

## Sources and timing of calcium mobilization during embryonic development of the corn snake, *Pantherophis guttatus*

James R. Stewart<sup>a,\*</sup>, Tom W. Ecaj<sup>b</sup>, Daniel G. Blackburn<sup>c</sup>

<sup>a</sup>Department of Biological Sciences, East Tennessee State University, Johnson City, TN 37614, USA

<sup>b</sup>Department of Physiology, Quillen College of Medicine, East Tennessee State University, Johnson City, TN 37614, USA

<sup>c</sup>Department of Biology, Trinity College, Hartford, CT 06106, USA

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### Abstract

Embryos of oviparous Reptilia (=turtles, lepidosaurs, crocodylians and birds) extract calcium for growth and development from reserves in the yolk and eggshell. Yolk provides most of the calcium to embryos of lizards and snakes. In contrast, the eggshell supplies most of the calcium for embryonic development of turtles, crocodylians and birds. The yolk sac and chorioallantoic membrane of birds recover and transport calcium from the yolk and eggshell and homologous membranes of squamates (lizards and snakes) probably transport calcium from these two sources as well. We studied calcium mobilization by embryos of the snake *Pantherophis guttatus* during the interval of greatest embryonic growth and found that the pattern of calcium transfer was similar to other snakes. Calcium recovery from the yolk is relatively low until the penultimate embryonic stage. Calcium removal from the eggshell begins during the same embryonic stage and total eggshell calcium drops in each of the final 2 weeks prior to hatching. The eggshell supplies 28% of the calcium of hatchlings. The timing of calcium transport from the yolk and eggshell is coincident with the timing of growth of the yolk sac and chorioallantoic membrane and expression of the calcium binding protein, calbindin-D<sub>28K</sub>, in these tissues as reported in previous studies. In the context of earlier work, our findings suggest that the timing and mechanism of calcium transport from the yolk sac of *P. guttatus* is similar to birds, but that both the timing and mechanism of calcium transport by the chorioallantoic membrane differs. Based on the coincident timing of eggshell calcium loss and embryonic calcium accumulation, we also conclude that recovery of eggshell calcium in *P. guttatus* is regulated by the embryo.

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### 1. Introduction

The macrolecithal eggs of oviparous Reptilia (=Chelonia, Lepidosauria and Archosauria) (Gauthier et al., 1988) are enclosed and protected by a proteinaceous eggshell reinforced structurally by mineralization, principally in the form of calcium carbonate (Packard and Packard, 1984; Packard, 1994). The eggs of anamniote vertebrates lack an eggshell yet are similar to the eggs of reptiles in that nutrients for

embryonic development, such as calcium, are stored in the form of yolk (Packard and Seymour, 1997). The calcium rich yolk of reptilian eggs was inherited from amniote ancestors but calcium deposited in the eggshell is an innovation of Reptilia (Packard, 1994; Packard and Seymour, 1997). The eggshell offers an additional source of calcium for embryonic growth and metabolism and different lineages of Reptilia have exploited this source to varying degrees (Packard and Packard, 1984; Packard, 1994). Yolk is the principal source of calcium during all phases of embryonic growth of oviparous squamates but calcium from the eggshell supplements yolk calcium late in the period of incubation (Packard et al., 1984a, 1985; Packard and

\* Corresponding author. Tel.: +1 423 439 6927; fax: +1 423 439 5958.  
E-mail address: stewartjr@etsu.edu (J.R. Stewart).

Packard, 1988; Shadrix et al., 1994). In contrast, the eggshell is the major source of calcium to embryos of turtles (Packard et al., 1984b; Packard and Packard, 1986, 1991a), crocodylians (Packard and Packard, 1989) and birds (Johnston and Comar, 1955; Packard and Packard, 1991b) and is withdrawn from this compartment earlier in development in crocodylians and birds compared to squamates (Packard and Packard, 1984; Packard, 1994). Because of the heavy dependence on calcium from yolk, the pattern of calcium mobilization of embryos of oviparous squamates has been proposed as the most appropriate model among extant oviparous amniotes for functional characteristics of the common ancestor of Reptilia and Synapsida (Packard and Seymour, 1997).

Metabolic functions of the amniote egg, including calcium transport, are mediated by extraembryonic membranes (Packard and Seymour, 1997; Stewart, 1997). In embryos of domestic fowl, calcium from yolk is transferred to the embryonic vascular system by the yolk sac splanchnopleure (Ono and Tuan, 1991; Tuan and Suyama, 1996) and calcium from the eggshell is transported by the chorioallantoic membrane (Terepka et al., 1969; Garrison and Terepka, 1972; Tuan, 1987). Endodermal cells of the yolk sac splanchnopleure express the calcium-binding protein, calbindin-D<sub>28K</sub> (Ono and Tuan, 1991; Sechman et al., 1994; Tuan and Suyama, 1996), which is a common characteristic for calcium transporting tissues (Bindels, 1993). Calbindin-D<sub>28K</sub> is thought to act as an intracellular calcium buffer and/or carrier facilitating calcium diffusion through the cytosol during transport (Bindels, 1993; Hoenderop et al., 2000). However, the chorioallantoic membrane transports calcium differently than the yolk sac splanchnopleure. Chorionic epithelial cells do not express calbindin-D<sub>28K</sub> (Sechman et al., 1994), but transport calcium within microvesicles (Akins and Tuan, 1993). Furthermore, the chorioallantoic membrane of chicken embryos is structurally specialized for calcium transport from the inner surface of the eggshell (Leeson and Leeson, 1963; Coleman and Terepka, 1972; Packard and Packard, 1984). In contrast, both the yolk sac splanchnopleure and chorioallantoic membrane of *Pantherophis guttatus* express calbindin-D<sub>28K</sub> (Ecay et al., 2004). Thus, the mechanisms of calcium transport by the yolk sac of squamates and birds may be similar, but that of the chorioallantoic membranes clearly differ. Lepidosaurs (tuatara, lizards and snakes) are the sister taxa to archosaurs (crocodylians and birds) (Gauthier et al., 1988) and understanding the mechanism of calcium mobilization of embryonic squamates will provide insight into the evolution of the specialized calcium recovery mechanism of avian embryos. Additionally, the calcium storage and recovery system of squamate eggs may reveal characteristics of an early stage in the evolution of the amniote egg (Packard, 1994; Packard and Seymour, 1997). We studied the pattern of calcium mobilization in *P. guttatus* to determine if the timing (age and embryonic stage) of calcium transport from the yolk and eggshell is

coincident with the expression of calbindin-D<sub>28K</sub> in the yolk sac splanchnopleure and chorioallantoic membrane (Ecay et al., 2004).

## 2. Materials and methods

*P. guttatus* eggs were obtained from six 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 days 11–18, then transferred to East Tennessee State University where they were 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  $-120 \pm 40$  kPa as measured by thermocouple psychrometry using a Wescor C52 chamber and Wescor HR33T microvoltmeter. The individual containers were kept at room temperature (22–23 °C) for 12 days and then 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 days 27–31. Clutches were sampled a second time at 35–37 days post-oviposition and all subsequent samples beginning at days 41–42 were at weekly intervals until hatching on days 82–84. A single egg or hatchling from each clutch was included in each of the samples. Embryos were separated from yolk and staged (Zehr, 1962) and internal yolk was dissected free of hatchlings. Embryos hatchlings and yolk were each placed in tared containers. Eggshells were rinsed in distilled water to remove adhering membranes, blotted dry and placed in tared containers. Wet mass was measured for all samples prior to freezing at  $-20$  °C. Embryos, yolks and eggshells were dried to constant mass in a Labconco Freeze Dryer 5 and ashed at 600 °C in a Fisher Isotemp muffle furnace. Ash samples were digested in hot nitric acid for 12 h, evaporated to near dryness and then stored in 2.5% hydrochloric acid. Lanthanum chloride (10% of sample volume) was added to each sample prior to reading calcium concentration (Kopp and McKee, 1979) using a flame atomic absorption spectrophotometer (Varian SpectraAA-10/20). The spectrophotometer was calibrated against samples of known calcium concentration. The percentage of calcium in each sample was estimated as the concentration of calcium in solution relative to the concentration of digestate. Total calcium was estimated as a percentage of sample mass.

Data were analyzed using a two-factor analysis of variance (SAS Institute) with embryo, yolk and eggshell components as dependent variables, incubation age (sample period) as a fixed factor and clutch as a random factor. Initial egg wet mass was entered as a covariate. Comparisons among adjusted means for dependent variables were

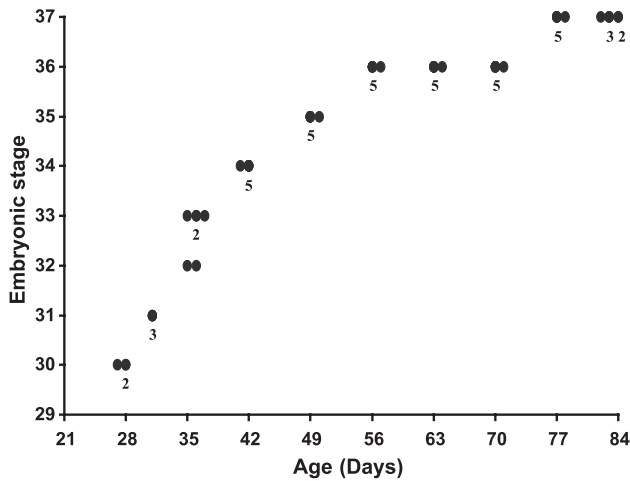


Fig. 1. Relationship between day of incubation and embryonic stage for eggs of six *Pantherophis guttatus*.

interpreted by plotting 95% confidence intervals of the adjusted means. Non-overlapping confidence intervals were considered significant differences between means.

### 3. Results

The first sample of embryos was composed of stages 30–31 (Zehr, 1962) and subsequent samples were progressively later stages to stage 36 on the fourth week (Fig. 1). Embryos were stage 37 on the seventh week of sampling and hatched 5–7 days later.

The organic composition of embryos, estimated as ash-free dry mass, which was approximately 50 mg in the initial samples, increased exponentially throughout the sampling interval (Fig. 2; Table 1). Embryos grew slowly for 33 days. The day 63 sample averaged 494 mg and there were significant differences in organic mass between each of the final four samples (Fig. 2). Hatchlings averaged 1.56 g ash-free dry mass. Mobilization of organic material from yolk

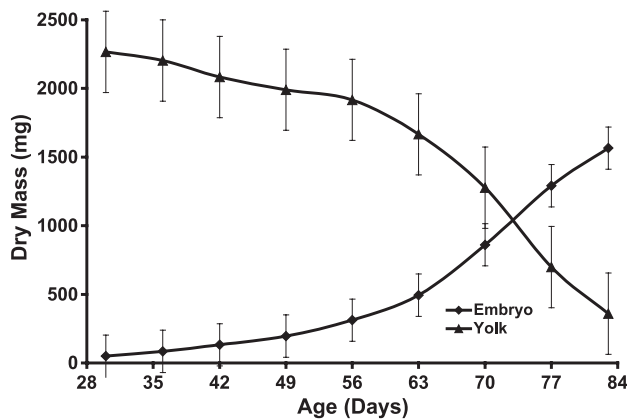


Fig. 2. Least squares means ( $\pm 95\%$  confidence intervals) for ash-free dry mass of egg compartments based on samples of eggs of six *Pantherophis guttatus*. Average age at the sample date is indicated on the y-axis.

Table 1  
F-values for the analysis of variance of the effect of age (sampling interval) on egg components of *Pantherophis guttatus*

	Degrees of freedom	F
<i>Embryo</i>		
Ash-free Dry Mass	8, 39	288.1*
Calcium-free Ash	8, 38	218.9*
Calcium	8, 38	276.5*
<i>Yolk</i>		
Ash-free Dry Mass	8, 39	118.2*
Calcium-free Ash	8, 38	137.4*
Calcium	8, 38	121.2*
<i>Eggshell</i>		
Ash-free Dry Mass	8, 39	0.57
Calcium-free Ash	8, 39	26.4*
Calcium	8, 39	53.2*
<i>Total calcium</i>		
(Embryo, Yolk, Eggshell)	8, 37	0.60

\*  $p < 0.0001$ .

was gradual from day 30 to day 63 with pronounced losses thereafter to hatching (Fig. 2; Table 1). The final sample, which consisted of yolk withdrawn into the coelomic cavity prior to hatching, averaged 360 mg. There was no significant effect of sampling date on ash-free dry mass of eggshells (Table 1). There were significant effects ( $p \leq 0.01$ ) for female (clutch) and initial egg wet mass for embryos, yolks and eggshells.

The pattern for embryonic assimilation of inorganics, measured as calcium-free ash and calcium, was similar to that for organic molecules (Figs. 3 and 4; Table 1). The greatest gains were during the last 3 weeks (days 63–84) of incubation during embryonic stages 36–37. Loss of inorganic mass from yolk tracked loss of organics and was greatest during the last 21 days of incubation in stage 36–37 embryos (Figs. 3 and 4; Table 1). There were also significant differences in calcium-free inorganic mass and in total calcium in eggshells among sampling

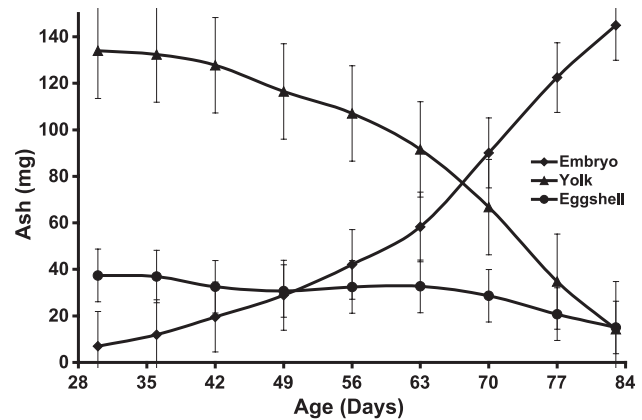


Fig. 3. Least squares means ( $\pm 95\%$  confidence intervals) for calcium-free ash of egg compartments based on samples of eggs of six *Pantherophis guttatus*. Average age at the sample date is indicated on the y-axis.

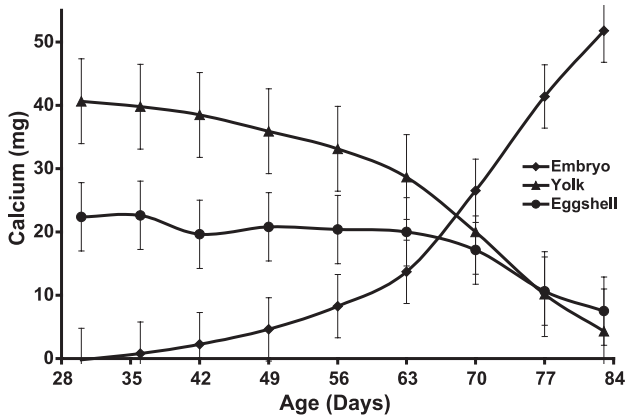


Fig. 4. Least squares means ( $\pm 95\%$  confidence intervals) for total calcium of egg compartments based on samples of eggs of six *Pantherophis guttatus*. Average age at the sample date is indicated on the y-axis.

times (Figs. 3 and 4; Table 1) with a pronounced reduction of calcium in each of the last two samples (Fig. 4). Total calcium in hatchlings, including internal yolk (56.1 mg) was significantly greater ( $p < 0.01$ ) than total calcium in embryos and yolks in our initial sample (40.4 mg). Approximately 15.7 mg (28%) of calcium in hatchlings and internal yolk was recovered from the eggshell. There was no significant effect of sampling day on total calcium in eggs (embryos, yolks and eggshells) (Table 1) and total calcium at hatching (63.5 mg) did not differ significantly ( $F_{8,37} = 0.61$ ) from total calcium at embryonic stage 30–31 (62.8 mg). There was a significant female (clutch) effect for embryonic, yolk and eggshell calcium-free ash ( $p \leq 0.05$ ) and for yolk and eggshell calcium ( $p = 0.0001$ ), but not for embryo calcium. There was also a significant effect ( $p \leq 0.01$ ) for initial egg wet mass on embryo and yolk calcium-free ash and calcium, but not for eggshell calcium-free ash or calcium. There were significant effects ( $p = 0.001$ ) for both clutch and initial egg wet mass on total calcium.

#### 4. Discussion

Oviparous squamates commonly oviposit eggs that have completed early stages of embryonic development within the oviduct (Shine, 1983). Embryonic growth and metabolism of squamate embryos are gradual during the initial two-thirds of incubation, then increase dramatically during the last trimester (Packard et al., 1984a; Shadrix et al., 1994; Thompson and Stewart, 1997; Thompson and Russell, 1999). Embryos of *P. guttatus* are stage 22 at oviposition (Blackburn et al., 2003). Under our incubation conditions, embryos began to grow markedly when they reached stage 35 and underwent the greatest increase in mass during the penultimate embryonic stage 36 (Figs. 1 and 2). The dramatic increase in embryonic mass resulted from increased mobilization of nutrients from yolk and mobilization of calcium from the eggshell.

The mineralized eggshell of modern Reptilia is thought to have evolved as a secondary specialization to enclose the calcium-rich yolk of ancestral eggs (Packard, 1994; Packard and Seymour, 1997). These eggshells commonly provide a source of calcium to developing embryos and in some lineages they are the richest source of calcium for embryos (Packard and Packard, 1984; Packard, 1994). The eggs of squamates, particularly snakes, are likely to be most similar to those of ancestral Reptilia because calcium-rich yolk, not the eggshell, provides most of the calcium for embryonic development (Packard, 1994; Packard and Seymour, 1997). Total hatchling calcium content, including internalized yolk, for *P. guttatus* was 56.1 mg, 15.7 mg higher than the content of yolk and embryo in our initial sample of eggs (embryonic stages 30–31). Hatchlings contained more calcium than was present in yolks and embryos on day 30 because the eggshell was a secondary source of calcium. The percentage contribution to hatchlings from eggshell, 28%, is similar to that of two previous studies of oviparous colubrid snakes, *Coluber constrictor*, 23% (Packard et al., 1984a) and *Pituophis melanoleucus*, 24% (Packard and Packard, 1988). In contrast, the hatchlings of archosaur reptiles receive 62–92% of their calcium from the eggshell (Packard, 1994). The eggshells of *P. guttatus* experienced no significant loss of calcium until day 70 of incubation, but calcium levels were successively lower in each of the final two samples (Fig. 4). The average calcium content of the eggshell in the day 70 sample was 17.1 mg. Hatchling eggshells average 7.5 mg of calcium. Thus, 9.6 mg (56%) of the calcium content of day 70 eggshells was mobilized during the last 14 days of incubation. During this same interval, 78% (15.7 mg) of the calcium in yolk was mobilized. The final two weeks of incubation were characterized by dramatic increases in embryonic calcium content and both yolk and eggshell contributed to these gains.

The pattern of embryonic calcium acquisition of oviparous Reptilia has been most thoroughly studied in domestic fowl. Yolk is the primary source of embryonic calcium prior to day 10 of incubation (Johnston and Comar, 1955; Ono and Tuan, 1991; Tuan and Suyama, 1996) during the time in which the definitive yolk sac, the yolk sac splanchnopleure, gradually becomes established around all but the abembryonic surface of the yolk (Romanoff, 1960). Endodermal cells of the yolk sac splanchnopleure mediate calcium transfer from yolk to the blood vascular system (Ono and Tuan, 1991; Tuan and Suyama, 1996). Mobilization of calcium from the eggshell begins at approximately day ten (Johnston and Comar, 1955) coincident with the expansion of the allantois around the perimeter of the yolk sac (Romanoff, 1960). The allantois completely encloses the contents of the egg by day 12 (Romanoff, 1960), slightly more than halfway through the 21-day incubation period. This is approximately the time during incubation at which calcium levels increase in the yolk as calcium uptake from the eggshell exceeds calcium recovery from yolk (Packard and Packard, 1984). The increase in calcium recovery from

the eggshell occurs because of the establishment and differentiation of the chorioallantoic membrane that functions to transport calcium from the eggshell to the embryonic circulatory system (Tuan, 1987). The chorioallantoic membrane of day 9 or day 10 chicks consists of a stratified squamous chorionic epithelium that overlies allantoic blood vessels (Coleman and Terepka, 1972) and is structurally similar to the chorioallantoic membrane of squamate reptiles (Stewart and Brasch, 2003). By incubation day 14, the outer layer of chorionic epithelial cells has differentiated into two large cell types specialized to facilitate release and transport of calcium from the eggshell (Leeson and Leeson, 1963; Coleman and Terepka, 1972; Packard and Packard, 1984). In addition, allantoic blood vessels contribute to a large blood sinus that extends between the chorionic epithelial cells to lie in close proximity to the eggshell membrane (Leeson and Leeson, 1963; Narbaitz, 1977; Packard and Packard, 1984). These specializations support three primary functions of this tissue, calcium recovery, calcium transport and respiratory exchange.

The mechanism of calcium transport by the chorionic epithelial cells of the domestic fowl differs from the endodermal cells of the yolk sac. The yolk sac endoderm, is similar to epithelia of other calcium transporting tissues, for example, kidney distal tubules and small intestine (Opperman et al., 1990; Feher et al., 1992; Bindels, 1993; Hoenderop et al., 2000), in that the cells express a cytoplasmic calcium binding protein, in this instance calbindin-D<sub>28K</sub> (Ono and Tuan, 1991; Tuan and Suyama, 1996). Calbindin-D<sub>28K</sub> is thought to facilitate calcium transfer through the cytoplasm while protecting cells from high levels of free calcium ions (Hoenderop et al., 2000). The mechanism of calcium transport by chick chorionic epithelial cells is different because these cells do not express calbindin-D<sub>28K</sub> (Sechman et al., 1994). Calcium is hypothesized to move through the cytoplasm of these cells within microvesicles (Akins and Tuan, 1993).

*P. guttatus* is typical of oviparous squamate reptiles in that the chorioallantoic membrane has formed over a region of the embryonic hemisphere and the yolk sac splanchnopleure surrounds part of the yolk at oviposition (Stewart and Florian, 2000; Blackburn et al., 2003). The topology of the membranes is comparable to incubation day 5 of chick development (Romanoff, 1960) when yolk is the primary source of calcium (Johnston and Comar, 1955; Ono and Tuan, 1991; Tuan and Suyama, 1996). Embryonic growth and calcium mobilization prior to oviposition have not been studied in squamates, but embryonic dry mass and calcium content are low at oviposition for *P. guttatus* as they are for other species of oviparous snakes (Packard et al., 1984a,b; Packard and Packard, 1988) and oviparous lizards (Packard et al., 1985; Florian, 1990; Stewart and Thompson, 1993; Shadrix et al., 1994; Thompson et al., 2001). Yolk is the primary source of calcium for growth of embryonic squamates following oviposition and total calcium in yolk

continues to decline throughout incubation (Packard et al., 1984a, 1985; Packard and Packard, 1988; Shadrix et al., 1994). The removal of calcium from the yolk of squamates, as exemplified by *P. guttatus*, is relatively slow during early embryonic stages but accelerates late in the incubation period when the embryonic growth rate is high (Fig. 4). The increase in calcium mobilization from yolk of *P. guttatus* occurred during embryonic stage 36, approximately 21 days prior to hatching and was coincident with a high level of calbindin-D<sub>28K</sub> expression in the yolk sac splanchnopleure (Ecay et al., 2004). The timing of increased calcium mobilization from yolk was approximately 1 week before increases in removal of eggshell calcium (Fig. 4). Squamate reptiles differ from archosaur reptiles in that calcium mobilized from the eggshell does not replenish yolk calcium stores and yolk withdrawn into the abdominal cavity at hatching contains little calcium (Packard, 1994). Although yolk calcium levels do not increase during incubation in squamate eggs, the mechanism of transcellular calcium movement in squamates may be similar to that of birds because cells of the yolk sac splanchnopleure of *P. guttatus* express calbindin-D<sub>28K</sub> as do those of birds (Tuan and Suyama, 1996; Ecay et al., 2004).

The heavily calcified eggshells of archosaurs are the primary source of calcium for embryonic development and recovery of calcium from this source begins earlier in development and is more extensive than calcium recovery from the eggshells of squamates (Packard, 1994). One possible explanation for variation in the timing of calcium removal from the eggshell is that the allantois grows more slowly relative to the length of the incubation period in squamates compared to domestic fowl (Stewart and Florian, 2000). Whereas the allantois of chickens surrounds the egg contents for the final 43% of the incubation period (Romanoff, 1960), this membrane alignment is not achieved until the final 32% of the incubation period in *E. fasciatus* (Shadrix et al., 1994; Stewart and Florian, 2000). The allantois does not surround the egg contents of *P. guttatus* until the penultimate embryonic stage 36 (Blackburn et al., 2003), roughly the last 33% of incubation for our specimens. Development of the chorioallantoic membrane of squamates also differs from domestic fowl in that the chorionic epithelial cells and allantoic blood vessels do not differentiate to form the specialized tissue characteristic of chickens. The efficiency of the recovery from the eggshell of chickens has been attributed to specializations of the chorionic epithelial cells (Coleman and Terepka, 1972; Packard and Packard, 1984; Tuan, 1987). Chorionic epithelial cells of squamates either remain squamous (Stewart and Florian, 2000) or become thinner in later developmental stages (Blackburn et al., 2003) and allantoic blood vessels remain at the base of a stratified squamous chorionic epithelium. Although the chorionic epithelial cells of squamates lack the structural characteristics associated with the functional specializations of birds, calcium extraction from the eggshell of *P. guttatus* is

regulated. There is no measurable loss of calcium from the eggshell of *P. guttatus* until the chorioallantoic membrane surrounds the egg contents during embryonic stage 36. Calcium loss from the eggshell is initiated two weeks before hatching and increases during the interval of development when calcium removal from the yolk and embryonic calcium gains are also high (Fig. 4). The chorioallantoic membrane of *P. guttatus* expresses calbindin-D<sub>28K</sub> early in development, indicating the potential for calcium transport, but expression is greatest during embryonic stages 36 and 37 when eggshell calcium levels drop dramatically (Ecay et al., 2004). These data suggest that calcium is extracted from the eggshell of squamates, as it is in birds, although the mechanism is unknown. In spite of differences in the structure of the chorioallantoic membrane, some features of the calcium extraction mechanism may be shared between squamates and birds. However, the mechanism of transport of calcium by chorionic epithelial cells differs because, unlike the chorioallantoic membrane of birds (Sechman et al., 1994), the chorioallantois of *P. guttatus* expresses calbindin-D<sub>28K</sub> (Ecay et al., 2004).

The suggestion that the pattern of calcium mobilization of embryonic squamates is a useful model for functional attributes of the egg of early amniotes was derived from two observations: (1) both anamniote and amniote vertebrates recover calcium from yolk during embryonic development, and (2) a mineralized eggshell is apomorphic for Reptilia (Packard, 1994; Packard and Seymour, 1997). Compared to the hypothetical ancestral pattern, birds have evolved specializations resulting in increased reliance on calcium from the eggshell but retain mechanisms for calcium recovery from yolk. One hypothesis derived from this scenario is that mobilization of calcium from yolk should be similar in squamates and birds. This hypothesis is supported because although the timing of calcium mobilization from yolk occurs relatively earlier in birds, at least some aspects of the mechanism of calcium transport are shared between corn snakes and chickens (Johnston and Comar, 1955; Ono and Tuan, 1991; Tuan and Suyama, 1996; Ecay et al., 2004). A second hypothesis, that birds have a specialized system for calcium recovery from the eggshell compared to that of squamates has received support from studies of domestic fowl (Narbaitz, 1977; Tuan, 1987; Akins and Tuan, 1993), but the specific attributes that differ have not been addressed because comparative data have not been available. Calcium mobilization from the eggshell apparently is actively regulated by embryos of both lineages. However, the mechanism of calcium transport by the chorioallantoic membrane of *P. guttatus* may be more similar to that of the yolk sac of chickens than to the chorioallantoic membrane of chickens because transcellular movement of calcium by both the yolk sac and chorioallantoic membrane of *P. guttatus* occurs in association with increased expression of calbindin-D<sub>28K</sub> (Ecay et al., 2004).

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