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Expression of Calbindin-D_{28K} by Yolk Sac and Chorioallantoic Membranes of the Corn Snake, *Elaphe guttata*

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ABSTRACT The yolk splanchnopleure and chorioallantoic membrane of oviparous reptiles transport calcium from the yolk and eggshell to the developing embryo. Among oviparous amniotes, the mechanism of calcium mobilization to embryos has been studied only in domestic fowl, in which the mechanism of calcium transport of the yolk splanchnopleure differs from the chorioallantoic membrane. Transport of calcium is facilitated by calbindin-D_{28K} in endodermal cells of the yolk splanchnopleure of chickens but the chorioallantoic membrane does not express calbindin-D_{28K}. We used immunoblotting to assay for calbindin-D_{28K} expression in yolk splanchnopleure and chorioallantoic membrane of the corn snake, *Elaphe guttata*, to test the hypothesis that the mechanism of calcium transport by extraembryonic membranes of snakes is similar to birds. High calbindin-D_{28K} expression was detected in samples of yolk splanchnopleure and chorioallantoic membrane during late embryonic stages. We conclude that calbindin-D_{28K} is expressed in these extraembryonic membranes to facilitate transport of calcium and that the mechanism of calcium transport of the chorioallantoic membrane of the corn snake differs from that of the chicken. Further, we conclude that calbindin-D_{28K} expression is developmentally regulated and increases during later embryonic stages in the corn snake. *J. Exp. Zool. (Mol. Dev. Evol.) 302B:517–525, 2004.* © 2004 Wiley-Liss, Inc.

INTRODUCTION

Embryos of oviparous reptiles have two sources of calcium for growth and metabolism; calcium sequestered in yolk during vitellogenesis and calcium carbonate deposited in the eggshell by the oviduct. The relative importance of these two sources varies greatly among species (Johnston and Comar, '55; Packard '94). Yolk is the principal source of calcium for embryonic development in lizards and snakes (Squamata), whereas the eggshell is the primary source for turtles (Chelonia) and crocodilians and birds (Archosauria).

The pattern and mechanism of embryonic calcium mobilization have been most thoroughly studied in domestic fowl, where calcium transport from extraembryonic sites to the embryo is a function of the yolk sac splanchnopleure and chorioallantoic membrane (Terepka et al., '69; Tuan, '87; Ono and Tuan, '91; Tuan and Suyama, '96). The mechanism of calcium mobilization from yolk by the yolk splanchnopleure involves a developmental increase in calbindin-D_{28K} expression (Tuan and Suyama, '96). Calbindin-D_{28K} acts as a cytosolic calcium buffer in calcium-transporting tissues such as the kidney and intestine and may facilitate transcellular calcium diffusion in these tissues (Bindels, '93). Calbindin-D_{28K} likely serves the same function in yolk sac splanchnopleure and its expression level in the yolk sac is controlled by vitamin-D3 as in the kidney and intestine (Ono and Tuan, '91; Bindels, '93; Bouillon et al., 2003).

A different mechanism of calcium transport controls chorioallantoic membrane delivery of eggshell calcium to the chicken embryo. The eggshell of chickens is heavily calcified and

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embryos receive approximately 80% of their calcium from the eggshell via a highly regulated process involving vascular and epithelial cell specializations (Packard and Packard, '84). Calcium transporting capacity of the chorioallantoic membrane is correlated developmentally with expression of a unique calcium binding protein, transcalcin, and with increased calcium ATPase activity (Tuan and Scott, '77; Tuan and Knowles, '84; Akins and Tuan, '93a,b). In contrast to the chicken yolk sac splanchnopleure and other calcium-transporting epithelia of vertebrates, calbindin-D_{28K} is not expressed in the chicken chorioallantoic membrane (Sechman et al., '94).

The pattern of calcium mobilization by embryonic squamates differs from birds because squamate yolk calcium is gradually depleted during development and less calcium is withdrawn from the eggshell (Packard and Packard, '84; Packard, '94). Yolk calcium content of bird embryos increases during later stages of development as calcium mobilized from the eggshell is deposited both in the yolk and in the embryo. The calcium content of bird hatchlings is derived primarily from the eggshell (72%–92%) (Packard, '94). In contrast, snake embryos obtain only 20% of their calcium from the eggshell (Packard et al., '84; Packard and Packard, '88). A poorly calcified eggshell coupled with heavy reliance on calcium stored in yolk are likely characteristics of early amniotes and thus squamate embryos provide an appropriate model for an early stage in the evolution of calcium deposition and mobilization in amniote eggs (Packard, '94; Packard and Seymour, '97). The mechanism of calcium mobilization by squamate embryos has not been studied. Recent research on development of the extraembryonic membranes of the corn snake, *Elaphe guttata*, has shown that the chorioallantoic membrane does not contain structural specializations similar to those of chickens (Blackburn et al., 2003). We used immunoblotting to assay for calbindin-D_{28K} expression in chorioallantoic membrane and yolk sac splanchnopleure during development of *E. guttata* to test the hypothesis that calcium transport by the extraembryonic membranes is similar to chickens and that calbindin-D_{28K} is differentially expressed in these tissues.

MATERIALS AND METHODS

Elaphe guttata eggs were obtained from seven captive females in the Trinity College Ophidian Research Colony. Eggs were incubated in plastic containers containing wet vermiculite at room temperature (approx. 25°C) from oviposition to day 22–30 (embryonic stages 30–31), then placed in plastic incubation chambers in a substratum composed of distilled water and vermiculite (1:2). The water potential estimated for this batch of vermiculite was -120 ± 40 kPa as measured by thermocouple psychrometry using a Wescor C52 chamber and Wescor HR33T microvoltimeter. The individual chambers were incubated in a Precision (model 818) low temperature incubator at a temperature of 26°C. Containers were rehydrated weekly to compensate for uptake of water by eggs and for evaporation and rotated within the incubator weekly to compensate for possible temperature variation within the chamber.

Ages of eggs in the initial sample were post-oviposition day 11 (embryonic stage 27) and periodic samples were taken until approximately one week before surviving eggs hatched on days 82 – 84 (Table 1). The number of eggs sampled for each stage is indicated in Table 1. All samples were analyzed separately. The embryonic series represented stages 27–37 in a staging system in which stage 37 is the ultimate stage (Zehr, '62).

Chorioallantoic membranes were surgically removed by cutting around the equator of the egg through the eggshell and underlying chorioallantoic membrane and then separating the chorioallantoic membrane from the eggshell. Yolk sac samples were dissected free of the embryo and other membranes and rinsed free of yolk in Ringer solution (118 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM glucose, 32 mM Tris-HCl (pH 7.2), and 5 mg/l phenol red). Samples of chorioallantoic membrane and yolk sac were placed in tissue solubilization buffer (4°C) at a wet weight:-volume ratio of 1:10 (grams/ml). Tissue solubilization buffer consisted of 50 mM mannitol, 1 mM

TABLE 1. Tissue samples of chorioallantoic membrane and yolk splanchnopleure of Elaphe guftaia (N=7 clutches)

Age (days)	Stage	No. of Eggs
11	27	2
12–20	28	3
19–25	30	3
31–33	32	3
36–40	33	4
41–45	34	4
49–50	35	4
56	36 E	4
64	36 M	2
72	36 L	2
76–78	37	5

EDTA, 5 mM Na-*N*-2-hydroxyethylpiperizine-*N*'-ethane-sulfonic acid (HEPES, pH 7.4), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml N-tosyl-1-phenylalanin-chloromethyl ketone (TPCK). Tissue samples were disrupted with 5-10 bursts (1 sec duration) with a probe sonicator.

Duplicate 25 μl aliquots were set aside for protein determination (BCA assay; Pierce) and the remainder strored at –80°C. Samples of disrupted tissue were diluted in electrophoresis sample buffer and proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, '70) and electrophoretically transferred (Towbin et al., '79) to PVDF membranes (Millipore). For calbindin-D_{28K} immunodetection, PVDF blots were incubated in blocking buffer (5% nonfat dry milk, 5% horse serum, 0.05% Tween-20 in Tris-buffered saline (TBS; pH 7.4)) for 1 hr at room temperature and then with a 1:1000 dilution of a rabbit polyclonal antibody against rat calbindin-D_{28K} (Sigma #C2724) in blocking buffer at 4°C overnight. Blots were then washed in multiple changes of blocking buffer and incubated for 2 hrs at room temperature in blocking buffer that contained a peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:20,000 dilution; Amersham). After extensive washing in TBS, immune complexes were visualized on x-ray film by chemiluminescense using the SuperSignal Pico West kit (Pierce). Immunoblotting results were quantified by scanning densitometry using a flatbed scanner (Hewlett Packard) and Intelligent Quantifier software (Bio Image).

RESULTS

Characterization of the anti-calbindin-D_{28K} antibody. Calbindin-D_{28K} is highly expressed in the central nervous system of all vertebrates and in amniote kidneys and other calcium-transporting tissues (Parmentier et al., '87; Bindels, '93; Mutema and Rhoten, '94). However, direct demonstration of calbindin-D_{28K} expression in snake kidney or other calcium transporting tissues has not been reported. We have used a commercial polyclonal antibody directed against a synthetic peptide (20-mer) synthesized from the rat calbindin-D_{28K} sequence. To determine whether snake calbindin-D_{28K} is recognized by this anti-rat calbindin-D_{28K} antibody we compared antibody reactivity in electroblots of mouse and *Elaphe guttata* kidney proteins (Fig. 1). The rat calbindin-D_{28K} antibody recognizes single bands of approximately 28 kilodaltons in both the mouse and *E. guttata* kidney samples. We conclude that this antibody crossreacts with snake calbindin-D_{28K}. In subsequent immunoblots, we included samples of mouse and *E. guttata* kidney homogenates as positive controls for calbindin-D_{28K} antibody activity.

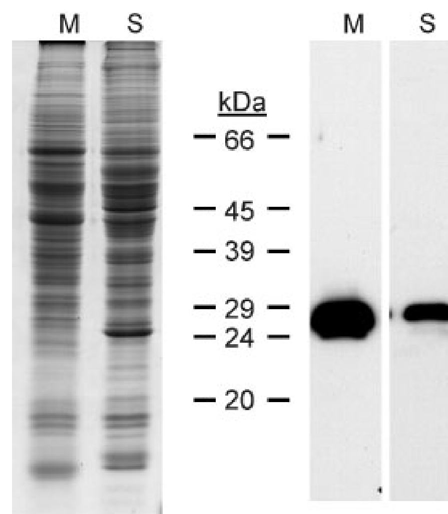

Fig. 1. Immunoblot analysis of calbindin-D_{28K} expression in mouse and *Elaphe guttata* kidneys. The two left lanes show total protein staining (20 μg/lane) of mouse (M) and *E. guttata* (S) kidney homogenates fractionated on 12.5–20% SDS-PAGE gels. The two right lanes are immunoblots of parallel gels using a rabbit polyclonal antibody against a rat calbindin-D_{28K} peptide. This antibody reacts with a single band of approximately 28 kDa in each tissue sample.

Yolk sac ontogeny

To analyze calbindin-D_{28K} expression in the yolk sac during development, SDS-PAGE gel lanes were loaded with 20 μg protein/lane of samples spanning stages 27–37. Figure 2 illustrates a pair of typical immunoblots from this study. Calbindin-D_{28K} expression in the yolk sac is undetectable (stages 27–32) or low (stage 33–34) during the early stages of embryonic development and increases significantly in stages 35–37. Scanning densitometry of these immunoblots indicates that calbindin-D_{28K} expression increases by 50–100 fold between stage 33, when it is first detected, and stage 37 when the highest level of expression is observed. We conclude that calbindin-D_{28K} expression is developmentally regulated in *Elaphe guttata* yolk sac splanchnopleure.

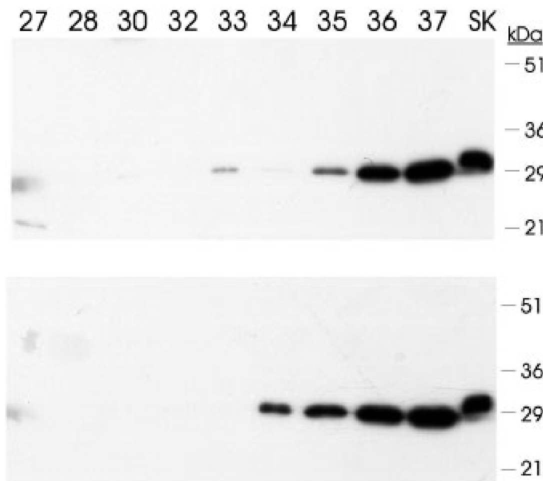

Fig. 2. Immunoblotting analysis of calbindin-D_{28K} expression in yolk sac during *Elaphe guttata* embryonic development. Yolk sac homogenates (20 μg protein/lane) from the indicated stages were fractionated on 12.5–20% gradient SDS-PAGE gels and electroblotted to PVDF membranes. A sample (20 μg protein) of *E. guttata* kidney homogenate (SK) was included in all gels as a positive control for the anti-calbindin-D_{28K} antibody. Shown are immunoblotting results from two separate series of developmental stages (18 samples total).

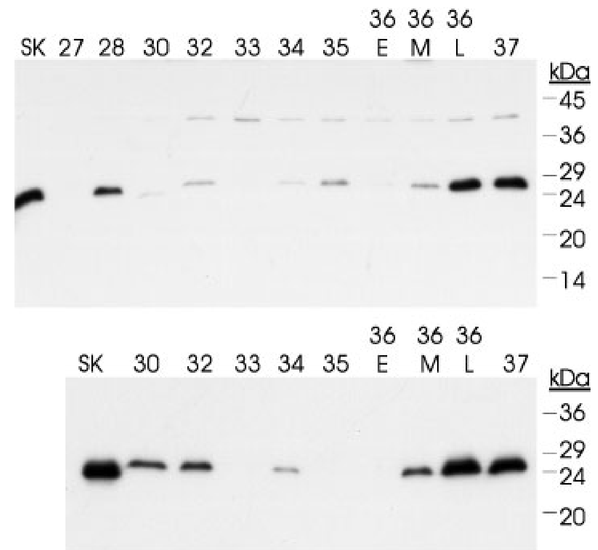

Fig. 3. Immunoblotting analysis of calbindin-D_{28K} expression in chorioallantois during *Elaphe guttata* embryonic development. Chorioallantoic mambrane homogenates (20 μg protein /lane) were fractionated on 12.5–20% gradient SDS-PAGE gels and electroblotted to PVDF membranes. A sample (20 μg protein) of *E. guttata* kidney homogenate (SK) was included in all gels as a positive control for the anti-calbindin-D_{28K} antibody. Because of its length, three samples representing the early (E), middle (M) and late (L) phases of stage 36 were analyzed. Shown are immunoblotting results from two separate series of developmental stages (20 samples total).

Chorioallantoic membrane ontogeny

Developmental series of chorioallantoic membrane samples were analyzed in a manner similar to the yolk sac samples discussed above. Figure 3 contains two typical immunoblots from the chorioallantoic membrane analysis. Unlike the yolk sac results, low levels of calbindin-D_{28K} expression were observed in at least one sample of each stage of chorioallantoic membrane, with the exception of stage 27, which did not exhibit detectable expression (N = 2). Within a stage, expression varied from undetectable to approximately 20% of the maximum expression at the latest stages. Beginning with mid-stage 36, calbindin-D_{28K} expression was consistently elevated, peaking at stage 37. Densitometric analysis of immunoblots showed that calbindin-D_{28K} expression was increased in late stage 36 and stage 37 at least 5–10 fold over earlier stages. We conclude that the chorioallantoic membrane expresses low levels of calbindin-D_{28K} at early stages and that expression is upregulated in the latter stages of development.

Early embryonic stage (27–30) samples of chorioallantoic membrane were isolated without regard to their position in the egg. Beginning with some stage 30 samples and all subsequent stages, chorioallantoic membranes were bisected along the plane separating the embryonic and abembryonic hemispheres of the egg. For the analysis shown in Figure 3, embryonic hemisphere samples were used in stages 32–37. The possibility of positional differences in calbindin-D_{28K} expression was analyzed by comparing samples from different hemispheres of the same egg on adjacent gel and immunoblot lanes. Figure 4 illustrates a typical result. Abembryonic expression was greater than embryonic expression in 14 of 18 chorioallantoic membrane pairs sampled and analyzed in this way. This observation suggests that, in addition to developmental regulation, calbindin-D_{28K} expression in the chorioallantoic membrane varies with position around the circumference of the egg.

DISCUSSION

The extraembryonic membranes of amniotes provide respiratory and nutritional support for the developing embryo but relatively little is known of the functional attributes or mechanisms of these tissues. The yolk sac has multiple functions. It is a significant site of angiogenesis and hematopoiesis during early embryonic development, is the source of primordial germ cells, and mediates yolk

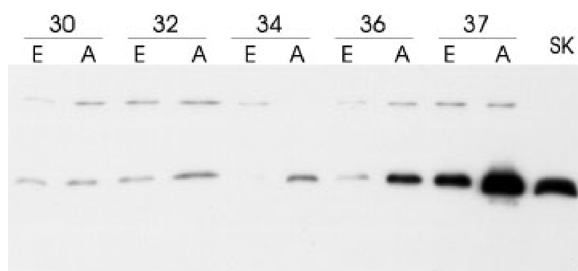

Fig. 4. Immunoblotting analysis of spatial or positional influence on the level of calbindin-D_{28K} expression in the chorioallantois. The chorioallantoic membrane was divided into the embryonic (E) and abembryonic (A) fractions prior to homogenization. Samples from the indicated developmental stages were fractionated on 12.5–20% gradient SDS-PAGE gels and electroblotted to PVDF membranes. A sample (20 μg protein) of *Elaphe guttata* kidney homogenate (SK) was included as a positive control for the anti-calbindin-D_{28K} antibody. Shown is a representative immunoblot.

metabolism. The chorioallantoic membrane is an important respiratory membrane that also contributes to water balance and nutrition. Both of these tissues have a significant role in calcium delivery to embryos. Calcium for embryonic development of oviparous reptiles is available from two sources, yolk reserves and the eggshell (Johnston and Comar, '55; Packard and Packard, '84), but the role of the extraembryonic membranes in calcium transport from these two compartments has been studied only in the domestic chicken, *Gallus gallus*.

Absorption and secretion of calcium are highly regulated in a variety of vertebrate tissues. The intestine, kidney and extraembryonic membranes, are primary sites of calcium absorption in amniotic vertebrates (Terepka et al., '69; Bronner et al., '86; Ono and Tuan, '91; Bronner and Pansu, '99; Hoenderop et al., 2000). Models of the mechanism of calcium transport by epithelial cells of the intestine, renal distal tubule, chorion (including trophoblast) and yolk sac implicate several pathways, primarily distinguished as either paracellular or transcellular (Bronner et al., '86; Feher et al., '92; Akins and Tuan, '93b; Bindels, '93; Tuan and Suyama, '96; Hoenderop et al., 2000; Larsson and Nemere, 2002; Peng et al., 2003). During paracellular transport, calcium passively diffuses across apical tight junctions between adjacent epithelial cells and moves down its electrochemical gradient (Bronner et al., '86; Feher et al., '92; Bindels, '93). Transcellular transport of calcium is mediated by calcium channels in the apical plasma membrane (Hoenderop et al., 2002; Peng et al., 2003). There are two possible mechanisms for the movement of intracellular calcium to the basolateral region of the cell, transport within membrane bound vesicles (Coleman and Terepka, '72b; Akins and Tuan, '93a,b) or facilitated diffusion as a calbindin-Ca^{2+} complex (Bronner et al., '86; Feher et al., '92; Bindels, '93; Tuan and Suyama, '96; Hoenderop et al., 2000; Larsson and Nemere, 2002). Calcium released from calbindin crosses the basolateral plasma membrane facilitated by calcium ATPase. Calbindin synthesis is regulated by vitamin D and the level of calbindin is correlated with calcium absorbing activity (Bronner et al., '86; Ono and Tuan, '91; Tuan and Suyama, '96; Bronner and Pansu, '99; Hoenderop et al., 2000; Peng et al., 2003).

Embryonic chickens mobilize calcium from yolk by endodermal cells of the yolk sac splanchnopleure relatively early in the incubation period (Tuan and Suyama, '96) followed by active calcium transport from the eggshell by the chorioallantoic membrane (Terepka et al., '69; Garrison and Terepka, '72 a,b; Tuan, '87). Calcium uptake from the eggshell is so great during later developmental stages that both embryonic and yolk components of the egg undergo increases in calcium content (Packard and Packard, '84). Yolk calcium content is thus enriched by calcium mobilized from the eggshell (Tuan et al., '91). Calcium mobilization from the yolk of squamates differs in that the yolk compartment does not gain calcium during incubation (Packard and Packard, '84, Packard, '94). However, the mechanism for calcium recovery from yolk may be similar in birds and squamates. The yolk of both taxa experiences a gradual loss of calcium relatively early in incubation and calcium transport continues throughout incubation (Packard and Packard, '84; Ono and Tuan, '91; Packard, '94; Sechman et al., '94; Packard and Clark, '96; Tuan and Suyama, '96). Endodermal cells of the yolk sac splanchnopleure of chickens transport calcium from yolk to the blood vascular system and intracellular transport of calcium is facilitated by the calcium binding protein, calbindin-D_{28K} (Ono and Tuan, '91; Tuan and Suyama, '96). Consistent levels of calbindin-D_{28K} mRNA and calbindin-D_{28K} protein occur in the yolk sac from incubation days eight to sixteen and each increases markedly during later development (Sechman et al., '94). Likewise, calcium mobilization from the yolk is gradual early in development of squamates and accelerates late in development (Packard et al., '84; Packard and

Packard, '88; Packard, '94) and the yolk sac splanchnopleure of *Elaphe guttata* expresses calbindin-D_{28K} during the last few weeks of incubation, embryonic stages 35–37. Although the overall developmental pattern of calbindin-D_{28K} expression in chicken and *E. guttata* yolk sac is similar, we found that *E. guttata* calbindin-D_{28K} expression increased 50 fold or more in contrast to the approximate doubling of expression in the chicken yolk sac (Sechman et al., '94). This difference in yolk sac calbindin-D_{28K} expression may reflect differences in the relative importance of the yolk as a source of calcium for embryonic development between these two species.

The eggshell of domestic fowl is heavily calcified and the mechanism of calcium transport by the chorioallantoic membrane differs from that of the yolk sac splanchnopleure. The chorionic epithelium of the chorioallantoic membrane contains two cell types that have been implicated in erosion and uptake of calcium from the eggshell (Coleman and Terepka, '72a,b; Packard and Packard, '84). Calcium is eroded from the eggshell by acidification resulting from the release of carbonic anhydrase by villus cavity cells of the chorionic epithelium (Simkiss, '80; Anderson et al., '81; Narbaitz et al., '81; Packard and Lohmiller, 2002). Capillary covering cells in the same epithelial layer are thought to take up calcium that has been released into the space between the eggshell and the chorionic epithelium (Coleman and Terepka, '72a,b), but calcium uptake has also been suggested for villus cavity cells (Narbaitz, '72), and both cell types may function in calcium uptake (Packard and Packard, '84). Calcium transporting capacity of the chorioallantoic membrane is correlated with expression of a calcium binding protein, transcalcin (Tuan and Scott, '77; Tuan et al., '78a; Tuan et al., '86; Tuan, '87) that has been localized immunohistochemically to calcium-transporting chorionic epithelial cells (Tuan and Knowles, '84; Tuan et al., '86). These cells also contain calcium-ATPase, which is closely associated with transcalcin and developmentally correlated with calcium transport activity by the chorioallantoic membrane (Tuan and Knowles, '84; Tuan et al., '86; Akins and Tuan, '93a,b). Unlike endodermal cells of the yolk sac splanchnopleure, chorionic epithelial cells of chicken embryos do not express calbindin-D_{28K} mRNA or protein during any stage of development (Sechman et al., '94).

Structural and functional characteristics of the chorioallantoic membrane of *Elaphe guttata* differ markedly from domestic chickens. The chorionic epithelium of *E. guttata* is stratified throughout development and allantoic blood vessels lie at the base of the epithelium (Blackburn et al., 2003). The outer epithelium consists of thin squamous cells during later embryonic stages (Blackburn et al., 2003) when calcium is mobilized from the eggshell (Stewart et al., 2005). In contrast to the chorioallantoic membrane of chickens, calbindin-D_{28K} is present in the chorioallantoic membrane of *E. guttata* and expression of this calcium binding protein is greatest during the final two embryonic stages (36–37). In addition to an overall increased synthesis of calbindin-D_{28K} in the chorioallantoic membrane during later embryonic stages, expression is greater in the abembryonic hemisphere of the egg compared to the embryonic hemisphere. Regulation of calbindin-D_{28K} is sensitive to developmental timing and is also subject to localized control mechanisms.

The expression of calbindin-D_{28K} in the yolk sac and chorioallantois of *Elaphe guttata*, is developmentally regulated, however the timing of calbindin-D_{28K} upregulation is different. In the yolk sac, increased expression begins by stage 35 (day 49–50), whereas in the chorioallantois, expression begins to increase around the middle of stage 36 (day 64). Thus, there is a 14 day lag in the upregulation of chorioallantoic calbindin-D_{28K} expression. Because calbindin-D_{28K} expression is vitamin-D3-dependent in many other calcium transporting tissues (Ono and Taun, '91; Bindels, '93; Bouillon et al., 2003), we suggest that the delayed increase in calbindin-D_{28K} expression reflects a lack of vitamin-D3 receptor expression in the chorioallantois until stage 36.

The eggs of the earliest amniotes most likely lacked a calcareous eggshell, as do the eggs of modern anamniotes and monotremes (Hughes, '77, '84), and were supplied with nutrient rich yolk that was the sole source of calcium for embryonic development (Packard, '94; Packard and Seymour, '97). In contrast, eggs of oviparous Reptilia have a calcareous eggshell and calcium is mobilized both from yolk and from the eggshell during embryogenesis (Packard, '94). One of the hypothetical early stages in the evolution of calcium mobilization by embryonic reptiles that were dependent on yolk calcium was utilization of calcium stored in the eggshell (Packard and Seymour, '97). Among extant oviparous reptiles, squamate embryos are most dependent on yolk for calcium and thus may exhibit a pattern of calcium mobilization that is similar to that of early reptiles (Packard, '94;

Packard and Seymour, '97). Both the yolk splanchnopleure and the chorioallantoic membrane of *Elaphe guttata* are similar to other specialized calcium transporting tissues, including avian yolk sac, in that both extraembryonic membranes express calbindin-D_{28K}, which is regulated by vitamin-D3. One explanation for the similarity in the mechanism of calcium transport by the yolk splanchnopleure of corn snakes and chickens is that this mechanism is a plesiomorphic trait for Reptilia. In contrast, chickens and corn snakes have different mechanisms for recovery and transport of calcium from the eggshell. The chorionic epithelium of *E. guttata* lacks the histological specializations characteristic of chickens and transports lower quantities of calcium. However, the chorioallantoic membrane of corn snakes has evolved a mechanism for calcium transport that is similar to other calcium transporting tissues and the timing of expression of calbindin-D_{28K} in the extraembryonic membranes of *E. guttata* suggests that calcium transport is developmentally regulated.

Although embryos of oviparous squamates are heavily dependent on calcium from yolk, some viviparous squamate embryos obtain most of their calcium from a uterine source (Stewart and Thompson, 2000, Thompson et al., 2000). Viviparity has evolved independently in numerous lineages of lizards and snakes (Blackburn, '82, '85; Shine, '85). Viviparous squamates have secondarily lost calcareous eggshells (Heulin, '90; Qualls, '96; Blackburn, '98) but do have chorioallantoic placentae formed by close apposition of the chorioallantoic membrane and uterine epithelium. The percentage of neonatal calcium derived from the placenta varies from 0% to 90% (Stewart and Thompson, 2000). Species for which most embryonic calcium is supplied directly from the uterus also receive most other nutrients for embryonic development from a placenta. These placentotrophic species have specializations for nutrient mobilization that are not present in oviparous ancestors. However, embryos of many viviparous species are primarily lecithotrophic, i.e., receive most nutrition from yolk, and yet placental transfer of calcium occurs in most of these species. Neither the pathway nor the mechanism of calcium delivery to embryos of viviparous squamates has been studied. If expression of calbindin-D_{28K} as occurs in *E. guttata* is characteristic of the chorioallantoic membranes of squamates, a mechanism for regulation of calcium transport by embryos would exist during the earliest stages of the evolution of viviparity and placentation. Regulation of calcium transport mediated by vitamin-D3 could supply the modest amounts of calcium to predominantly lecithotrophic viviparous embryos but could also provide the mechanism of calcium delivery for species that have evolved a substantial level placentotrophy.

The mechanism of calcium transport of embryonic *Elaphe guttata* reveals that squamates do have specializations for calcium recovery from both the yolk and the eggshell and that these specializations would enhance the potential for placental transfer during extended intrauterine gestation in the evolution of viviparity.

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