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PATTERNS OF MATERNAL PROVISION AND EMBRYONIC MOBILIZATION OF CALCIUM IN OVIPAROUS AND VIVIPAROUS SQUAMATE REPTILES

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***Abstract*.—Embryos of oviparous squamate reptiles obtain all organic and most inorganic nutrients from yolk; yolk provides 19–86% of hatchling calcium content. The remaining calcium is extracted from the eggshell. Yolk calcium provision to viviparous embryos also is variable and includes three patterns. The contribution of yolk to embryonic development for most viviparous squamates is similar to oviparous species, but the attenuated eggshell of viviparous species is a poor source of calcium because it lacks an outer layer of calcium carbonate, and embryos supplement yolk calcium via placental transfer. In a second pattern, yolk provides all organic nutrients and calcium. The final pattern occurs in viviparous species that are substantially placentotrophic and placental transfer accounts for most organic and inorganic nutrients, including calcium. The many independent evolutionary transitions to viviparity among squamates have inspired interest in a possible link to patterns of embryonic calcium nutrition. A prominent model predicts that the pattern of maternal provision and embryonic uptake of calcium unique to squamates facilitates the evolution of viviparity. A primary assumption of the model is that the evolution of viviparity precedes the evolution of calcium placentotrophy. An alternative model predicts that viviparity and placentotrophy evolve concurrently because mechanisms for nutrient provision and mobilization are not dependent on reproductive mode. These hypotheses have not been tested directly but review of the literature indicates that neither fully explains the diversity of squamate embryonic calcium nutrition. Viviparous species differ from oviparous species primarily in the timing of uterine calcium secretion and structure of eggshell calcium. Future studies should focus on the mechanisms that promote these differences.**

Key Words.—calcium; calbindin-D_{28K}; lecithotrophy; oviparity; placentotrophy; viviparity

The earliest amniotes were oviparous, i.e., oviposited eggs that developed outside the body of the female, (Packard and Seymour 1997; Stewart 1997; Wilkinson and Nussbaum 1998; Laurin et al. 2000; Wilkinson et al. 2002) and were provisioned with calcium-rich yolk that supplied all nutrients for embryonic development (Packard and Seymour 1997). One of the key innovations of these eggs was the formation of a fibrous tertiary shell membrane that encapsulated the egg contents. The subsequent evolution of calcareous deposits in the shell membrane of Reptilia provided structural support for the large-yolked oviparous egg and also a secondary source of calcium for developing embryos (Packard and Seymour 1997). The eggs of ancestral reptiles likely had lightly calcified eggshells and embryos were heavily dependent on calcium stored in yolk (Packard and Seymour 1997). Many oviparous squamates share these characteristics (Packard 1994).

Embryos of most oviparous reptiles obtain calcium from both yolk and an eggshell, but patterns of calcium mobilization vary (Packard and Packard 1984; Packard 1994). Oviparous squamate reptiles generally have lightly calcified eggshells and embryos mobilize most calcium from yolk, whereas embryonic turtles, crocodilians and birds are highly dependent on calcium from the eggshell. Viviparity has evolved independently in more than 100 lineages of squamate reptiles (Blackburn 1982, 1985a, 2006; Shine 1985) and these lineages provide a unique opportunity to study the evolutionary relationship between reproductive mode and pattern of embryonic calcium nutrition. Embryos of viviparous squamates receive calcium from yolk, a placenta, or in most cases, both (Thompson et al. 2000). Calcium placentotrophy is widespread and exhibits a high level of homoplasy, i.e., an independently derived functional relationship between females and offspring. The magnitude of similar, yet independent, transformations in the evolution of placental calcium transport is remarkable and provides a natural system to study evolutionary patterns and mechanisms.

SOURCES OF CALCIUM FOR EMBRYOS OF OVIPAROUS SQUAMATE REPTILES

There is a long history of interest in the physiological and nutritional characteristics of chicken eggs and to a lesser extent the eggs of turtles (see Romanoff 1967; Simkiss 1967; Packard and Clark 1996 for reviews).

TABLE 1. Dry mass and calcium content of recently ovulated egg yolks and hatchlings of lizards. Values are reported as means.

Species	Yolk Dry Mass (mg)	Yolk Ca (mg)	Yolk Ca (mg)/ Dry Mass (g)	Hatch Dry Mass (mg)	Hatch Ca (mg)	Hatch Ca (mg)/ Dry Mass (g)	% Yolk Ca in Hatch
Oviparous							
Iguana iguana[i]	--	59.0	--	--	101.0[*]	--	58
Pogona barbata[h]	710	9.42	13.3	500	15.8[*]	31.6	60
Lacerta vivipara[n]	54.4	0.17	3.1	46.8	0.89[*]	19.0	19
Podarcis muralis[o]	93.4	0.64	6.8	70.7	1.40	19.8	46
Eumeces chinensis[m]	300	2.07	6.9	200	2.65[*]	13.2	78
Plestiodon fasciatus[c]	84.1	0.72	8.6	72.3	1.19[*]	16.5	61
Saiphos equalis[p]	71.7	0.47	6.6	52.0	0.89	17.1	53
Bassiana duperreyi[a]	79.8	0.44	5.5	68.0	1.01	14.9	44
Lampropholis delicata[b]	26.6	0.19	8.9	20.7	0.44	21.3	43
Lampropholis guichenoti[b]	41.6	0.30	8.0	31.2	0.64	20.5	47
Saproscincus mustelinus[l]	44.9	0.33	7.3	31.1	0.60	19.6	55
Viviparous							
Lacerta vivipara[n]	57.6	0.14	2.4	40.2	0.65	16.2	22
Mabuya sp.[k]	0.40	0.0007	1.75	191.8	6.71	35.0	1
Eulamprus tympanum[q]	187.2	1.63	8.7	156.9	4.77	30.4	34
Saiphos equalis[p]	107.2	0.74	6.9	77.1	1.03	13.4	72
Pseudemoia entrecasteauxii[a]	32.5	0.30	9.2	54.6	1.09	20.0	28
Pseudemoia pagenstecheri[l]	18.0	0.16	8.9	50.2	1.10	21.9	15
Pseudemoia spenceri[d]	55.8	0.71	12.7	68.6	1.72	25.1	41
Niveoscincus coventryi[e]	31.9	0.16	5.0	25.6	0.52	20.3	31
Niveoscincus ocellatus[f]	59.4	2.19	36.9	100.0	4.76	47.6	46
Niveoscincus metallicus[j]	45.7	0.39	8.5	34.5	0.85	24.6	46
Niveoscincus metallicus[g]	41.8	0.66	15.8	37.9	0.72	19.0	100[**]

[*]Includes internal yolk.
[**]Yolk and hatchling calcium values do not differ significantly.
[a]Stewart and Thompson (1993); [b]Thompson et al. (2001c); [c]Shadrix et al. (1994); [d]Thompson et al. (1999c); [e]Thompson et al. (2001a); [f]Thompson et al. (2001b); [g]Thompson et al. (1999a); [h]Packard et al. (1985); [i]Packard et al. (1992); [j]Thompson et al. (2000); [k]Ramirez-Pinilla (2006); [l]Stewart et al. (2009a); [m]Ji et al. (1996); [n]Stewart et al. (2009b); [o]Ji and Brana (1999); [p]Linville et al. (2010); [q]Thompson et al (2001d).

The initial recognition that the pattern of calcium mobilization by embryos of squamates may differ from turtles and birds resulted from a comparative review of the role of calcium in reproductive physiology of tetrapods (Simkiss 1967). At the time of this review, the data for squamates were limited to two viviparous snakes; *Vipera berus* and *Thamnophis sauritus* (Dessauer and Fox 1959), both of which had yolk calcium concentrations that exceeded greatly the values for two turtles and the domestic chicken. Simkiss (1967) speculated that the yolk of these squamates was so rich in calcium that no additional source was necessary for developing embryos. This hypothesis gained further support from a more comprehensive study of *Vipera berus* in conjunction with an analysis of yolk calcium content of four lizards, two of which were oviparous and two viviparous (Jenkins and Simkiss 1968).

The hypothesis that yolk was the sole source of calcium for embryonic squamates (Simkiss 1967; Simkiss and Jenkins 1968) was refuted by research that revealed that embryos of both lizards and snakes extract calcium from the eggshell (Packard et al. 1984a, 1985). Nonetheless, embryos of oviparous lizards and snakes rely heavily on yolk, which provides for lizards, 19–78%, and for snakes, 72–86% of calcium for development (Tables 1, 2).

Following Simkiss' (1967) monograph, there have been two reviews of the sources and patterns of mobilization of calcium to embryos of oviparous amniotes (Packard and Packard 1984; Packard 1994) and one review of regulation of calcium metabolism in embryos (Packard and Clark 1996). The excellent summary of Packard (1994) recognized several characteristics that were phylogenetically informative. The review by Packard (1994) included two snakes (Packard et al. 1984a; Packard and Packard 1988), three lizards (Packard et al. 1985, 1992; Shadrix et al. 1994), four turtles (Packard et al. 1984b; Packard and Packard 1986, 1991a; Miller and Jones 1990), one crocodilian (Packard and Packard 1989) and three birds (Johnston and Comer 1955; Romanoff 1967; Packard and Packard 1991b, 1993; Hart et al. 1992). Three characteristics contributed to variation among these species: (1) distribution of calcium in oviposited eggs; (2) sources of calcium for embryos; and (3) ontogenetic pattern of mobilization of calcium by embryos.

Eggshells of chelonians and archosaurs differ structurally from and are more heavily calcified than

TABLE 2. Dry mass and calcium content of recently ovulated egg yolks and hatchlings of snakes. Values are reported as means.

Species	Yolk Dry Mass (g)	Yolk Ca (mg)	Yolk Ca (mg)/ Dry Mass (g)	Hatch Dry Mass (g)	Hatch Ca (mg)	Hatch Ca (mg)/ Dry Mass (g)	% Yolk Ca in Hatch
Oviparous							
Coluber constrictor[d]	1.4	30.0	21.4	1.0[*]	39.0[*]	39.0	77
Elaphe carinata[g]	8.4	93.8	11.2	6.8	135.0[*]	19.8	69
Elaphe taeniura[h]	6.6	90.1	13.6	5.6	139.9[*]	25.0	64
Pituophis melanoleucus[e]	4.8	88.0	18.2		116.0[*]		76
Pantherophis guttatus[f]	2.3	40.4	17.6	1.9	56.1[*]	29.5	72
Naja naja atra[i]	4.7	53.9	11.5	3.5	62.4[*]	17.8	86
Viviparous							
Nerodia rhombifera[a]	3.5	87.0	24.8	2.4[*]	83.6[*]	34.8	100[**]
Thamnophis ordinoides[b]	.59	10.7	18.1	.44[*]	13.9[*]	31.6	77
Virginia striatula[c]	.13	2.6	20.0	.097[*]	3.2[*]	33.0	81

[*]Includes internal yolk.
[**]Yolk and hatchling calcium values do not differ significantly.
[a]Stewart and Castillo (1984); [b]Stewart et al. (1990); [c]Stewart (1989); [d]Packard et al. (1984a); [e]Packard and Packard (1988); [f]Stewart et al. (2004a); [g]Ji et al. (1997b); [h]Ji et al. (1999); [i]Ji et al (1997a).

those of squamates (Packard and DeMarco 1991; Packard 1994). For most squamates, the investment of calcium in eggshells is considerably less than for yolk, whereas, chelonians and archosaurs deposit relatively little calcium in yolk (Fig. 1). The relative amount of calcium that embryos recover from yolk compared to eggshells likewise is variable within major taxa, but generally is correlated with relative abundance (Tables 1 and 2; Fig. 2). Bird embryos are highly dependent on calcium from the eggshell, whereas, the proportion of hatchling calcium contributed from eggshell is lowest in snakes. Values for lizards, turtles, and crocodilians overlap broadly. Utilization of the available calcium also differs because squamate embryos mobilize proportionally more of the available calcium in the egg (yolk + eggshell) than do turtles and archosaurs, which discard most of the eggshell calcium at hatching. The characteristics identified by Packard (1994) that distinguish squamates from other oviparous reptiles remain generally supported by recent data; most calcium in the egg is contained in yolk relative to eggshell, calcium-rich yolk provides most of the calcium for embryonic development, and embryos use most of the calcium deposited in the egg, i.e., from both yolk and eggshell. However, the range of variation in maternal provision and embryonic mobilization for squamates, particularly lizards, is higher than previously recognized (Tables 1 and 2).

SOURCES OF CALCIUM FOR EMBRYOS OF VIVIPAROUS SQUAMATE REPTILES

Viviparity has evolved independently in numerous lineages of lizards and snakes (Blackburn 1982, 1985a, 2006; Shine 1985). The eggs of viviparous squamates lack calcareous eggshells (Heulin 1990; Qualls 1996; Blackburn 1998) and embryos of viviparous squamates develop in the maternal uterus. The chorioallantoic membrane, which contacts the inner surface of the eggshell of oviparous species, is closely apposed to the uterine epithelium to form a chorioallantoic placenta in viviparous species. Most viviparous squamates also have some form of yolk sac placentation. Thus, for viviparous species, calcium is transported directly to the embryo via either the chorioallantoic placenta or yolk sac placenta in lieu of storage in the eggshell.

Embryos of many viviparous squamates, like their oviparous counterparts, are highly dependent on yolk calcium, but the range of proportional contribution of yolk calcium to hatchlings is greater in viviparous species (Fig. 3). Whereas, embryos of all oviparous species take up some calcium from the eggshell, some viviparous embryos depend entirely on yolk calcium (Jenkins and Simkiss 1968; Stewart and Castillo 1984; Thompson et al. 1999a); others mobilize a relatively small percentage of their calcium from yolk (Thompson and Stewart 1994; Thompson et al. 1999b) or are entirely dependent on placental calcium transport (Ramirez-Pinilla 2006).

ONTOGENETIC PATTERNS OF CALCIUM MOBILIZATION BY OVIPAROUS SQUAMATE EMBRYOS

Ontogeny of the distribution of calcium in egg compartments (yolk, embryo, shell) is known for six oviparous lizard and three oviparous snake species (Packard et al. 1984a, 1985, 1992; Packard and Packard 1988; Shadrix et al. 1994; Stewart et al. 2004a, 2009a,b; Linville et al. 2010). There is little change in dry mass or calcium distribution in eggs during early embryonic

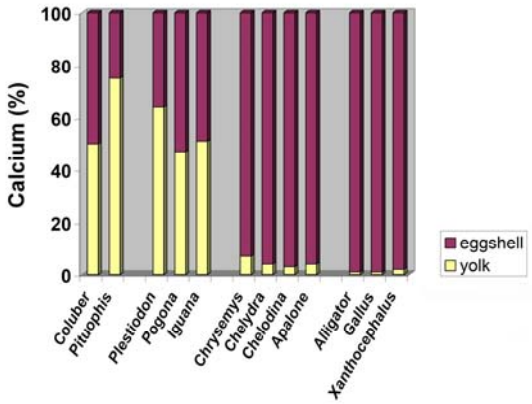

FIGURE 1. Distribution of calcium in oviposited eggs of oviparous amniotes. Data from Packard (1994).

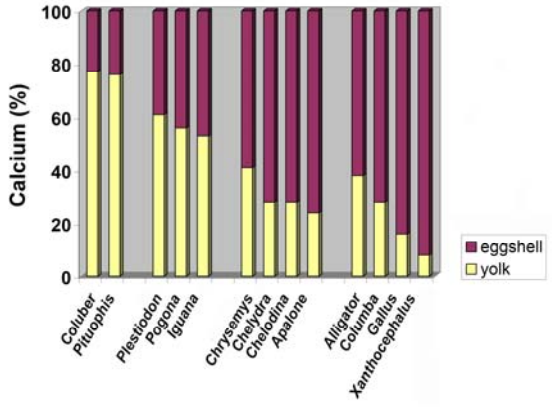

FIGURE 2. Sources of calcium in hatchlings of oviparous amniotes. Data from Packard (1994).

stages. Embryonic differentiation is followed by a dramatic increase in embryonic dry mass and a corresponding decrease in yolk dry mass. Embryonic metabolism and growth increase following stage 35 (staging system of Dufaure and Hubert 1961) in the lizard, *Plestiodon fasciatus* (Shadrix et al. 1994; Thompson and Stewart 1997) and remain high until hatching (Fig. 4). A similar pattern occurs in the snake, *Pantherophis guttatus*, with a dramatic increase in embryonic mass following embryonic stage 34 (staging system of Zehr, 1962; Stewart et al. 2004a; Fig. 5). Calcium content of embryos increases concomitant with the increase in dry mass. Mobilization of yolk reserves accounts for embryonic calcium gains during the first phase of growth because total calcium content of yolk plus embryo remains constant. However, prior to hatching, total calcium in yolk plus embryo exceeds the quantity in oviposited eggs as calcium is extracted from the eggshell (Figs. 4 and 5). Yolk calcium levels continue to drop throughout the embryonic growth phase indicating that calcium from the eggshell is taken up by the embryo and not temporarily stored in yolk. Indeed, calcium is selectively withdrawn from yolk during late stages of incubation because yolk calcium concentration drops (Packard et al. 1984a, 1985; Packard and Packard 1988; Stewart et al. 2009a,b).

MECHANISMS OF CALCIUM TRANSPORT DURING EBRYONIC DEVELOPMENT

Understanding the multiple mechanisms for calcium transport used by maternal and embryonic tissues, as well as the regulation of their functional expression during development, will provide important insights into the roles of these activities during the transition from oviparity to viviparity. Maternal calcium can be provisioned to squamate eggs or developing embryos at three distinct times and in three different forms (i.e. bound to yolk proteins and lipids, as eggshell calcium carbonate, and free ionic calcium during placental transport). Calcium delivery involves regulated calcium transport across a boundary layer of maternal cells (ovarian follicle, eggshell gland, uterine epithelium). Similarly, embryos access stored calcium (yolk and shell) or delivered calcium (placenta) at the time when appropriate tissues (yolk sac endoderm, chorionic epithelium, or placenta) have developed the functional capacity for calcium transport to the embryonic circulation. At present, there have been few studies of the cellular and biochemical mechanisms controlling calcium transport across any of the reproductive and embryonic tissues of squamates. Thus, our current understanding of this process is largely modeled on extensive mechanistic studies of the calcium transporting properties of homologous reproductive /embryonic tissues in chickens and other avian species, which in turn have been modeled on other avian and vertebrate tissues and organs involved in calcium homeostasis.

The intestine, kidney, and extraembryonic membranes, are primary sites of calcium absorption in amniotes (Terepka et al. 1969; Bronner et al. 1986; Ono and Tuan 1991; Bronner and Pansu 1999; Hoenderop et al. 2000; reviewed by Hoenderop, et al. 2005; Perez et al. 2008). The basic pattern of calcium absorption across epithelial cells of the intestine, renal distal tubule, chorion (including trophoblast) and yolk sac follows two pathways: passive diffusion across epithelial tight junctions and between adjacent cells along the paracellular pathway or energy consuming transport on a transcellular path through epithelial cells (Bronner et al. 1986; Feher et al. 1992; Akins and Tuan 1993b; Bindels 1993; Tuan and Suyama 1996; Hoenderop et al. 2000; Larsson and Nemere 2002; Peng et al. 2003). During paracellular transport, passive calcium diffusion is governed by chemical and electrical gradients across epithelial cells (Bronner et al. 1986; Feher et al. 1992;

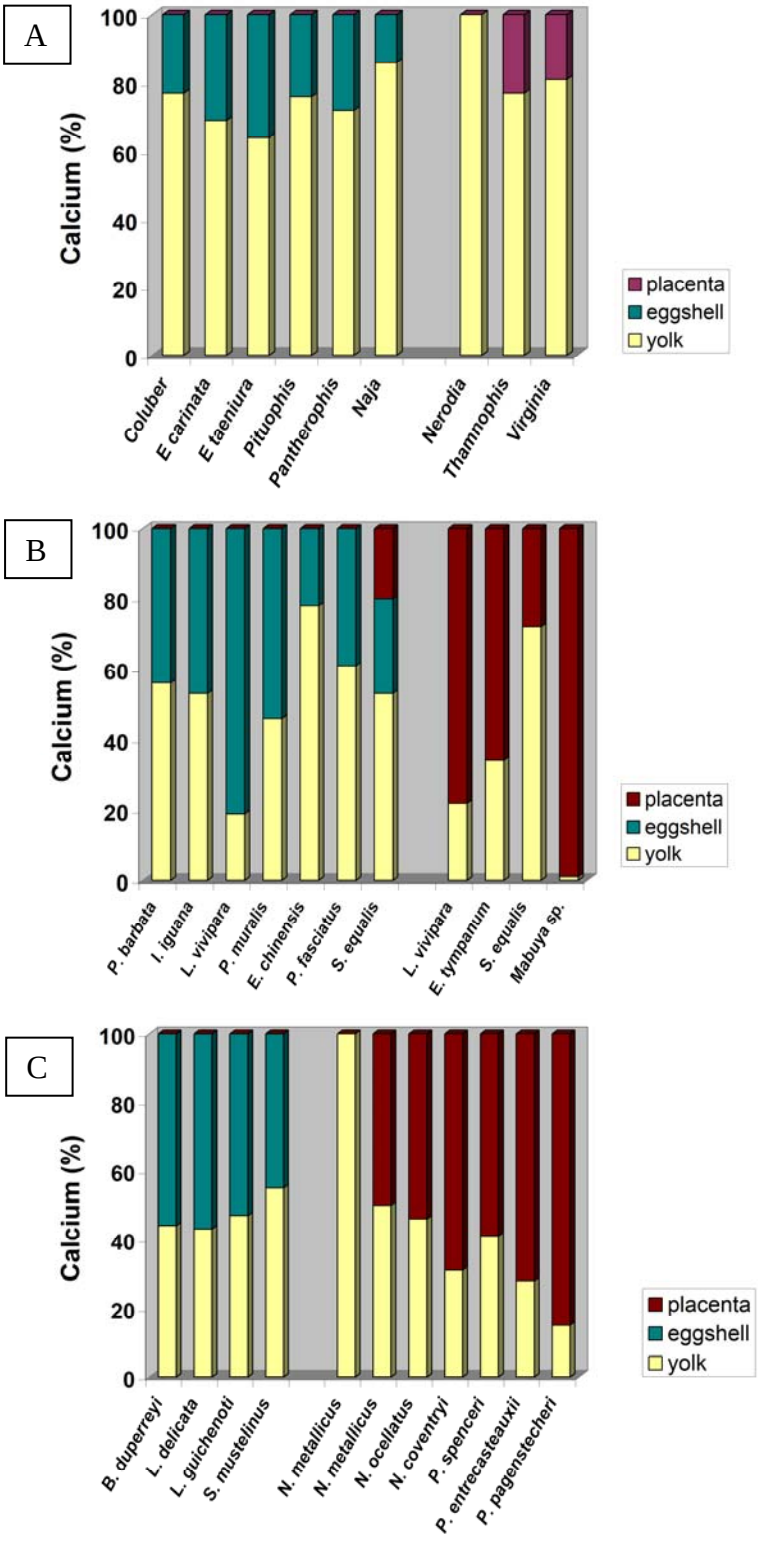

FIGURE 3. Sources of calcium in (A) hatchling/neonatal snakes (Data from Table 2), (B) hatchling/neonatal lizards (Data from Table 1), and for (C) hatchling/neonatal *Eugongylus* group skinks (Data from Table 1).

Bindels 1993). Transcellular transport depends on the expression of specific plasma membrane and intracellular calcium transport proteins. These include the highly calcium-specific ion channels of the transient receptor potential vanilloid family (TRPV5 and TRPV6), cytosolic calcium binding proteins (calbindin-D9K and calbindin-D28K), a plasma membrane sodium/calcium exchanger (NCX) and/or a plasma membrane calcium transporting ATPase (Ca^{2+}-ATPase; Hoenderop et al. 2005; Perez et al. 2008). Transcellular calcium transport has been modeled as a three-step process with calcium entry into the cytoplasm by way of luminal membrane TRPV5 and TRPV6 channels as the first step. Calcium next binds one of the calbindins, which act to buffer cytosolic calcium and enhance calcium diffusion across the cell (Bronner et al. 1986; Feher et al. 1992; Bindels 1993; Tuan and Suyama 1996; Hoenderop et al. 2000; Larsson and Nemere 2002). Evidence in some systems supports calcium movement through the cytoplasm within membrane delimited compartments such as the endoplasmic reticulum (Coleman and Terepka 1972b; Akins and Tuan 1993a,b; Khanal and Nemere 2008). In the final step, calcium is transported outward across the serosal membrane by a Ca^{2+}-ATPase and/or a sodium/calcium exchanger (Hoenderop et al. 2005; Perez et al. 2008).

Calbindin-D28K expression levels are positively correlated with calcium absorbing capacity in several tissues including mammalian kidney (Bindels 1993), chicken duodenum (Bar et al. 1979), and chicken eggshell gland (Bar et al. 1984). In addition, calbindin mRNA and protein synthesis in kidney and intestine are regulated by 1,25-dihydroxyvitamin D3, the steroid hormonal form of vitamin D (Bronner et al. 1986; Ono and Tuan 1991; Tuan and Suyama 1996; Bronner and Pansu 1999; Hoenderop et al. 2000; Peng et al. 2003). Vitamin D3 regulates co-expression of the TRPV5/6 channels and calbindin-D28K in mammalian kidney and co-expression of TRPV5/6 with calbindin-D9K in mammalian intestine, thereby linking these transporters to the endocrine control of calcium homeostasis (Hoenderop et al. 2000; Bar 2008; Khanal and Nemer 2008). Estrogens regulate expression of TRPV 5/6 channels and calbindins in placenta as well as in intestine and kidney (reviewed in Hoenderop et al. 2005; Lafond and Simoneau 2006; Khanal and Nemer 2008). Estrogen, but not vitamin D, regulates calbindin-D28K expression in chicken eggshell glands (reviewed by Bar 2009).

STORAGE AND RELEASE OF YOLK CALCIUM

Calcium is first supplied to the squamate egg prior to ovulation and fertilization during the process of vitellogenesis and the yolk produced is an important source of calcium for both oviparous and viviparous squamate embryos (Tables 1 and 2). Yolk calcium is bound to the highly phosphorylated phosvitin domain of vitellogenin, which is synthesized in the liver and taken up into the oocyte by receptor-mediated endocytosis in the follicle (reveiwed in Romano et al. 2004 and Finn 2007). During embryonic development, yolk platelets, including phosvitin-calcium complexes and lipid-lipovitellin complexes, are engulfed by endodermal cells

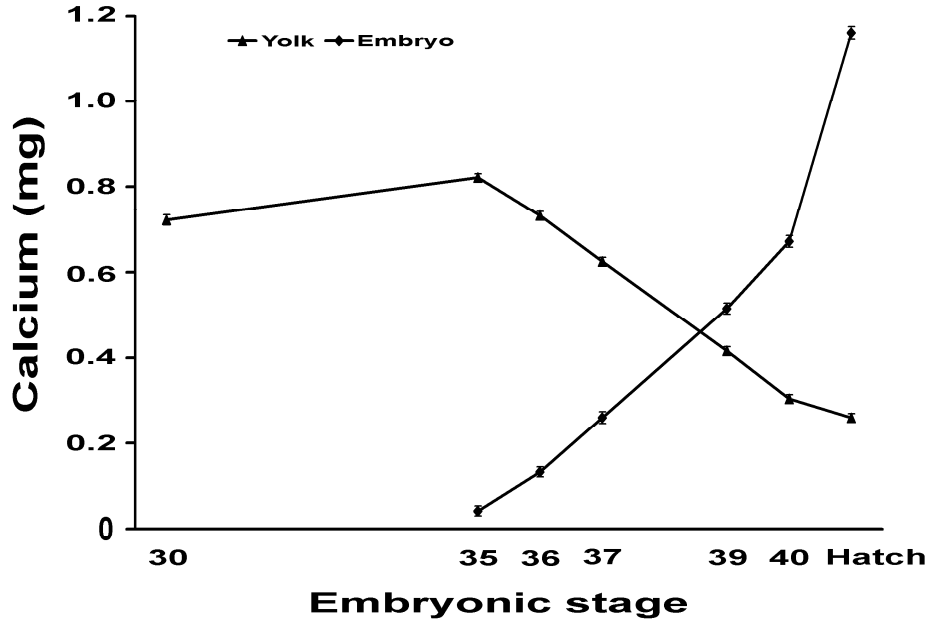

FIGURE 4. Ontogeny of calcium content of yolks and embryos for eggs of *Plestiodon fasciatus* (from Shadrix et al. 1994). Embryonic staging according to Dufaure and Hubert (1961).

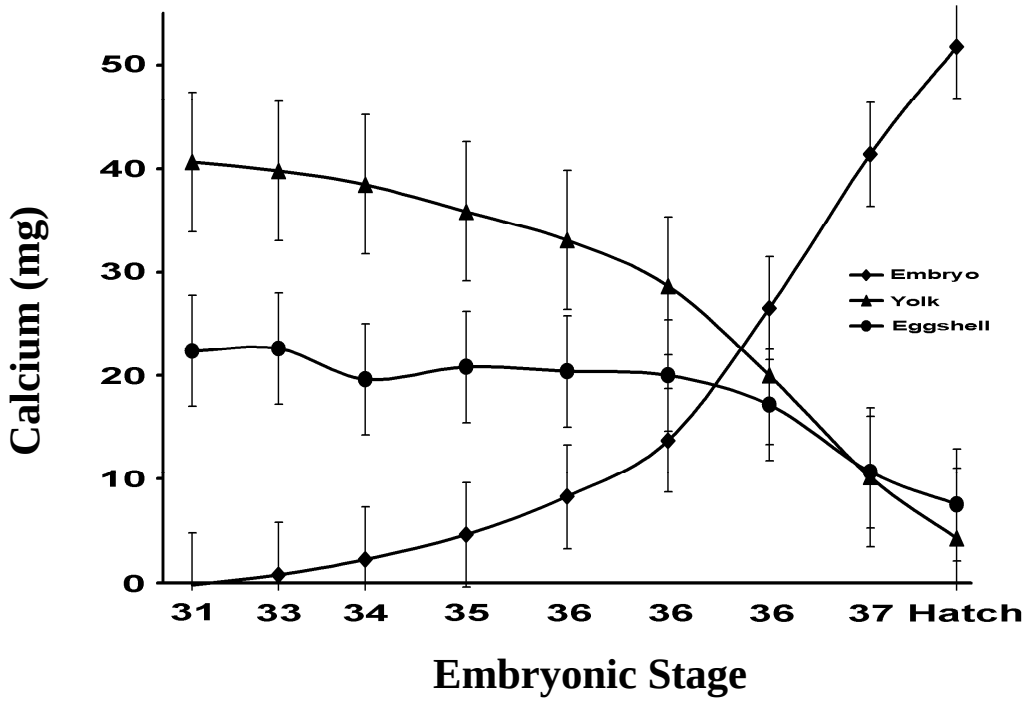

FIGURE 5. Ontogeny of calcium content of yolks, eggshells, and embryos for eggs of *Pantherophis guttatus* (from Stewart et al. 2004a). Embryonic staging according to Zehr (1962).

of the yolk sac membrane and processed for delivery to the embryo (Komazaki et al. 1993). Embryonic chickens mobilize calcium from yolk by endodermal cells of the yolk sac splanchnopleure relatively early in the incubation period (Johnston and Comar 1955; Romanoff 1967). However, from the middle of incubation to hatching (days 9–21), chicken yolk accumulates calcium from the eggshell (Romanoff 1967; Packard 1994). Squamates, in contrast, exhibit a slow decline in yolk calcium that accelerates in the last third of development, coincident with embryonic growth and squamate yolk has not been observed to accumulate calcium at any stage of development (Packard 1994, Stewart et al. 2004a, 2009a,b). Thus, yolk sac endoderm of squamates exhibits net calcium transport only in the yolk to embryo direction while chicken yolk endoderm reverses the direction of net calcium transport in the latter half of development to favor yolk calcium accumulation.

Calbindin-D28K expression has been observed in chicken endoderm as early as day 3 of incubation (Tuan and Suyama 1996) but most studies of chicken yolk sac calbindin-D28K expression have focused on the latter half of development (day 9 to 21). Late in development, chicken yolk sac calbindin-D28K expression and calcium transport are highly sensitive to exogenous vitamin D3, but the physiological significance of this observation has not been determined (Clark et al. 1989; Ono and Tuan 1991; Tuan and Suyama 1996). Calbindin-D28K levels in chicken yolk sac do not change in the middle third of incubation (day 9 to 14) but do increase two fold during the last third of development (day 16 to 20; Sechman et al. 1994; Tuan and Suyama 1996). Calbindin-D28K has also been observed in the yolk sac of the oviparous snake, *Pantherophis guttatus* (Ecay et al. 2004). Expression was undetectable early (stages 27–32 in Fig. 5) and increased more than 10 fold in the last stages of development (stages 35–37 in Fig. 5) when calcium transport across the yolk sac is highest (Stewart et al. 2004a). This level of induction is similar to vitamin D3-stimulated calbindin-D28K expression in chicken yolk sac (Ono and Tuan 1991) but the role of vitamin D in *Pantherophis guttatus* yolk sac has not been determined. Expression of additional calcium tranport proteins (TRPV5/6 channels, plasma membrane Ca^{2+} ATPase, sodium/calcium exchanger, etc.) in chicken or squamate yolk sac endoderm have not been reported.

Late in development, selective calcium over protein and lipid absorption from yolk has been noted in several squamate species (Packard et al. 1984a, 1985; Packard and Packard 1988; Stewart et al. 2009a,b). This phenomenon may be explained by selective degradation of calcium/phosvitin complexes. In the oviparous lizard

Podarcis sicula, lipovitellins (proteolipid complexes originating from vitellogenins) appear stable throughout development but phosvitin undergoes gradual and continuous proteolytic breakdown (Romano et al. 2002). Calcium binding capacity is reduced in short phosvitin fragments (Goulas et al. 1996) and thus proteolytic cleavage may cause selective calcium release from yolk. These observations suggest that the embryonic demand for calcium late in development, presumably for skeletogenesis, exceeds metabolic demands met by other yolk nutrients and the potential for calcium mobilization from the eggshell.

DEPOSITION AND RECOVERY OF EGGSHELL CALCIUM

Another source of calcium for embryonic development is supplied during eggshell formation. Secretion of calcium is highly regulated in the amniote oviduct. The calcareous eggshell of Reptilia is secreted during passage of the egg along the oviduct by epithelial cells located in shell glands. Studies of birds indicate that intracellular transport of calcium during eggshell formation is correlated with the concentration of calbindin-D28K within shell gland epithelial cells (Bar et al. 1984) and Ca^{2+}-ATPase activity, which is expressed on the apical surfaces of gland cells, is co-localized with calbindin-D28K in the shell gland (Wasserman et al. 1991). Ca2+ -ATPase expression in the shell glands is increased by estrogen treatment but unaffected by vitamin D (Nys and de Laage 1984; Qin et al. 1993). Similarly, calbindin-D28K expression in the shell gland is unaffected by vitamin D but does respond to estrogen stimulation (Bar 2009). Expression of the TRPV5/6 calcium channels in avian eggshell glands or oviductal epithelia has not been reported. There is little information on the mechanism of calcified shell formation in non-avian reptiles, but a recent study suggests that some features of egg shelling in squamate reptiles may be similar to birds because the apical surface of epithelial cells of the shell glands of the oviparous lizard *Lampropholis guichenoti* express Ca^{2+}-ATPase during egg shelling (Thompson et al. 2007).

Recovery of shell calcium is one activity of the chorioallantois, which lies in close opposition to the inner surface of the shell. The mechanism of calcium transport by the chorioallantoic membrane of chickens 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 1972a,b; Packard and Packard 1984). Calcium is eroded from the eggshell by acid released from villus cavity cells of the chorionic epithelium, which results from increased carbonic anhydrase activity in villus cavity cells (Simkiss 1980; Anderson et al. 1981; Narbaitz et al. 1981; 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 1972a,b), but calcium uptake has also been suggested for villus cavity cells (Narbaitz 1972), and both cell types may function in calcium transport (Packard and Packard 1984). Unlike endodermal cells of the yolk sac splanchnopleure, chorionic epithelial cells of chicken embryos do not express calbindin-D28K mRNA or protein during any stage of development (Sechman et al. 1994). However, the calcium transporting capacity of the chorioallantoic membrane has been correlated with expression of a novel extracellular calcium binding protein, transcalcin (Tuan and Scott 1977; Tuan et al. 1978, 1986; Tuan 1987) that has been localized immunohistochemically to calcium-transporting chorionic epithelial cells (Tuan and Knowles 1984; Tuan et al. 1986). These cells also contain Ca^{2+}-ATPase, which is closely associated with transcalcin and developmentally correlated with calcium transport activity by the chorioallantoic membrane (Tuan and Knowles 1984; Tuan et al. 1986; Akins and Tuan 1993a,b). Members of the annexin family of calcium binding proteins have been immunolocalized to chorionic cells and their expression increases from incubation days 8 to 12 in chicken eggs (Matschke et al. 2006). Vitamin D upregulates calcium transport by the chorioallantoic membrane in quail eggs (Elaroussi et al. 1994; Elaroussi and DeLuca 1994) but a direct affect on chorioallantoic calcium transport has not been demonstrated (Tuan and Ono 1986; Tuan 1987; Packard et al. 1998). Interestingly, carbonic anhydrase activity of the chicken chorioallantois, which is linked to calcium transport (Tuan 1984), is upregulated by vitamin D3 (Narbaitz et al. 1981) suggesting an indirect link between vitamin D3, transport protein expression and calcium transport activity.

In contrast to birds, the oviparous snake *Pantherophis guttatus* expresses calbindin-D28K in the chorioallantois and expression levels increase at late stages of development coincident with calcium transport from the eggshell (Ecay et al. 2004). Thus, the oviparous squamate chorioallantois may more closely resemble typical calcium absorbing epithelia compared to the chicken chorioallantois. This may reflect the high calcium transport demand of the bird chorioallantois, which must transport 80% or more of the calcium required for embryonic development (Packard 1994). Calbindin-D28K expression in *P. guttatus* was consistently greater in chorioallantoic membrane of the abembryonic hemisphere of the egg, compared to the embryonic hemisphere, suggesting embryonic regulation of calcium transport activity, but further aspects of regulation have not been investigated (Ecay et al. 2004).

PLACENTAL CALCIUM TRANSPORT

For viviparous squamates, placental transport can provide a substantial amount of hatchling calcium (Tables 1 and 2; Fig. 3). As for eggshell and yolk calcium transport there is little direct evidence pointing to a basic mechanism for squamate placental calcium transport. In mammals, the uterus of mice, pigs, and rats express one or both calbindin-D9K and D28K during pregnancy (reviewed in Lafond and Simoneau 2006). Also expressed in mammalian placenta are isoforms of a plasma membrane Ca^{2+}-ATPase and sodium/calcium exchanger (Lafond and Simoneau 2006). Using knockout mice, Suzuki et al. (2008) demonstrated the direct role of TRPV6 in mouse maternal to fetal calcium transport. While there is some evidence that mammalian placental calcium transporter expression and function may be controlled by both vitamin D3 and estrogens (Lafond and Simoneau 2006), their abundance and function in chicken eggshell gland appears to be independent of vitamin D3 status and regulated by estrogens (Bar 2009). Estrogen response elements have been identified in the promoter regions of calbindin-D28K (Gill and Christakos 1995) and TRPV6 (Weber et al. 2001).

Shell glands of the oviparous lizard *Lampropholis guichenoti* express a plasma membrane Ca^{2+}-ATPase (PMCA) at their lumenal surface at the time of eggshell production but not at other times in the reproductive cycle (Thompson et al. 2007). The viviparous scincid lizards *Niveoscincus metallicus*, *N. ocellatus*, and *Pseudemoia spenceri* also express PMCA in uterine glandular and surface epithelia during pregnancy as determined by immunofluorescent staining (Herbert et al. 2006). The timing of expression of PMCA in these species is lineage specific. *Pseudemoia spenceri* exhibits expression of PMCA throughout the reproductive cycle (vitellogenesis to late pregnancy), while in *N. metallicus* and *N. ocellatus* uterine expression of PMCA is evident only in early stages of pregnancy (Herbert et al. 2006).

CALCIUM PLACENTOTROPHY AND THE EVOLUTION OF VIVIPARITY

The potential influence of the pattern of embryonic calcium mobilization on the evolution of viviparity was first articulated by Packard et al. (1977) who noted that if eggshells are a requisite source of calcium for developing embryos, loss of the calcareous layer of the eggshell would impose a constraint on the evolution of viviparity. Loss of the eggshell would be an obligate correlate of extended oviductal egg retention, an intermediate stage in the evolution of viviparity, because this structure would impede embryonic respiration. Packard et al. (1977) also argued that the loss of the eggshell would not result in a nutritional deficit for squamate embryos if yolk was the sole source of calcium for embryonic development as predicted (Simkiss 1967; Jenkins and Simkiss 1968). This reasoning led to the hypothesis that viviparity would evolve only in lineages, such as Squamata, that have calcium-rich yolk and that do not depend on the eggshell as a source of calcium (Packard et al. 1977; Packard and Packard 1984). The eggshell calcium constraint hypothesis has two premises; the evolution of viviparity precedes the evolution of placentotrophy and oviparous squamate embryos are not dependent on eggshell calcium. The latter premise has been proven false because oviparous squamate embryos do extract calcium from the eggshell (Packard 1994), including lineages in which viviparous populations or species also occur (Stewart and Thompson 1993; Shadrix et al. 1994; Thompson et al. 2001c; Stewart et al. 2009a,b; Linville et al. 2010). Nonetheless, the yolk of squamates is rich in calcium (Tables 1 and 2) and, if this source is sufficient to sustain development, the evolution of viviparity may be facilitated.

If the evolution of viviparity precedes the evolution of calcium placentotrophy, as posited by Packard et al. (1977), initial stages in the evolution of viviparity should be solely dependent on yolk calcium, even if the oviparous ancestor extracted calcium from the eggshell. Thus, some viviparous squamate embryos should obtain all calcium for development from yolk. The absence of net placental uptake of calcium in a viviparous snake (*Nerodia rhombifera*; Stewart and Castillo 1984) and a viviparous lizard (*Niveoscincus metallicus*; Thompson et al. 1999a) is consistent with this transition sequence.

The concept of incipient matrotrophy (Blackburn 1985b) is central to an alternative scenario to the model of Packard et al. (1977). The incipient matrotrophy model predicts that mechanisms are in place for nutrient exchange between maternal and embryonic tissues as a result of extended oviductal egg retention and that placentotrophy evolves concurrently with, and is a necessary correlate of viviparity (Blackburn 1985b, 1992, 1995, 2006). The model follows observations that: (1) uterine provision of nutrients such as eggshell calcium precedes the evolution of viviparity, (2) viviparity entails a close anatomical and physiological relationship between embryos and mothers, and (3) placental transport of either inorganic or organic molecules (or both) occurs in most viviparous squamates. Thus, embryos of species that have recently evolved viviparity should receive calcium from placental transfer if their immediate ancestors received calcium from the eggshell.

Experimental manipulations would provide the strongest evidence to distinguish between these competing models, but we can also test predictions derived from the models through comparisons between genetically similar organisms. *Lacerta vivipara* and

Saiphos equalis are species of lizards that exhibit geographic variation in reproductive mode (Heulin et al. 1993; Smith et al. 2001; Surget-Groba et al. 2001, 2006). Populations of *L. vivipara* either oviposit eggs with calcareous eggshells with embryos in the limb bud stage or give birth to free-living young (Heulin et al. 1993). All populations of *S. equalis* retain eggs in the uterus to late embryonic stages (prolonged egg retention) but there are differences in the length of post-ovipositional incubation. Some populations oviposit eggs with calcareous eggshells that take up to a week or more to hatch, whereas other populations oviposit eggs that hatch shortly after oviposition, i.e., give birth to free-living young (Smith and Shine, 1997). Comparisons between two populations with different reproductive modes within each species led to similar conclusions concerning the relationship between reproductive mode and pattern of calcium provision to embryos (Stewart et al. 2009b; Linville et al. 2010). Embryos of oviparous (prolonged egg retaining for *S. equalis*) populations extract a substantial amount of calcium from eggshells. Eighty one percent of the calcium in hatchling *L. vivipara* and 27% in hatchling *S. equalis* are mobilized from the eggshell. Embryos of viviparous populations of each species receive a substantial amount of calcium via placental transfer (76% of neonatal calcium in *L. vivipara* and 28% in *S. equalis*). In addition, embryos from prolonged egg retaining populations of *S. equalis* obtain 20% of their calcium via placental transfer and thus have three sources of calcium, yolk, eggshell, and placenta. There are insufficient data to predict polarity in the evolution of reproductive mode for these populations in either species. However, the pattern of calcium provision to embryos apparently has not constrained the evolution of reproductive mode in either species.

Intraspecific comparisons of these two reproductively bimodal species are best explained if the evolution of nutritional provision of calcium to embryos was coincident with evolution of reproductive mode. In addition, patterns of embryonic calcium uptake indicate that the transition is associated with altered uterine function. Whereas the timing of embryonic acquisition of calcium relative to embryonic development is similar in intraspecific comparisons of oviparous and viviparous populations of each species, the timing of uterine secretion of calcium differs between reproductive modes (Stewart et al. 2009b; Linville et al. 2010). Thus, if evolution of calcium placentotrophy occurs concurrently with evolution of viviparity, plasticity in the mechanism of uterine secretion facilitates the transition.

An additional finding of the comparisons between populations of *Lacerta vivipara* and *Saiphos equalis* has further implications for the evolution of calcium placentotrophy. Substantial placental calcium transfer occurs in viviparous populations of both species. Although placental structures of *L. vivipara* lack histological specializations (Stewart et al. 2004b), the relative contribution of placental calcium to neonates is comparable to species with structurally specialized placentae (Thompson et al. 2000; Stewart et al. 2009b). However, viviparous neonates of both species have lower calcium densities than oviparous hatchlings (Stewart et al. 2009b; Linville et al. 2010). Thus, histological specializations are not a requirement for substantial placental transfer of calcium, but in the absence of placental specializations calcium transfer is not as effective in providing calcium to embryos as extraction from the eggshell. In summary, intraspecific comparisons of the reproductively bimodal species, *Lacerta vivipara* and *Saiphos equalis*, suggest two characteristics that may influence significantly the evolution of calcium placentotrophy; there is considerable plasticity in the timing of uterine calcium secretion and placental specializations for calcium delivery to embryos are required for placental transfer to be as effective as calcium extraction from the eggshell.

EVOLUTION OF CALCIUM PLACENTOTROPHY

In contrast to *Lacerta vivipara* and *Saiphos equalis*, species of the viviparous genus *Pseudemoia*, which are Australian scincid lizards, are highly placentotrophic for all nutrients and have structurally specialized placentae (Stewart and Thompson 1993, 2009; Thompson and Stewart 1994; Thompson et al. 1999b,c). *Pseudemoia* is a monophyletic genus and phylogenetic relationships among the six species within the genus are well resolved (Smith 2001). The identity of the oviparous sister taxon is uncertain, although the most likely candidates are two lineages, one including the genus *Bassiana* and the other the genera *Lampropholis* and *Saproscincus* (Greer and Kluge 1980; Greer 1989; Smith 2001; Smith et al. 2007). There are two primary lineages within *Pseudemoia*. *Pseudemoia spenceri* is the sister taxon to a lineage containing the remaining five species, which include *P. entrecasteauxii* and *P. pagenstecheri*. These three species are placentotrophic for both organic and inorganic molecules, including calcium, and placental transfer of calcium is proportional to placental provision of organic molecules (Stewart and Thompson 1993; Thompson and Stewart 1994; Thompson et al. 1999b,c; Stewart et al. 2009a). Egg size is larger and placental transfer less substantial in *P. spenceri* compared to *P. entrecasteauxii* and *P. pagenstecheri*. The relative contribution of yolk calcium to neonates of *P. spenceri* is comparable to oviparous species of *Eugongylus* group skinks (Table 1, Fig. 3C), whereas embryos of *P. entrecasteauxii* and *P. pagenstecheri* recover relatively less calcium from yolk. All three species of *Pseudemoia* have morphologically specialized placentae (Weekes 1935; Stewart and Thompson 1996, 1998, 2009) but *P.*

entrecasteauxii and *P. pagenstecheri* ovulate smaller eggs relative to the mass of neonates (Table 1). The combination of morphological specialization and substantial placentotrophy is rare among squamates and indicates considerable evolutionary change in this lineage compared to oviparous sister taxa.

Oviparous species of the scincid lizard subfamily Lygosomatinae (*Bassiana duperreyi*, *Lampropholis delicata*, *L. guichenoti*, *Saproscincus mustelinus*, and *Saiphos equalis*) ovulate yolk that provides 43–55% of the calcium content of hatchlings and thus the contribution of calcium from the eggshell is substantial (Table 1, Fig. 3C). The proportional contribution of yolk calcium to hatchlings for two species in the scincid subfamily Scincinae (*Eumeces chinensis* and *Plestiodon fasciatus*) are higher but indicate a substantial contribution of eggshell calcium to hatchlings (Table 1). This pattern of embryonic reliance on calcium from eggshells may be widespread among Scincidae, a lineage with the highest number of independent origins of viviparity among squamates (Blackburn 1982, 1999). These data do not support the hypothesis that viviparity evolves only in lineages in which embryos do not depend on calcium from the eggshell (Packard et al. 1977).

The pattern of embryonic calcium mobilization for viviparous *Pseudemoia pagenstecheri* reveals that, as with viviparous *Lacerta vivipara* and *Saiphos equalis*, uterine calcium secretion occurs primarily during the latest stages of embryonic development (Stewart et al. 2009a,b; Linville et al. 2010). In contrast to intraspecific comparisons of the reproductively bimodal species, placental transfer of calcium in *P. pagenstecheri* exceeds calcium recovery from the eggshell by embryos of the oviparous *S. mustelinus*. As a result, neonates of *P. pagenstecheri* have higher calcium concentrations than hatchling *S. mustelinus* (Stewart et al. 2009a). One of the functions of the morphologically specialized placentation of *P. pagenstecheri* is calcium transport.

Model for the Evolution of Calcium Placentotrophy

Variation in patterns of embryonic calcium nutrition among squamates suggests a model for the evolution of calcium placentotrophy. Oviparous species and many viviparous species ovulate eggs that provide a substantial amount of calcium to hatchlings. Embryos of oviparous eggs supplement yolk nutrients by extracting calcium from the eggshell. Embryonic uptake of calcium is correlated with embryonic growth and mobilization of calcium from the eggshell occurs during the peak embryonic growth phase late in development. Eggshell calcium is requisite to optimal embryonic growth in some species, but also provides an optional source of nutrition. The eggshells of many squamates contain more calcium than is extracted by embryos indicating that the supply of calcium at least meets minimal embryonic requirements. Calcium withdrawn from eggshells in excess of the minimal requirement and stored in embryonic tissues, such as bone or endolymphatic sacs (Simkiss 1967; Packard and Packard 1988), would provide an important source of nutrition for free-living hatchlings. Thus, extraction of eggshell calcium may be either obligatory or facultative. For species with calcium-rich yolk, calcium extraction from the eggshell is facultative and augments hatchling nutritional status. Species with calcium-poor yolk are nutritionally dependent on calcium from the eggshell, but also extract excess calcium to augment nutritional requirements.

Most viviparous species supplement calcium from yolk with placental transfer but there is a wide range of placental calcium provision relative to yolk (0–99%). Yolk calcium concentrations for most viviparous species are similar to those of closely related oviparous species (Tables 1 and 2). The relative contribution of yolk calcium to hatchling nutrition for predominantly lecithotrophic viviparous species is similar to oviparous species but supplemental calcium is provided by transfer across a placenta rather than extraction from an eggshell. In contrast, placentotrophic viviparous species with reduced yolk mass have high levels of placental calcium transfer in conjunction with high levels of organic nutrient transfer. Placental calcium nutrition for these embryos compensates for a loss of both eggshell and yolk calcium. The pattern of embryonic calcium mobilization of viviparous species is similar to oviparous species because embryonic uptake of calcium is correlated with embryonic growth and both extraction of calcium from eggshells and placental transfer of calcium occur during the peak embryonic growth phase late in development.

Four general patterns characterize embryonic calcium nutrition for squamate reptiles: (1) viviparous species totally dependent on yolk calcium; (2) supplementation of yolk calcium by extraction from the eggshell; (3) supplementation of yolk calcium by placental transfer; and (4) compensation for the loss of yolk calcium by placental transfer. The first pattern includes viviparous species that do not produce calcareous eggshells and lack placental transfer of calcium. For the second and third pattern, yolk provides all or nearly all of the organic nutrients for embryonic development, but contains insufficient calcium to meet the requirements for growth based on the available organic molecules. The final pattern results from placental specialization in conjunction with reduction in total yolk provision. A model for the evolution of calcium placentotrophy must address possible transitions between these patterns.

While we can define characteristics of nutritional provision of calcium to embryos in relation to

reproductive mode, most comparisons are between taxa that have been separated from a common ancestor long enough to have evolved novel structural and functional specializations. With adequate taxon sampling we will be able to distinguish plesiomorphic and apomorphic characters, and thus evolutionary patterns, but these analyses will not address questions concerning the mechanisms underlying the transitions. Species with mixed reproductive strategies, i.e., geographic variation in reproductive mode, are the appropriate models for comparative and experimental techniques to test hypotheses for the mechanisms that influence embryonic calcium provision during transitions between reproductive modes. Recent research on two such species suggests a relationship between oviductal egg retention and uterine calcium secretion.

Reproductive mode varies geographically in the lizards, *Lacerta vivipara* and *Saiphos equalis*, and embryos obtain calcium from both yolk and uterine secretion irrespective of reproductive mode. All populations of *S. equalis* retain eggs in the oviduct until late developmental stages. Uterine secretion of calcium contributes to an eggshell during early embryonic development in populations of *S. equalis* with prolonged oviductal egg retention, but uterine calcium secretion continues throughout gestation and embryos also obtain calcium via placental transfer later in development. Thus, both an eggshell and placental transfer provide embryonic nutrition in some populations of *S. equalis*. Viviparous females of *S. equalis* secrete thin shell membranes that do not support an outer layer of calcium carbonate and placental calcium is delivered directly to embryonic tissues. Females from one population of *L. vivipara* oviposit eggs with calcareous eggshells when embryos are at an early developmental stage, whereas females that retain eggs to give birth to free living neonates produce thinner eggshells that contain less calcium. The uterus of these females also secretes calcium during late developmental stages that is delivered to embryos via a placenta. Thus, uterine calcium secretion in both species is correlated with the length of time eggs are in the uterus and the thinner eggshell membranes of viviparous females do not promote calcium carbonate crystal formation.

Uterine epithelial cells of both oviparous and viviparous lizards express plasma membrane Ca^{2+}-ATPase (Herbert et al. 2006; Thompson et al. 2007). Expression in an oviparous species, *Lampropholis guichenoti*, occurs during egg shell formation, but expression occurs throughout gestation in a viviparous placentotrophic species, *Pseudemoia spenceri*. The regulation of eggshell formation and calcium secretion in squamates has not been determined, but a combination of hormonal regulation and mechanical stress on the uterus has been implicated in domestic fowl (Bar 2009). If the regulation of uterine calcium secretion in squamates also involves a complex of hormonal and mechanical factors, the evolution of extended egg retention could result in concurrent evolution of extended calcium availability to embryos. One stimulus for uterine calcium secretion may be the presence of eggs in the uterus.

The protein layers of the eggshell of oviparous squamates are secreted shortly after ovulation, followed by the secretion of calcium, which forms crystals on the outside of the protein matrix (Guillette et al. 1989; Packard and Demarco 1991; Guillette 1992; Qualls 1996; Heulin et al. 2005). Calcification of the eggshell is initiated by formation of small crystals of calcium carbonate scattered on the surface of the fibrous shell membrane and these initial sites enlarge as they accumulate more crystalline material (Packard and Demarco 1991). The mechanism that initiates crystal formation is not known, but it is likely that the surface morphology and/or chemical structure of the outer layer of the eggshell membrane may be involved (Packard and DeMarco 1991; Hernandez-Hernandez et al. 2008). The protein layers of the eggshells of viviparous species are thinner than oviparous species and calcium crystals do not form on these membranes (Heulin 1990; Qualls 1996; Heulin et al. 2005). If the thinner shell membranes of viviparous species have evolved through loss of the outer layer of the shell membrane of oviparous ancestors, they may also have lost the feature that triggers crystal formation. These thinner, structurally modified membranes could then allow diffusion of calcium between maternal and embryonic tissues. Thus, calcium secreted during later stages of gestation would pass through the thin shell membrane and be taken up by the embryo as occurs in some populations of *Lacerta vivipara* and *Saiphos equalis*.

Squamate embryos take up calcium as they grow and, in contrast to bird embryos, do not sequester calcium extracted from eggshell in yolk (Packard 1994). Oviparous squamate embryos are dependent on yolk calcium for early stages of differentiation and growth and only extract calcium from the eggshell late in incubation as embryonic growth accelerates (Packard 1994; Stewart et al. 2004a, 2009a; Linville et al. 2010). Viviparous species have a similar pattern of embryonic growth and calcium mobilization, but placental transfer, not an eggshell, is the source of calcium during the embryonic growth phase (Stewart et al. 2009a; Linville et al. 2010). The primary difference in calcium provision between oviparous and viviparous species is the timing of uterine secretion and it is likely that most of the specializations of the extraembryonic membranes of oviparous species to recover calcium from yolk and mobilize calcium from the eggshell are conserved in viviparous descendents.

We predict that the evolution of calcium placentotrophy is influenced by three characteristics of

squamate reproduction and development: (1) uterine calcium secretion is stimulated by the presence of eggs in the uterus; (2) structure and biochemistry of eggshell proteins influence the properties of eggshell calcium; and (3) embryonic uptake of calcium is driven by embryonic metabolism. Prolonged uterine egg retention would promote continued calcium secretion by positive feedback stimuli to uterine tissues, thus co-opting a mechanism that is a proto-adaptation for placental calcium secretion. Layers of eggshell proteins may be structurally and functionally diversified, as they are in birds (Hernandez-Hernandez et al. 2008). Reduction in eggshell thickness in squamates with prolonged egg retention may eliminate the proteins that promote calcium carbonate crystal formation, transforming the shell membrane into a more porous system for calcium diffusion. If uptake of calcium by extraembryonic tissues is promoted through chemical signals related to embryonic growth and metabolism, the mechanism of calcium uptake by embryos would be independent of reproductive mode but the source of calcium would depend on maternal provisioning. The evolution of viviparity and calcium placentotrophy would be concurrent, as predicted by Blackburn (2006), if yolk calcium reserves are not sufficient to sustain embryonic growth as provided by organic components in yolk. Under these conditions, embryos would take up calcium from placental secretion to supplement yolk calcium. However, if yolk contains all the calcium required to sustain growth, the evolution of viviparity would precede the evolution of calcium placentotrophy (as modeled by Packard et al. 1977). Given the diversity of squamate patterns of embryonic nutrition and numerous origins of viviparity, both transitions are likely to have occurred.

There is only indirect evidence for any of the mechanisms we have proposed. However, the assumptions of our model are testable and squamate reptiles are uniquely suited to study this evolutionary transition because placental transfer of calcium has evolved independently in numerous lineages, the relative contributions of placental and yolk calcium to embryonic nutrition vary among species, and the structure of the maternal–embryonic relationship is highly conserved resulting in numerous parallel evolutionary trajectories.

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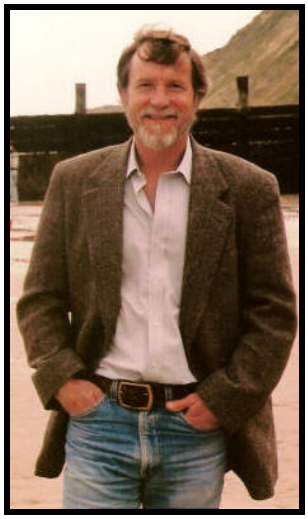

JAMES R. STEWART is a Senior Research Scientist at East Tennessee State University who caught an alligator lizard (*Elgaria coerulea*) in northern California that gave birth in the collecting sac, and he has been trying to understand how that could have happened ever since. Currently his pursuit of this interest involves study of the relationship between reproductive mode (oviparity, viviparity) and pattern of embryonic calcium nutrition of squamate reptiles. Photographer unknown.

TOM W. ECAY has worked on the role of nutrition, including calcium, in the growth of captive bred mice (seventh grade), calcium regulation of mouse oocyte maturation (Boston College – Ph.D.), fluid and ion transport in shark rectal gland and human colon (University of Texas – Houston and East Tennessee State University, postdoctoral and faculty appointments). His current interests include how momma snakes and lizards put calcium into eggs (yolk and eggshell) and how growing embryos get it back out again. He is an Associate Professor in the Department of Physiology at East Tennessee State University. Photographer unknown.