The Journal of Experimental Biology 214, 2999-3004
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doi:10.1242/jeb.059337

RESEARCH ARTICLE

Developmental expression of calcium transport proteins in extraembryonic membranes of oviparous and viviparous *Zootoca vivipara* (Lacertilia, Lacertidae)

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Accepted 21 June 2011

SUMMARY

The eggshell of oviparous lizards is a significant source of calcium for embryos, whereas the eggshell of viviparous lizards, when present, contains little calcium. In view of the potential cost to embryonic nutrition occasioned by the loss of eggshell calcium, the large number of independent origins of viviparity among lizards is surprising. Concomitant evolution of viviparity and calcium placentotrophy would ameliorate the loss of eggshell calcium, but a mechanism linking these events has yet to be discovered. *Zootoca vivipara*, a lizard with geographic variation in its mode of parity, is an excellent model for studying mechanisms of calcium transport to oviparous and viviparous embryos because each is highly dependent on calcium secreted by the uterus (eggshell or placenta) and ontogenetic patterns of embryonic calcium mobilization are similar. We compared developmental expression of the calcium transport protein calbindin-D_{28K} in yolk splanchnopleure and chorioallantoic membranes of oviparous and viviparous embryos to test the hypothesis that the mechanism of calcium transport does not differ between modes of parity. We found that the ontogenetic pattern of protein expression is similar between reproductive modes and is correlated with calcium uptake from yolk and either eggshell or placenta. Calbindin-D_{28K} is localized in the chorionic epithelium of embryos of both reproductive modes. These findings suggest that the embryonic calcium transport machinery is conserved in the transition between reproductive modes and that an adaptation of oviparous embryos for calcium uptake from eggshells functions similarly to transport calcium directly from uterine secretions.

Key words: placentotrophy, viviparity, calcium, calbindin-D_{28K}.

INTRODUCTION

Oviparous reptiles oviposit eggs with calcareous eggshells that provide a substantial amount of calcium to embryos (Packard and Demarco, 1991; Packard, 1994; Stewart and Ecay, 2010). In contrast, viviparous species produce eggshells that are considerably thinner and lack an outer layer of calcium carbonate (Blackburn, 1993; Heulin, 1990). In the absence of an alternative source of calcium, the evolution of viviparity would result in a nutritional deficit to developing embryos (Packard et al., 1977). Recent research on lizard species with geographic variation in their mode of parity reveals that embryos of viviparous populations receive roughly the same amount of calcium from placental transport as oviparous embryos extract from eggshells (Stewart et al., 2009; Linville et al., 2010). In addition, the ontogenetic pattern of embryonic calcium mobilization does not differ between reproductive modes. These studies suggest that specializations for calcium uptake by embryos are conserved in the evolution of reproductive mode. Functional continuity in the acquisition of calcium by embryos during transitions between modes of parity would contribute to the propensity of squamates to evolve viviparity and to the widespread structural similarity of extraembryonic tissues among placental squamates.

Recovery of calcium from the eggshell of squamates is mediated by the chorioallantoic membrane (Packard, 1994), which expresses proteins shared with other calcium-transporting tissues (Stewart and Ecay, 2010). For example, embryonic uptake of calcium from eggshell in the oviparous snake *Pantherophis guttatus* is correlated with the expression of the calcium-binding protein calbindin-D_{28K} and plasma membrane calcium ATPase (PMCA) in chorioallantoic membrane (Ecay et al., 2004; Ecay et al., 2006; Stewart et al., 2004a). Calbindin-D_{28K} facilitates cytosolic transfer of calcium from mucosal to serosal plasma membranes and PMCA transports calcium across the basolateral plasma membrane in a variety of vertebrate tissues (Feher et al., 1992; Bindels, 1993; Peng et al., 2003; Hoenderop et al., 2005). The pathway for mobilization of calcium from yolk by embryonic squamates has not been studied. However, endodermal cells of the yolk sac splanchnopleure of chicken embryos transport calcium from the yolk to the blood vascular system and intracellular transport of calcium is facilitated by calbindin-D_{28K} (Johnston and Comar, 1955; Ono and Tuan, 1991; Tuan and Suyama, 1996). The mechanism may be similar for squamates because expression of calbindin-D_{28K} is correlated with loss of calcium from yolk in the oviparous snake *P. guttatus* (Ecay et al., 2004; Stewart et al., 2004a).

Zootoca vivipara is a reproductively bimodal lizard. Oviparous females oviposit eggs with thick eggshells containing an outer layer of calcium carbonate (Heulin, 1990; Heulin et al., 2002) that provide a substantial amount (81%) of the calcium contained in hatchlings (Stewart et al., 2009). Viviparous females produce thin eggshells that lack an outer layer of calcium carbonate and give birth to

independent neonates (Heulin, 1990; Heulin et al., 1991). These females provide most of the calcium (76%) contained in neonates *via* placental transport and the pattern of embryonic acquisition of calcium does not differ from that of oviparous embryos (Stewart et al., 2009). We studied the expression of proteins implicated in calcium transport (calbindin-D_{28K}, PMCA) in yolk splanchnopleure and chorioallantoic membrane of a developmental series of oviparous and viviparous *Z. vivipara* embryos to address two questions. (1) does the mechanism of calcium transport by the extraembryonic membranes share functional attributes with other vertebrate calcium-transporting tissues? (2) Is the mechanism of calcium transport by embryos conserved in the evolution of reproductive mode?

MATERIALS AND METHODS

Zootoca vivipara (Jacquin 1787) were collected under permits 02/2006/Dpt64, 05/2006/Dpt35, 47/2007/Dpt64 and 04/2007/Dpt35, and export permits 35-87 and 35-90, and cleared for importation into the USA by the US Fish and Wildlife Service (Dec Control no. 2009AT670542). All procedures related to animals maintained at East Tennessee State University were approved by the University Committee on Animal Care.

Tissues were harvested from *Z. vivipara* embryos from populations that were the subject of a previous study of embryonic patterns of calcium acquisition (Stewart et al., 2009). Oviparous females were collected in September 2008 (N=15) from Louvie, France (43°06′N, 0°23′W, elevation 370 m). Viviparous females were collected in September 2008 (N=16) 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 elsewhere (Heulin et al., 2005), until the following spring. Females were removed from hibernation conditions, 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 2009. Animals were housed in glass terraria with nest boxes filled with moist sphagnum moss. Room lights provided 14 h:10 h photophase:scotophase and 40 W incandescent cage lights provided additional heat for 8 h a day. Water was provided *ad libitum* and crickets dusted with phosphorus-free calcium with vitamin D (Rep-Cal) were offered twice a week. Oviposited eggs were incubated at 25°C in vermiculite:water (2:1), estimated to provide a water potential of –120±40 kPa as measured by thermocouple psychrometry using a Wescor C52 chamber and Wescor HR33 T microvoltimeter (Wescor, South 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. Embryos were assigned to developmental stages based on the staging system of Dufaure and Hubert (Dufaure and Hubert, 1961). Eggs of both modes of parity were treated similarly and either fixed in 10% neutral buffered formalin or dissected to provide tissue samples, which were placed in cell lysis buffer. Immunoblots were prepared for samples of yolk splanchnopleure and chorioallantoic membrane for reproductive stages 33–40 for each reproductive mode. Samples examined with immunohistochemistry included two eggs of each reproductive mode for each of three embryonic stages: 37, 39 and 40.

Western blotting

Tissue samples of yolk splanchnopleure and chorioallantoic membrane were dissected free and placed in mammalian cell lysis buffer (MCL1, Sigma, St Louis, MO, USA) at a wet mass:volume ratio of 1:5 (mg μl^{-1}) and stored at –80°C. Samples were thawed on ice, diluted with an equal volume of fresh lysis buffer and homogenized in 1.5 ml microcentrifuge tubes using 1 mm glass beads and a Disruptor Genie cell disrupter (Scientific Industries, Inc., Bohemia, NY, USA). Following three 5 min rounds of cell disruption at 4°C, homogenates were centrifuged briefly to pellet beads and resistant tissue fragments.

The protein content of tissue homogenates was determined by BCA assay (Pierce, Rockford, IL, USA). Tissue samples were diluted to 2 $\mu g\,\mu l^{-1}$ total protein in 2× electrophoresis sample buffer prior to electrophoretic separation on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) (Laemmli, 1970). Gel lanes were loaded with 10 µl samples (20 µg protein/lane). One lane was loaded with 5 µl broad range standards (Bio-Rad, Hercules, CA, USA) to estimate molecular mass. Following SDS-PAGE, proteins were electroblotted to PVDF (Millipore, Billerica, MA, USA) (Towbin et al., 1979) and the pattern of electroblotted proteins visualized with 0.2% Ponceau S. Electroblots were bisected at the 60 kDa point to facilitate separate incubation with antibodies against high and low molecular mass proteins.

Primary antibodies used for immunoblots were a rabbit polyclonal against recombinant corn snake calbindin-D_{28K} (T.W.E. and J.R.S., unpublished), a mouse monoclonal (clone 5F10) against human PMCA (Sigma A7952) and a mouse monoclonal (clone C4) against chicken gizzard actin (Millipore). Secondary antibodies were peroxidase-conjugated goat anti-rabbit IgG (Pierce) and sheep anti-mouse IgG (GE Healthcare, Chalfont St Giles, Bucks, UK).

For immunodetection, protein blots were cleared of non-specific binding sites by incubation in blocking buffer [5% non-fat dry milk, 2% horse serum, 0.05% Tween-20, 0.02% sodium azide in Tris-buffered saline (TBS; pH 7.4)] for 1 h at room temperature. Primary antibodies were diluted in blocking buffer and incubated with blots overnight at 4°C on an orbital shaker. Primary antibodies were removed with six 5 min washes in blocking buffer (without azide), and peroxidase-conjugated secondary antibodies diluted in blocking buffer (without azide) were added for 2 h at room temperature. Blots were washed (6×5 min) in TBS and incubated with Immobilon chemiluminescent reagent (Millipore) for 5 min followed by visualization on X-ray film. Subsequent to immunodetection, blots were washed twice in TBS for 5–10 min, cleared of adherent antibodies with two 10 min washes in stripping buffer [0.05 mol l^{-1} glycine HCl (pH 2.5), 0.2% SDS, 0.1% Tween-20 in distilled H_2O], washed twice again in TBS for 10 min. Stripped blots were either immediately incubated with blocking buffer (30 min) followed by new primary antibody as above or stored dry for later reprobing. Actin immunoblotting was used as an internal standard for sample preparation and gel protein loading. Immune complexes revealed by chemiluminescence on X-ray film were quantified by scanning densitometry using a flatbed scanner (Epsom V500) and Un-Scan-It Gel™ 5.3 digitizing software (Silk Scientific, www.silkscientific.com).

Differences in densitometry readings of calbindin-D_{28K} and plasma membrane calcium ATPase in samples of chorioallantoic membrane for each reproductive mode were compared for two developmental intervals (early: embryonic stages 33–36; late: embryonic stages 37–40) using a mixed model analysis of variance (SAS 9.2, Cary, NC, USA). Actin values were entered as a covariate and individual immunoblots were entered as a fixed factor. For the oviparous population, eight females contributed one egg to each of the two developmental categories. Viviparous females contributed a single egg to one of the two developmental intervals (N=10 for

embryonic stages 33–36; N=6 for embryonic stages 37–40). Densitometry readings of calbindin-D_{28K} in samples of yolk splanchnopleure were analyzed similarly. Sample sizes were the same for oviparous females. For viviparous females, N=8 for embryonic stages 33–36 and N=6 for embryonic stages 37–40).

Immunohistochemistry

For formalin-fixed eggs, embryos were dissected free from other tissues after a brief immersion in formalin and the remainder of the egg contents was fixed overnight, dehydrated, cleared and embedded in paraffin. Paraffin sections (7 μm) were placed on glass slides, deparaffinized, hydrated, treated with 0.5% Triton X-100 in phosphate-buffered saline (PBS), washed in PBS and then blocked with 5% normal goat serum in PBS. Sections were incubated in primary antibody (monoclonal anti-bovine calbindin-D_{28K} from mouse, clone CB-955, Sigma C9848) for 2 h at 15 μg ml^{-1} in 5% normal horse serum. Paired sections were treated with 5% normal horse serum lacking antibody as negative controls. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in PBS prior to treatment with secondary antibody. Secondary antibody was biotinylated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) in 5% normal horse serum/PBS (5.5 μg ml^{-1}) for 1 h followed by peroxidase-conjugated streptavidin (Jackson ImmunoResearch) in 5% normal horse serum/PBS (2 μg ml^{-1}) for 1 h. Sections were reacted with 3,3′-diaminobenzidine (DAB) peroxidase substrate tablets (Sigma D4293) and counterstained with Harris hematoxylin. Sections of lizard kidney were used as positive controls.

Tissue sections were visualized using a Leica DMLB light microscope using a Leica DFC 420 image capture system with Leica Application Suite basic software. Images were cropped and labeled with Adobe Photoshop CS3 software.

RESULTS

Western blotting

Antibodies against PMCA and actin reacted with samples of chorioallantoic membrane for all embryonic stages (33–40) of both oviparous and viviparous embryos, whereas there was a weak reaction for antibodies against calbindin-D_{28K} in early embryonic development (stages 33–36) and a distinct reaction in later embryonic stages (37–40) (Figs 1 and 2). Comparisons of densitometry readings between early developmental stages and late developmental stages for calbindin-D_{28K} indicated significantly higher protein expression in late developmental stages for both modes of parity (oviparous: $F_{1,6.2}$=21, P=0.003; viviparous: $F_{1,12}$=43, P<0.0001). In contrast, expression of PMCA did not differ between developmental intervals for either oviparous ($F_{1,4.8}$=0.57, P=0.49) or viviparous ($F_{1,12}$=1.39, P=0.26) embryos.

Antibody reactions against actin and calbindin-D_{28K} in yolk splanchnopleure were similar to those for chorioallantoic membrane. Samples from all embryonic stages reacted consistently for actin, but only later embryonic stages exhibited consistent reactions for calbindin-D_{28K} (Figs 3 and 4). PMCA immunoreactivity in yolk splanchnopleure samples was below the limit of detection with the available antibodies. Densitometry readings for calbindin-D_{28K} expression indicated significantly higher values for later developmental stages for both oviparous ($F_{1,17.3}$=55, P<0.0001) and viviparous ($F_{1,6.7}$=9.6, P<0.02) embryos.

Immunohistochemistry

Reaction product was present in chorionic epithelial cells of histological sections of chorioallantoic membrane treated with antibody against calbindin-D_{28K} for embryos of both modes of parity for embryonic stages 37, 39 and 40 (Fig. 5). No discernable pattern was evident in the distribution of positively staining cells. However, a higher number of cells stained in stage 40 embryos and contiguous staining of cells was more common. Epithelial cells of lizard kidney distal tubules stained positively. Neither kidney distal tubule epithelial cells nor chorionic epithelial cells stained in any of the negative controls.

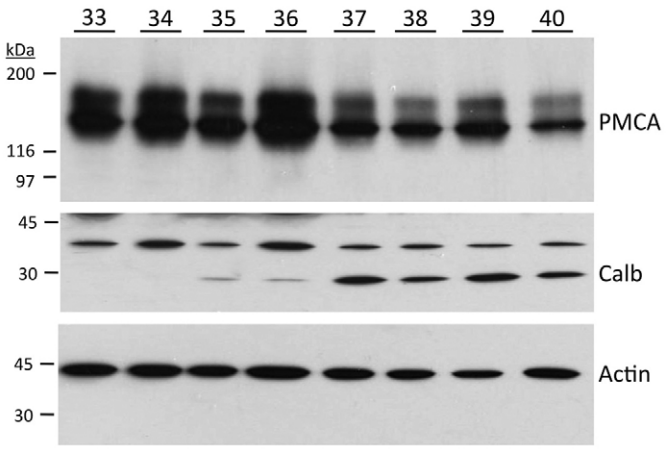

Fig. 1. Immunoblot of developmental expression of plasma membrane calcium ATPase (PMCA), calbindin-D_{28K} (Calb) and actin for the chorioallantoic membrane of oviparous *Z. vivipara* embryos. Embryonic stages were assigned according to Dufaure and Hubert (Dufaure and Hubert, 1961) (see Materials and methods) and are indicated at the top. Positions of molecular mass standards are on the left. Electroblots were bisected at the 60 kDa point to allow separate incubation with antibodies against high and low molecular mass proteins. The upper part was reacted with PMCA antibodies and the lower with calbindin-D_{28K} antibodies. The lower part of the blot was then stripped and reprobed with actin antibodies.

DISCUSSION

Vertebrate calcium-transporting tissues typically share a suite of characteristics that suggest the mechanism for transcellular transport of calcium is highly conserved. The extraembryonic membranes of *Z. vivipara* exhibit characteristics in common with a variety of vertebrate calcium-transporting tissues and the pattern of protein expression is similar for the two modes of parity. The prevailing model for calcium transport includes three primary components: calcium channels in the apical plasma membrane of transporting cells, a calcium-binding protein to facilitate diffusion through the cytoplasm, and a calcium-ATPase (PMCA) in basolateral plasma membranes (Hoenderop et al., 2000; Hoenderop et al., 2005; Larsson and Nemere, 2002; Peng et al., 2003). The two tissues responsible for calcium mobilization from yolk and eggshell in oviparous reptiles are yolk splanchnopleure and chorioallantoic membrane (Packard, 1994), and expression of calcium transport proteins in these tissues of *Z. vivipara* and the oviparous snake *Pantherophis guttatus* (Ecay et al., 2004; Ecay et al., 2006) is consistent with the general model for the mechanism of transcellular calcium transport. Further, expression of the calcium-binding protein calbindin-D_{28K} is closely correlated with calcium transport activity in kidney, intestine and egg shell gland of birds (Bar, 2009), as it is in yolk splanchnopleure and chorioallantoic membrane of *Z. vivipara* and *P. guttatus* (Ecay et al., 2004; Stewart et al., 2004a; Stewart et al., 2009). Calbindin-D_{28K} is localized in chorionic epithelial cells, which implicates these cells as major components

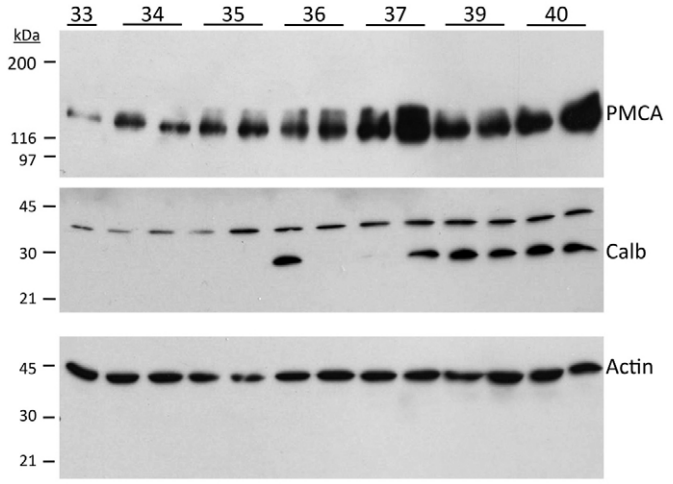

Fig. 2. Immunoblot of developmental expression of plasma membrane calcium ATPase (PMCA), calbindin-D_{28K} (Calb) and actin for chorioallantoic membrane of viviparous *Z. vivipara* embryos. Embryonic stages were assigned as for Fig. 1 and are indicated at the top. Positions of molecular mass standards are on the left. Duplicate samples from separate embryos at each stage were run in adjacent lanes. The upper part of the blot was reacted with PMCA antibodies and the lower with calbindin-D_{28K} antibodies. The lower part of the blot was then stripped and reprobed with actin antibodies.

of a transcellular calcium-transporting system. In addition, the similarity between reproductive modes of *Z. vivipara* in the ontogenetic pattern of expression of calbindin-D_{28K} and PMCA and the localization of calbindin-D_{28K} in chorionic epithelium indicates that the embryonic response to calcium availability is conserved in the evolution of reproductive mode.

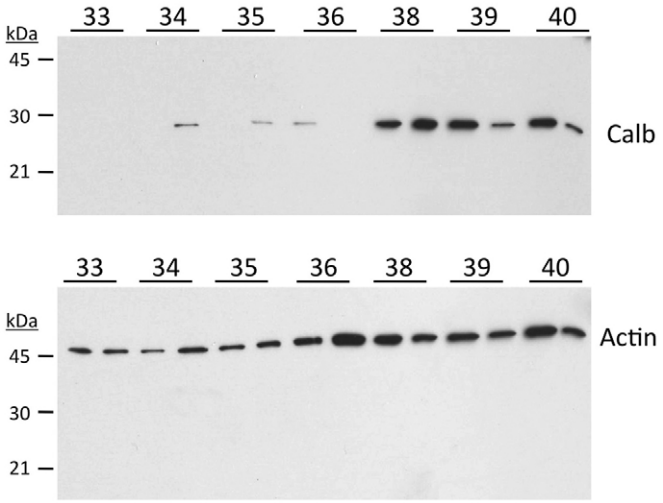

Fig. 3. Immunoblot of developmental expression of calbindin-D_{28K} and actin for yolk splanchnopleure of oviparous *Z. vivipara* embryos. Embryonic stages were assigned as for Fig. 1 and are indicated at the top. Positions of molecular mass standards are on the left. Duplicate samples from separate embryos at each stage were run in adjacent lanes. The upper part of the blot was reacted with PMCA antibodies (not shown) and the lower with calbindin-D_{28K} antibodies. The lower part of the blot was then stripped and reprobed with actin antibodies.

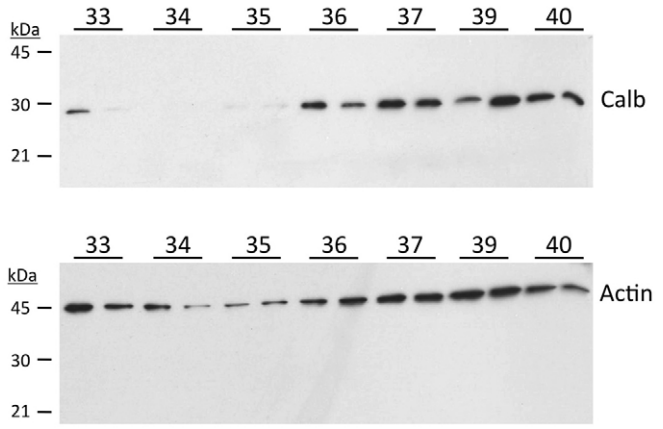

Fig. 4. Immunoblot of developmental expression of calbindin-D_{28K} and actin for yolk splanchnopleure of viviparous *Z. vivipara* embryos. Embryonic stages were assigned as for Fig. 1 and are indicated at the top. Positions of molecular mass standards are on the left. Duplicate samples from separate embryos at each stage were run in adjacent lanes. The upper part of the blot was reacted with PMCA antibodies (not shown) and the lower with calbindin-D_{28K} antibodies. The lower part of the blot was then stripped and reprobed with actin antibodies.

An important feature of the reproductive and developmental biology of *Z. vivipara* is that each reproductive mode has characteristics that are similar to those of other squamate species that share that reproductive mode, yet viviparity has evolved relatively recently, and perhaps on multiple occasions, within the species (Surget-Groba et al., 2006). In addition to the potential for important contributions to understanding the role of embryonic calcium nutrition in the evolution of reproductive mode, if calcium transport mechanisms are highly conserved, *Z. vivipara* is an appropriate model for oviparous and viviparous squamate species generally.

Eggs of oviparous squamates typically are oviposited when embryos are in the limb bud stage, at which time they have completed approximately 25–40% of the total time to hatching (Shine, 1983; DeMarco, 1993; Andrews and Mathies, 2000). Oviparous *Z. vivipara* females, which oviposit eggs containing stage 31–33 embryos, have a similar pattern of egg retention. Calcareous eggshells are a shared, derived trait for Reptilia (Packard and Seymour, 1997; Stewart, 1997) and the eggshell of oviparous squamates consists of three primary layers; the inner boundary layer, fibrous protein matrix and outer calcareous layer (Packard and DeMarco, 1991). The eggshell of oviparous populations of *Z. vivipara* contains all three layers characteristic of oviparous squamates with a thickness of 36–72 μm (Heulin, 1990; Heulin et al., 2002; Stewart et al., 2004b).

Viviparous squamates produce thinner eggshells than oviparous species and give birth to free-living neonates (Blackburn, 1993). Typically, the outer calcareous layer of the eggshell is absent and the layers of fibrous proteins are either greatly reduced or absent (Heulin, 1990). Eggshells of viviparous *Z. vivipara* lack the outer layer of calcium and the layer of fibrous protein is considerably thinner (7–9 μm) than that of oviparous females (Heulin, 1990; Stewart et al., 2004b). As a result, viviparous eggshells contain less calcium than oviparous eggshells (0.08 *vs* 1.2 mg) (Stewart et al., 2009).

Although there are substantial differences in eggshell structure and the length of intrauterine gestation for the two populations of

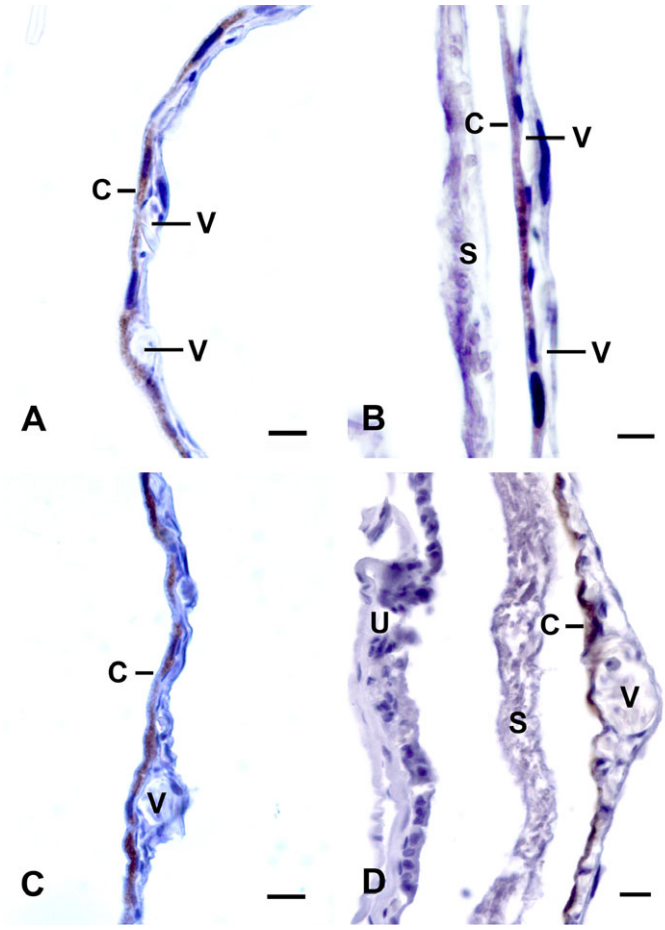

Fig. 5. Immunohistochemical localization of calbindin-D_{28K} in chorioallantoic membrane of oviparous and viviparous *Z. vivipara* embryos. Embryonic stages were assigned as for Fig. 1. The reaction appears as a brown precipitate in chorionic epithelial cells. (A) Oviparous, stage 37; (B) viviparous, stage 37; (C) oviparous, stage 40; (D) viviparous, stage 40. c, chorionic epithelium; s, eggshell; u, uterus; v, blood vessel. Scale bar 15 μm.

Z. vivipara, patterns of embryonic calcium mobilization are similar (Stewart et al., 2009). The pattern of embryonic growth of oviparous *Z. vivipara* is similar to that of other oviparous lacertid lizards (Ji and Brana, 1999; Stewart et al., 2009). Embryonic mass remains relatively low for the initial phase of post-ovipositional incubation, but undergoes a considerable increase during the last trimester of development. Embryonic calcium mass is correlated with total mass of embryos and the greatest increase in embryonic calcium mass and loss of calcium from eggshell occurs during later embryonic stages (37–40). Oviparous *Z. vivipara* produce yolk that contains less calcium than embryos can utilize and subsequently 81% of the calcium contained in hatchings is mobilized from the eggshell (Stewart et al., 2009). The yolk of viviparous females likewise is relatively low in calcium and the pattern of embryonic growth and calcium acquisition of viviparous embryos is similar to that of oviparous embryos. However, viviparous embryos do not have calcareous eggshells from which to extract calcium and yet 76% of neonatal calcium content comes from a source other than yolk (Stewart et al., 2009). The most likely source of calcium during late developmental stages is placental transport.

Phylogeographic analysis of populations of *Z. vivipara* indicate that either viviparity has evolved within the species on multiple occurrences or, subsequent to an initial transition to viviparity, there have been several reversals from viviparity to oviparity (Surget-Groba et al., 2006). Evolution of reproductive mode is relatively facile in this lineage given the pattern of maternal provision of calcium to eggs. The combination of low yolk calcium concentration and embryonic dependence on calcium from the eggshell has long been considered to constrain the evolution of viviparity (Packard et al., 1977). However, as this species reveals, the mechanism of embryonic calcium acquisition functions independently of the immediate source of calcium, and uterine secretions of calcium in viviparous females at least partially offset the absence of a calcareous eggshell.

The pattern of embryonic calcium mobilization of squamate reptiles is a useful model for the early reptilian egg (Packard, 1994; Packard and Seymour, 1997) and the available data are consistent with a hypothesis that embryonic mechanisms for calcium acquisition are plesiomorphic traits common to vertebrate calcium-transporting tissues. Adaptation of the chorioallantoic membrane for calcium transport may have closely followed the evolution of calcium deposition in eggshells. Calcium transport is one of the primary functions of the chorioallantoic membrane in modern reptiles and the transport mechanism is capable of responding to increases in embryonic metabolism and perhaps variation in calcium availability as well. General properties of this mechanism provide functional utility in response to a diversity of sources of calcium, including eggshells and direct uterine secretions. As a result, embryonic acquisition of calcium is not compromised by a transition between modes of parity because the extraembryonic membranes retain functional continuity in either reproductive mode, i.e. developmental patterns and mechanisms are conserved in the evolution of mode of parity. For species with geographic variation in their mode of parity, maternal sources of calcium are similar for oviparous and viviparous females, but the timing of uterine calcium delivery differs (Stewart et al., 2009; Linville et al., 2010). The relationship between uterine egg retention and secretion of calcium supports the hypothesis that calcium placentotrophy evolves concurrently with viviparity as a maternal response to the presence of eggs in the uterus (Stewart and Ecay, 2010).

ACKNOWLEDGEMENTS

We are grateful to G. Perron and C. Arthur (Parc National des Pyrénées) for providing authorization to work in the Ossau Valley. Funding was provided by a grant from The National Science Foundation (IOB-0615695).

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