootoca vivipara is one of three lizard species that demonstrate geographic variation in reproductive mode (Heulin et al., 1993). As with other oviparous reptiles, the eggshell of oviparous *Z. vivipara* consists of an inner matrix of fibrous protein covered with a well-developed outer layer of calcium carbonate crystals from which the underlying chorioallantoic membrane extracts calcium (Heulin, 1990; Heulin et al., 2002, 2005). Viviparous *Z. vivipara* eggs are surrounded by a thin proteinaceous eggshell positioned between the uterine epithelium and the chorioallantoic membrane (Heulin, 1990; Heulin et al., 2005). Uterine calcium secretions do not accumulate as a precipitated calcium carbonate layer on this eggshell, but pass through to the chorioallantoic membrane (Stewart et al., 2009a). Embryos of each reproductive mode accumulate calcium slowly until late in development, when uptake increases greatly, coincident with increased expression of calbindin-D_{28K} and PMCA in the chorioallantoic membrane (Stewart et al., 2011, Stewart et al., 2009a). The timing of embryonic uptake of calcium may be driven by differences in calcium availability, or alternatively, by the developmental expression of physiological mechanisms for calcium transport. The timing of uterine calcium delivery differs in each reproductive mode as the oviparous uterus calcifies an eggshell early in development and the viviparous uterus provides calcium at later stages coincident with embryonic demand and transport capacity of the chorioallantoic membrane.

We have surgically removed and cultured embryos from viviparous female *Zootoca vivipara* in incubation media with differing calcium content while simultaneously monitoring calcium flux between the embryo and incubation media to test the hypothesis that calcium uptake (chorioallantois calcium transport) is dependent on stage of development and not calcium availability.

2. Methods and materials

2.1. Incubation

Female viviparous *Zootoca vivipara* were collected from Paimpont France in September 2009. Lizards were maintained through a hibernation cycle, males and females paired for mating, and shipped to East Tennessee State University in March 2010. Eggs were removed from females ($N = 6$) prior to the phase of greatest embryonic growth (embryonic stages 36, 37, 37, 37, 37, 38). Embryonic development was staged by the system of (Dufaure and Hubert (1961). Eggs were assigned to one of three treatments, 1) initial sample, 2) incubation in nominally calcium-free media (composition in mM; 137 NaCl, 2.7 KCl, 1 $MgCl_2$, 12 $NaHCO_3$, and 0.3 NaH_2PO_4), or 3) incubation in media + 2.0 mM calcium chloride.

Following the protocols developed by Panigel (1956) and Bleu (2011) eggs were positioned on sterile cotton in a 60 ml plastic culture dish with 20 ml of medium and incubated at 25 °C in a humidified chamber. A media sample (10 ml) was removed for calcium determination and replenished with fresh incubation media (10 ml) at two day intervals until day 11, when the experiment was terminated. Calcium concentration in recovered media was measured with a calcium electrode (MI-600, Microelectrodes Inc., Bedford, NH) attached to an Accumet AB15 pH meter recording in mV mode. Culture dishes with cotton and media but no eggs were used as blanks to control for evaporative concentration of calcium, which was determined to be negligible. The calcium concentration in media samples was measured at the time of collection and electrode calibration curves were obtained at the same time using calcium standards from 100 to 0.01 mM. Calcium flux between compartments (egg and dish) was determined as the difference between the electrode measured calcium and the predicted calcium based on the measured media concentration corrected for carry over from the previous sample period.

2.2. Calcium analysis

Yolks, embryos and eggshells from the initial sample of eggs ($N = 6$ clutches, one egg per clutch per treatment) and from day 11 eggs (embryonic stage 40) ($N = 6$ clutches, one egg per clutch per treatment) were placed in separate tared vials, weighed and frozen (−10 °C). Samples were lyophilized to constant mass in a Labconco Freezone 4.5 freeze dryer. Preparation of samples for calcium analysis followed the method of (Shadrix et al., 1994). Samples were digested in borosilicate glass test tubes containing 3 ml concentrated nitric acid for 3 h at 125 °C. Digestates were cooled to room temperature for 1 h and 1 ml 30% hydrogen peroxide was added. The temperature was returned to 125 °C and maintained for approximately 15 h. The digestates were evaporated gently to near dryness on a hot plate, diluted in hydrochloric acid (1:1 in distilled H_2O) 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 Shimadzu model AA-6300 atomic absorption spectrophotometer calibrated against samples of known calcium concentration.

2.3. Immunoblotting

Chorioallantoic membranes were isolated from the initial sample of eggs (stage 36–38 embryos) and from eggs after 11 days in culture (stage 40 embryos). Tissue samples were homogenized and fractionated by polyacrylamide gel electrophoresis as described previously (Stewart et al., 2011). Electroblotted proteins were incubated with a rabbit polyclonal antibody to recombinant corn snake calbindin-D_{28K} (Ecay and Stewart, unpublished) at 1:50,000 dilution followed by peroxidase-conjugated anti-rabbit IgG (1:200,000). Immune complexes were visualized on X-ray film by chemiluminescence (Immobilon; Millipore). Blots were stripped and reprobed with anti-actin IgG (1:50,000 dilution; Sigma) as an internal control for sample loading. Films were digitized on a flatbed scanner and quantified by densitometry (Silk Scientific, Orem, UT).

2.4. Statistics

Differences in variance in calcium flux in incubation media between treatments (saline, 2 mM calcium) were tested by a two sample test for variances. We tested for homogeneity of variances within treatments with Bartlett's test. Variation in calcium flux in incubation media with 2 mM calcium was analyzed by one-way analysis of variance and calcium flux in incubation media with saline by both one-way analysis of variance and the Kruskal-Wallis non-parametric test. We analyzed variation in calcium content of egg shells, yolks and embryos with treatment (initial sample, saline, 2 mM calcium) and tissue as fixed factors, tissue dry mass as a covariate and clutch (female) as a random factor. We analyzed variation in calcium content of shell-free eggs (embryos + yolk) among treaments by analysis of variance with treatment as a fixed factor and clutch (female) as a random factor.

Differences in expression of calbindin-D_{28K} among treatments, as indicated by densitometry readings, were tested by mixed model analysis of variance with treatment and immunoblot identity as fixed factors, actin densitometry reading as a covariate and clutch as a random factor. Sheffe's multiple range test was used for a posteriori tests of differences among treatments. An alpha value of 0.05 was accepted as statistical significance. All statistics were generated with SAS 9.2 statistical software.

3. Results

3.1. Calcium in incubation media

Calcium flux between incubation media and eggs of both treatments (2 mM calcium, calcium-free saline) varied significantly with day of sample (2 mM calcium, $F_{5, 23} = 7.5$, $P < 0.001$; saline, $F_{5, 23} = 44.4$, $P < 0.001$). Because Bartlett's test revealed that variances were non-homogeneous for eggs incubated in saline, we also tested for a day effect with Kruskal-Wallis non-parametric analysis of variance. This statistic also indicated that calcium flux in saline incubation media varied significantly with day ($\times^2 = 27.9$, df = 5, $P < 0.0001$). Eggs in 2 mM calcium lost calcium to the media for the initial 8 days of culture but gained calcium thereafter (Fig. 1). Eggs in calcium-free saline lost calcium between the initiation of the experiment and the day two sampling, but calcium flux remained low for the remainder of incubation. The pattern of calcium flux through time reflected in mean values does not reveal the most intriguing difference between treatments, as illustrated by comparison of maximum and minimum values (Fig. 2). Variance in calcium flux was significantly higher for the 2 mM calcium media (mean = 0.053 mg, $s^2 = 0.026$, $n = 35$) than for calcium-free saline (mean = 0.048 mg, $s^2 = 0.005$, $n = 35$) ($F_{34, 34} = 4.9$, $P = 0.0001$) (Supplemental Table 1). Thus, individual variation in the interaction between the incubation medium and the egg was significantly greater for eggs in media with 2 mM calcium.

3.2. Calcium in eggs

Tissue calcium content was correlated with tissue dry mass and the distribution of calcium differed significantly by treatment and tissue. There also was a significant interaction effect (Supplemental Table 2). The eggshell contained the greatest quantity of calcium among egg compartments in eggs sampled at the initiation of the experiment and these eggshells contained significantly more calcium than eggs sampled at day 11 of incubation (Table 1). Embryos from eggs incubated in media with 2 mM calcium contained relatively more calcium (adjusted for embryonic dry mass) than embryos from eggs incubated in saline. Yolk calcium content, adjusted for yolk dry mass, did not differ among treatments (initial, saline, 2 mM calcium). Total calcium in shell-free

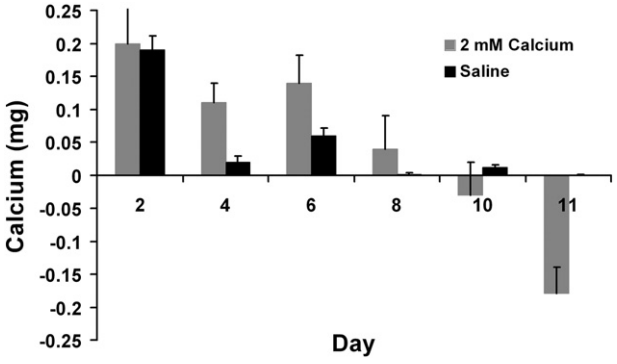

Fig. 1. Mean calcium flux (mg) over two day intervals in incubation media (calcium-free saline, 2 mM calcium + saline) containing *Z. vivipara* embryos from 6 females. Embryos were stage 36–38 on day 0 and stage 40 when the experiment was terminated on day 11. Positive values indicate loss of calcium from eggs, negative values indicate egg uptake of calcium from the medium. Error bars reported as + SEM.

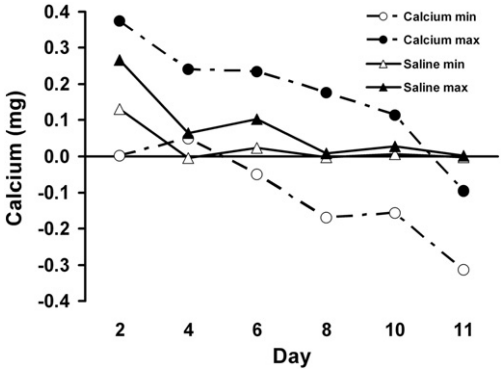

Fig. 2. Absolute maximum and minimum calcium flux (mg) over two day intervals in incubation media (calcium-free saline, 2 mM calcium + saline) containing *Z. vivipara* embryos from 6 females. Embryos were stage 36–38 on day 0 and stage 40 when the experiment was terminated on day 11. Positive values indicate loss of calcium from eggs, negative values indicate uptake of calcium from the medium.

eggs (embryos + yolks) from calcium-free saline incubation media (0.21 ± 0.02 mg) did not differ significantly from total calcium in eggs at day 0 (0.18 ± 0.02 mg), whereas eggs incubated in saline + calcium media (0.29 ± 0.02 mg) contained significantly more calcium ($F_{2,8} = 19.6$, $P < 0.01$).

3.3. Immunoblotting

Calbindin-D_{28K} was expressed in chorioallantoic membranes of embryos sampled at day 0 (stage 36–38) and in day 11 embryos (stage 40) from each treatment group (Fig. 3). Densitometry readings indicated significantly greater expression in day 11 embryos from eggs incubated in saline + 2 mM calcium compared to both day 0 embryos and day 11 embryos incubated in calcium-free saline (Fig. 4). Embryos incubated in calcium-free saline had greater expression of calbindin-D_{28K} than embryos at day 0. The inclusion of actin densitometry readings in the model did not affect the results.

4. Discussion

4.1. Characteristics of the eggshell

Eggs of viviparous *Zootoca vivipara* develop in an aqueous environment in contact with the uterine epithelium. At the time of ovulation, egg calcium is accounted for by maternally provisioned yolk, which contributes approximately 25% of neonatal calcium (Stewart et al., 2009a). The remaining 75% derives from uptake of calcium from uterine secretions. Not long after ovulation and prior to any measurable embryonic acquisition of uterine calcium, the egg is surrounded by a thin eggshell that itself accumulates calcium during early stages of intrauterine gestation (Stewart et al., 2009a). The functional role of the eggshell in calcium uptake by the embryo is not known. The structural alignment of calcium relative to the organic matrix of the eggshell during eggshell formation has not been described, but at parturition small crystals of calcite are scattered widely on the outer surface of eggshells (Heulin, 1990). In the experiments described here, the calcium content of eggshells in the initial sample of eggs (embryonic stage 36–38) was similar to comparable stage embryos from a previous study (Stewart et al., 2009a). In that prior study, we concluded that calcium moves through the eggshell during late stages of intrauterine gestation as embryonic calcium content increases, yet eggshell calcium content remains stable (Stewart et al., 2009a). The calcium content of eggshells containing stage 40 embryos incubated in either calcium-free saline or saline with 2 mM calcium was similar and lower than the initial sample of eggs. Thus, our *ex utero* incubation conditions altered the physical properties of the eggshell resulting in release of bound calcium or dissolution of precipitated

Table 1
Least squares means of calcium (mg) adjusted for dry mass (covariate) in egg compartments of *Zootoca vivipara* sampled at embryonic stage 36 or 37 (initial sample) and after 11 days of incubation (embryonic stage 40) in either calcium-free saline or saline with 2 mM calcium. Sample sizes are number of females (clutches). Values reported as ±SEM.

Treatment	Sample day	N	Shell	Yolk	Embryo
Initial sample	0	5	0.31 ± 0.02^{a}	-0.01 ± 0.03	0.09 ± 0.01^{c}
2 mM calcium	11	5	0.10 ± 0.02^{b}	0.03 ± 0.01	0.20 ± 0.02^{d}
Calcium-free saline	11	5	0.09 ± 0.02^{b}	0.03 ± 0.01	0.13 ± 0.02^{c}

$^{a,b}P < 0.0001$ between symbols within columns.
$^{c,d}P < 0.05$ between symbols within columns.

calcium carbonate crystals. Calcium binding and/or formation of calcium carbonate crystals on the eggshell would be promoted by a threshold concentration of calcium, bicarbonate ions and a suitable acid-base balance in the uterine compartment. Under our incubation conditions, at least one of these variables did not mimic uterine conditions. Thus, both experimental treatments allow assessment of embryonic response to an environment not regularly encountered.

4.2. Ca^{2+}-free media

Eggs incubated in calcium-free saline provide insight into how embryos respond to an extreme environmental condition. Embryos reared in calcium-free saline experienced a considerable calcium concentration gradient (embryo to incubation media) yet were able to protect against loss of calcium over the 11 days of the experiment. Total calcium in shell-free eggs (embryo + yolk) sampled on day 11 did not differ significantly from total calcium in sibling eggs at the initiation of the experiment. Estimates of calcium release into the incubation media support the results of analyses of egg calcium content. With the exception of the first sampling period (day 2), there was no significant difference between subsequent sampling days, i.e., media did not gain calcium after day 2. The increase in calcium in the media on day 2 can be accounted for by loss of calcium from the eggshell. Additionally, variation in media calcium flux was low throughout the experiment, indicating a relatively uniform response among eggs. If the chorioallantois expresses a paracellular pathway for calcium uptake late in development, this pathway must be either rectifying (allowing only unidirectional calcium flow into the embryo) or regulated to close under conditions of low external (uterine) calcium and preventing calcium efflux from the embryo. These results support a hypothesis that embryos have the capacity to conserve calcium. This physiological response would buffer embryos from calcium deficiencies, if for example, uterine fluid calcium concentrations fluctuate during gestation, resulting in an unfavorable calcium diffusion gradient for embryos at periodic intervals. Maintenance of embryonic calcium homeostasis will be dependent on the capacity of embryos to protect against calcium loss.

4.3. Media with Ca^{2+}

Our incubation media contained 2 mM calcium, which we predict is similar to circulating calcium in embryos as it is comparable to the ionized calcium content of plasma of the lizard, *Iguana iguana* (Dennis et al., 2001). The physiological response of embryos incubated in media with 2 mM calcium differed from those in calcium-free saline. Eggs in 2 mM calcium lost a similar amount of calcium on day 2 as eggs in 0 mM calcium, consistent with loss from the eggshell. However, eggs in 2 mM calcium continued to lose calcium on days 4 through 8. This calcium must have come from yolk and embryo and suggests that 2 mM calcium does not support an inwardly directed calcium gradient for paracellular uptake and conditions to maintain eggshell calcium binding and/or calcium carbonate precipitation. Additionally, variation in calcium flux among eggs in 2 mM calcium was significantly higher than for eggs in calcium-free media. This suggests that, on average, these embryos were losing calcium to the media and may vary in their capacity to protect against calcium loss under these environmental conditions. We propose this reflects multiple calcium sequestration/release mechanisms (eggshell binding, paracellular transport, yolk calcium mobilization, embryonic circulating calcium) with individual eggs adapting or modifying these in response to incubation conditions. After day 8 the direction of calcium movement reversed such that eggs gained and media lost calcium on days 10 and 11. The amount of calcium taken up through the last sampling times exceeded the calcium lost after day 2 and eggs incubated in 2 mM calcium contained significantly more calcium on day 11 (stage 40) than the initially sampled eggs (day 0, stage 36–38) and day 11 eggs in 0 mM calcium (stage 40). This demonstrates that eggs gain the active capacity to take up calcium from uterine secretions at the latest stage of development even against a concentration gradient that might favor passive efflux from the eggs.

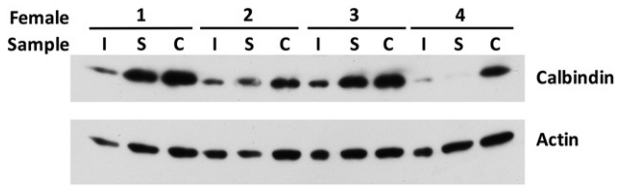

Fig. 3. Immunoblotting analysis of calbindin-D_{28k} expression in samples of the chorioallantoic membrane of *Zootoca vivipara* at the initiation of the experiment (I) and after 11 days incubation in calcium-free saline (S) or saline with 2 mM calcium (C). The blot was stripped and re-probed with anti-actin IgG as an internal control.

4.4. Calcium transporting mechanism

This experiment confirms earlier research demonstrating relatively low levels of expression of calbindin-D_{28K}, a marker for epithelial calcium transport activity (Hoenderop et al., 2005), in the chorioallantoic membrane of *Zootoca vivipara* prior to embryonic stage 40 (Stewart et al., 2011). Expression of this cytosolic calcium-binding protein, which is localized in chorionic epithelial cells, increases significantly during embryonic stage 40 when embryonic uptake of calcium is high. Similar timing of calbindin-D_{28K} expression was reported in oviparous and viviparous *Zootoca vivipara* embryos (Stewart et al., 2011) and observed here for eggs incubated in 0 mM and 2 mM calcium. We conclude that increased calbindin-D_{28K} expression is developmental stage dependent.

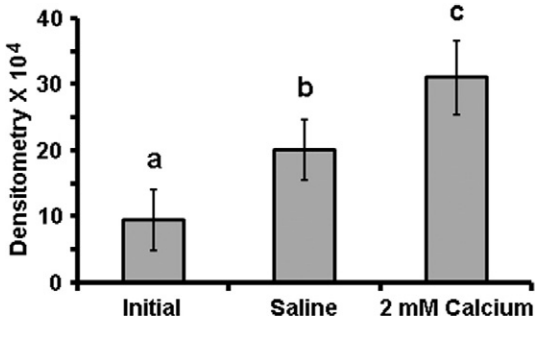

Fig. 4. Least squares means of densitometry readings of immunoblots of expression of calbindin-D_{28K} in chorioallantoic membranes of embryos sampled at the initiation of the experiment and after 11 days incubation in calcium-free saline or saline with 2 mM calcium. $N = 6$ females (clutches). Values reported as ±SEM. Letter symbols indicate significant difference between sample means; a,b $P < 0.05$, a,c $P < 0.0001$, b,c $P < 0.002$.

However, we observed here that calbindin-D_{28K} expression was greater in stage 40 eggs yet significantly higher in eggs incubated in 2 mM calcium, suggesting that calcium availability also regulates the expression of calbindin-D_{28K}. Thus, a positive feedback mechanism may characterize calcium transport by the chorioallantoic membrane such that once the terminal developmental stage is reached and calbindin-D_{28K} expression begins to increase, availability of transportable eggshell or uterine calcium stimulates additional expression of calbindin-D_{28K} and perhaps other components of the calcium transport machinery.

Epithelial tissues with a significant role in calcium transport share elements of a common transcellular transport machinery that includes a calcium channel (TRPV 5/TRPV 6), a cytosolic calcium binding protein (calbindin-D_{9K}/calbindin-D_{28K}) and plasma membrane calcium ATPase (PMCA) (Hoenderop et al., 2005). The chorioallantoic membrane of lizards and snakes, which has a significant function in delivering calcium to embryos, expresses calbindin-D_{28K} and PMCA (Ecay et al., 2004; Fregoso et al., 2012; Stewart et al., 2011; Stinnett et al., 2012). In addition, many vertebrate cells that function in calcium homeostasis detect extracellular calcium via calcium-sensing receptors (CaSRs) (Brennan et al., 2013). The response of embryos of *Zootoca vivipara* to both reduce calcium loss in the absence of exogenous calcium and to increase expression of calbindin-D_{28K} when calcium is available suggests that there is a mechanism to detect calcium, likely a CaSR, in the chorioallantoic membrane.

In summary, our results suggest several properties of the chorioallantoic membrane calcium transport system: 1) embryos have the capacity to protect against calcium loss in the absence of exogenous calcium, 2) embryos have a mechanism for monitoring uterine (or extraembryonic) calcium levels, 3) the detection of calcium influences permeability of the extraembryonic membranes to calcium and initiates transcription of transcellular calcium transporting proteins, 4) net uptake of calcium across the chorioallantoic membrane is dependent on maturation of the calcium transporting mechanism late in embryonic development.

4.5. Implications for the evolutionary transition from oviparity to viviparity

A recent model, influenced by studies of species with geographic variation in reproductive mode, proposes that the evolution of placental calcium transport in squamate reptiles precedes, or is coincident with, the evolution of viviparity (Stewart, 2013). Such a relationship between these two reproductive characteristics would be a contributing factor to the high incidence of evolutionary origins of viviparity in squamates because the transition between reproductive modes would result in little or no compromise in embryonic calcium nutrition. One prediction of the model is that embryonic response to calcium availability is independent of incubation environment, i.e., reproductive mode.

Oviparous embryos of *Zootoca vivipara* are highly dependent on the eggshell for calcium nutrition. Although the eggshell is secreted prior to substantial embryonic development, oviparous embryos acquire little calcium from eggshell until late developmental stages. Calcium mobilized from the eggshell is transported into the embryo and not into other egg compartments, such as yolk (Stewart et al., 2009a). Thus, embryonic uptake of calcium is correlated with embryonic growth and skeletal formation, i.e., when embryonic compartments are capable of calcium metabolism. Characteristics of calcium nutrition of oviparous embryos, i.e., embryonic dependence on uterine calcium, the ontogenetic pattern of embryonic uptake of calcium and expression of calbindin-D_{28K} in the chorioallantoic membrane are conserved in viviparous embryos of *Z. vivipara*, as well as in embryos of highly placentotrophic lizards (Stewart et al., 2009a, 2009b, 2011; Stinnett et al., 2012).

The results from this experiment reveal two characteristics of the regulatory mechanism of calcium transport to viviparous embryos: 1) embryos can protect against calcium loss in the absence of exogenous calcium, and 2) embryos have a mechanism for sensing calcium and that stimulation of the calcium sensor alters the physiological response of the chorioallantoic membrane by promoting expression of a transcellular calcium transporting mechanism, and perhaps, altering the paracellular permeability of the chorioallantoic membrane to calcium. We suggest that these two attributes are fundamental properties of the calcium transporting mechanism of *Z. vivipara* and thus also occur in oviparous embryos. As with most oviparous squamates (Packard and DeMarco, 1991), the eggshell of oviparous *Z. vivipara* is primarily composed of a loosely organized layer of calcium carbonate on the outside of a relatively thick organic matrix (Heulin, 1990; Heulin et al., 2002). Most squamate eggs experience water flux during development, with a net inflow over the course of incubation. Calcium likely travels in the water column within the interstices of the eggshell and thus is distributed throughout the eggshell protein matrix. The existence of a calcium-sensing receptor on chorionic epithelial cells would be an efficient mechanism to link the calcium transporting machinery to the availability of calcium as it is released from the eggshell. This property also would be advantageous to embryos in the evolutionary transition between oviparity and viviparity to coordinate embryonic response to the uterine environment and would remain advantageous in viviparous embryos.

Although embryonic calcium transport and metabolism may be conserved in the evolutionary transition between oviparity and viviparity, uterine structure and function differs. The protein matrix of the eggshell is greatly reduced in viviparous females in correlation with reduced size of uterine shell glands (Heulin et al., 2005) and the quantity of calcium is likewise reduced (Stewart et al., 2009a). Uterine calcium secretion is initiated soon after the protein matrix is secreted, as occurs in oviparous females, but the quantity incorporated onto the surface of the eggshell fibers is much less in viviparous females. In contrast to oviparous females, in which uterine calcium secretion terminates early in embryonic development, the uterine cycle of calcium secretion by viviparous females is prolonged and then increases dramatically late in gestation. This variation in the uterine cycle of calcium secretion is a major event in the evolutionary transition from oviparity to viviparity. Understanding the regulation of uterine calcium secretion and its response to prolonged intrauterine gestation will contribute importantly to understanding a critical stage in the evolution of viviparity and placentation.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2017.01.011.

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