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Does reduction of the eggshell occur concurrently with or subsequent to the evolution of viviparity in phrynosomatid lizards?

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Viviparity and placentation have evolved many times within squamate reptiles, but the sequence in which the attendant morphological modifications occur remains unclear. In particular, it is unknown whether a reduction of the egg shell occurs concurrently with longer periods of egg retention (i.e. increasingly advanced stages of embryogenesis at oviposition) or whether such thinning occurs after viviparity has evolved. To investigate this question, we evaluated the prediction that shell morphology and permeability vary systematically with the capacity to support embryonic development *in utero* (as judged by the maximum embryonic stage attainable *in utero*) in five species of oviparous sceloporine lizards and one lizard species in the sister genus *Urosuarus*. Despite major differences among species in the capacity to support embryogenesis, shell morphology (structure, thickness) and physiology (permeability to water vapour) did not vary as predicted. These results raise the intriguing possibility that other features associated with simple placentation (e.g. increased oviductal and chorioallantoic vascular density) evolve concurrently with longer periods of egg retention and viviparity and that shell thinning may occur subsequent to the evolution of viviparity, at least in sceloporine lizards.

ADDITIONAL KEY WORDS:—egg retention – embryonic development – placentation – reptiles – squamates.

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INTRODUCTION

The sequence of events during the transition from oviparity (egg laying) to viviparity (young fully formed at birth) in squamate reptiles has been an ongoing point of discussion for over half a century (Weekes, 1929, 1935; Panigel, 1951; Tinkle & Gibbons, 1977; Packard, Tracy & Roth, 1977; Shine, 1985; Guillette, 1993; Blackburn, 1995; Qualls, Andrews & Mathies, 1997). Particular attention has focused on the timing of the evolutionary reduction in the eggshell. Such 'thinning' of the eggshell is thought to occur because it would facilitate the exchange of respiratory gases by bringing the chorioallantois of the embryo and the uterine tissues of the female into close apposition, an arrangement termed a simple placenta (reviewed in Blackburn, 1993). Indirect support for this rational comes from the observation that the eggshells of viviparous species are either extremely thin or absent (Blackburn, 1995).

One view holds that shell thinning occurs gradually over evolutionary time, but does not begin until *after* viviparity has evolved (Panigel, 1951; Neill, 1964; Tinkle & Gibbons, 1977; Billet, Gans & Maderson, 1985). The rationale behind this view is that a progressive thinning of the shell prior to viviparity would leave eggs increasingly prone to desiccation while in the nest (Weekes, 1933, 1935; Packard, 1966; Blackburn 1998). The benefits of longer periods of egg retention would therefore tend to be countered by decreasing hatching success. The alternate and most widely accepted view holds that shell thinning begins *prior* to viviparity and occurs *concurrently* with longer durations of egg retention (Packard *et al.*, 1977; Shine, 1985; Guillette, 1991; but see Blackburn, 1998). This view is based primarily on the assumption that retaining fully shelled eggs presents serious problems for embryonic gas exchange and that these problems occur relatively early on during development (Packard *et al.* 1977). Several considerations lend support: First, embryonic oxygen consumption increases dramatically as embryogenesis proceeds, especially during the later developmental stages (Clark 1953; Dmi'el, 1970; Guillette, 1982; Birchard *et al.*, 1984; DeMarco & Guillette, 1992). Second, a thick eggshell would impede gas exchange because diffusion rates are inversely related to the lengths of the diffusion pathways through the shell (Ar *et al.*, 1974). Third, diffusion through the shell would be relatively slow while eggs are in the oviducts because the passages through shells are fluid filled and respiratory gases diffuse slower through liquid than air (Packard *et al.*, 1977; Deeming & Thompson, 1991).

We evaluated these two scenarios for the timing of shell thinning by focusing on the relationship between the structure and physiology of eggshells and the capacity to support uterine embryogenesis among closely related species of lizards. If shell thinning occurs concurrently with longer periods of egg retention, then we can predict that species that oviposit eggs with embryos at the modal stage of embryonic development for squamates should produce eggshells that are typical in morphology and permeability to respiratory gases. In contrast, species that oviposit eggs with

TABLE 1. Embryonic stage at normal oviposition and maximum embryo stage attainable *in utero* for six species of phrynosomatid lizards

	Stage at oviposition				Maximum stage attainable *in utero*	
Taxa	*n*	Mean	± SD	Reference	Maximum	Reference
Sceloporus undulatus hyancinthinus	9	28.6	0.68	Mathies, 1998	30.0	Mathies, 1998
Sceloporus undulatus consobrinus	9	29.2	1.06	Mathies, 1998	31.0	Mathies, 1998
Sceloporus virgatus	12	31.4	1.44	Mathies, unpub. data	37.0	Mathies, unpub. data
Sceloporus scalaris	5	32.8	1.04	Mathies & Andrews, 1995	40.0	Mathies, unpub. data
Sceloporus clarkii	1	29.5	...	This paper	29.5	This paper
Urosaurus ornatus	12	29.5	0.43	Mathies & Andrews, 1999	30.5	Mathies & Andrews, 1999

Note. Stage at oviposition: *n* refers to the number of clutches from which embryos were obtained. Maximum stage attainable *in utero*: sample sizes were variable.

embryos at relatively advanced stages of development should produce eggshells that are relatively permeable and exhibit morphological features associated with increased shell permeability (e.g. thin eggshells).

To test this prediction we characterized eggshell structure and permeability for six species of phrynosomatid lizards. Differences in the capacity to support uterine embryonic development among these species are substantial (Andrews & Rose, 1994; Mathies & Andrews, 1996; Andrews, 1997; this paper), ranging from embryo Stage 30, the modal stage at oviposition for lizards (Shine, 1983; DeMarco, 1993; Blackburn, 1995), to Stage 40, the stage at hatching or parturition. Five of the six species belong to the genus *Sceloporus*, a monophyletic lineage that includes both oviparous and viviparous species (Reeder & Weins, 1996; Méndez-de la Cruz, Villagrán-Santa Cruz & Andrews, 1998). We also include observations for one member the genus *Urosaurus*, the sister genus to *Sceloporus* (Reeder & Wiens, 1996) and a lineage in which oviparity is fixed. These species therefore provide a robust framework for evaluating whether the capacity to support embryonic development is correlated with eggshell structure and permeability.

The species we examined, listed in order of increasing capacity support embryonic development, were *Sceloporus clarkii*, *Sceloporus undulatus hyancinthinus*, *Urosaurus ornatus*, *Sceloporus undulatus consobrinus*, *Sceloporus virgatus*, and *Sceloporus scalaris* (Table 1). The specific prediction we tested was that shell thickness, density, and degree of mineralization would decrease, and shell permeability to water vapour would increase, in this same order.

MATERIAL AND METHODS

Experimental design

The sceloporine species we examined included representatives from three species groups. Members of the *undulatus* species group were *Sceloporus virgatus* Smith, *Sceloporus undulatus hyancinthinus* Green, and *S. undulatus consobrinus* Bosc & Daudin. Because *S. u. consobrinus* is more closely related to *S. virgatus* than it is to *S. u. hyancinthinus* (Wiens & Reeder, 1997), we treat these subspecies of *S. undulatus* as distinct species. Members

of the *scalaris* and *clarkii* species groups included *Sceloporus scalaris* Wiegmann and *Sceloporus clarkii* Baird & Girard, respectively. The species of *Urosaurus* we examined was *Urosaurus ornatus* Baird & Girard.

Gravid females were collected in spring just prior to the time of natural oviposition. Female *S. u. hyancinthinus* were collected on the southern slope of Brush Mountain in Montgomery County, Virginia, between 28 and 31 May 1997. Female *S. virgatus* were collected between 25 and 30 June 1997 in Cochise County, Arizona. Female *S. scalaris* were collected between 25 June and 2 July 1993 at the Appleton-Whittel Research Ranch Sanctuary, Santa Cruz County, Arizona. Female *S. u. consobrinus*, *S. clarkii*, and *U. ornatus* were collected between 25 and 30 June 1997 between Rodeo and Hachita, Hidalgo County, New Mexico. Females collected in Virginia were brought into our animal care facilities at Virginia Polytechnic Institute on the day they were collected. Females collected in Arizona and New Mexico in 1993 and 1997 were housed temporarily in terraria at the Southwestern Research Station in Portal, Arizona, and were transported to Virginia Polytechnic Institute on 4 July and 1 July of those years, respectively.

Eggs used to determine the normal embryonic stage at oviposition were obtained in two ways. Female *S. u. hyancinthinus* and *S. u. consobrinus* were placed into individual terraria and allowed to oviposit naturally. Eggs of *S. virgatus*, *S. scalaris*, and *U. ornatus* were obtained at the time oviposition normally occurs in the field by inducing oviposition with an intraperitoneal injection of oxytocin. Eggs were weighed to 0.1 mg, usually within 1–2 hours of laying. All eggs were stored briefly at room temperature in closed plastic containers containing moistened vermiculite. On the day each clutch was obtained, one egg from each clutch was selected for determination of embryonic stage at oviposition. Embryos were assigned stages following the criteria of Dufaure & Hubert (1961) with the modification that half stages were assigned if the embryos had characteristics intermediate between two developmental stages.

Eggs used to determine the maximum stage of embryonic development that is attained *in utero* for each species were obtained by inducing a second set of females of each species to facultatively retain eggs past the normal time of oviposition. Facultative egg retention was induced by keeping terraria substrates dry (for details see Mathies & Andrews 1996). The maximum stage attainable *in utero* was determined by periodically sampling retained eggs of different females or by examining eggs obtained at the time individual females 'dumped' or attempted to oviposit eggs in the dry terraria substrate. The maximum stage attainable *in utero* for *S. clarkii* is based on one female that was gravid when it was collected on 28 June. This female did not oviposit although she was housed under conditions that facilitated oviposition in a sympatric species (*S. u. consobrinus*). On 25 July, oviposition by this female was therefore induced using oxytocin. Values for the normal embryonic stage at oviposition and the maximum stage attainable *in utero* are presented in Table 1.

Eggs used to determine shell structure and gas permeability were obtained at the normal time of oviposition and eggshells were therefore fully-formed. For the sceloporine species this was made obvious by the presence of a thin cuticle covering the crystalline layer of all eggshells (see Results). Eggshells obtained from another set of *U. ornatus* females that were induced to retain eggs considerably past the normal time of oviposition did not differ from those used herein (Mathies & Andrews, 1999). Eggs used to measure gas permeability were sampled on the day they were obtained and only after the eggshell became opaque. In all species the opaque

appearance becomes complete within about an hour of oviposition which presumably indicates that the water content of the shells has declined to steady-state levels.

Measuring and describing the structure of eggshells

The terminology used to describe eggshells follows Packard & DeMarco (1991). The term 'eggshell' refers to all layers of the shell. The inner boundary refers to the thin, innermost layer of the shell. The shell membrane comprises a relatively thick layer of proteinaceous fibres of variable diameter overlying the inner boundary. A crystalline layer (in most squamates, the crystalline layer is composed of calcium carbonate in the form of calcite: Packard *et al.*, 1982, Packard & DeMarco, 1991), if present, overlies the shell membrane and is generally variable in morphology.

Scanning electron microscopy was used to characterize structural features of eggshells. Observations on eggshells of *S. scalaris* and preparation methods used are from Mathies (1994); additional micrographs of these shells are presented here. Eggshells of the other species were fixed by placing eggs in 3% gluteraldehyde ($\sim$1 h) and then in 70% ethyl alcohol ($\sim$1 h). Each shell was cut into two halves and the half including the embryonic pole was rinsed clean with distilled water and any remaining extraembryonic membranes were carefully dissected away. A strip of shell was cut from the equator region directly adjacent to the embryo. The shell strips were air dried in specially constructed shell holders that minimized curling and shrinkage of the strips. To obtain a radial view of the shell, strips were removed from their shell holder, dipped briefly into liquid nitrogen, and then snapped across their short axes into two pieces. One piece was used for SEM studies directly. The crystalline layer of other piece was removed by placing it in dilute (1N) HCL overnight. Treated pieces were then rinsed and dried again as above.

The thicknesses of untreated and treated shells were measured to 0.1 μm with dial calipers by taking five evenly spaced measurements from the Polaroid prints of each specimen. Means of the five measurements were used to represent the thickness of each shell strip half. Thickness of the shell membrane was the mean thickness of a treated shell strip half. The thickness of the crystalline layer was not measured because this material was too unevenly distributed over the shell membrane. Shell thickness of shells of *S. u. consobrinus* and *S. virgatus* varied considerably because the crystalline material was organized into discrete clumps. This arrangement was made obvious, in part, because the shell strips naturally tended to fracture between the clumps of crystalline material. Thus, for these species mean shell thickness was calculated as the overall mean of the mean minimum shell thickness (mean thickness of five adjacent troughs) and the mean maximum shell thickness (mean thickness of five adjacent peaks).

Shell density was calculated as the mass of the shell (mg) per unit shell membrane volume (cm^3). To determine shell mass, shells were rinsed clean with distilled H_2O, dried at 50 °C for 24 h, and weighed to the nearest 0.01 mg. Shell volume was calculated by multiplying the surface area (cm^2) of the egg times mean shell thickness (cm) measured for an egg from the same clutch. The surface area of eggs was estimated from the relationship $A = 4.835M^{0.662}$ derived from data for avian eggs where M is the mass (g) of the egg (Paganelli, Olszowka & Ar, 1974). This relationship provided a good approximation for surface area as judged by comparison with the relationship between egg surface area and mass for other species of small lizards

(Ackerman, Dmi'el & Ar, 1985). Sufficient data was available to calculate density for all species except *S. scalaris* and *S. clarkii.*

The degree of shell mineralization was calculated as the mass of the crystalline material per unit egg surface area. To determine the mass of the mineral layer, shells were cleaned, dried and weighed as above. The crystalline layer was removed by placing shells in HCL as above, and shells were then rinsed, redried, and reweighed. The mass of the crystalline layer was calculated as the dry mass of the intact shell minus the dry mass of the demineralized shell. Surface area of the egg was calculated as above.

Measuring the water vapour permeability of eggshells

The permeability of eggshells to water vapour was measured for *S. u. hyancinthinus, S. u. consobrinus*, *S. virgatus*, and *U. ornatus.* In birds, the permeability of the shell to water vapour varies monotonically with its permeability to oxygen (Paganelli, Ackerman & Rahn, 1978; Paganelli, 1991). However, this relationship, when used to estimate the oxygen permeability of squamate eggs, yields values that are consistently higher than actual values (Deeming & Thompson, 1991). Nonetheless, we assume that for squamates this relationship is a positive monotonic function and that the ranked differences between oxygen and water permeabilities are identical among species.

Each egg was placed on a thin wire stand in a vented dessicator containing a saturated solution of NaCL. Before placement into the desiccator, eggs were equilibrated to the desiccator temperature by placing them in sealed glass jars (containing moist vermiculite) in the water bath. The temperature of the shell surface, relative humidity, and barometric pressure within the desiccator were measured directly. Temperature at the surface of the shell was measured by placing the egg against 1 mm of the tip of a 30-ga. copper-constantan thermocouple connected to an Omega data logger that recorded temperature every 5 min. Surface temperatures averaged 30.8 (SE = 0.1)°C. Relative humidity within the dessicator was measured every 8 seconds using HOBO-RH (Onset Instruments Corp. Pocasset, MA). Relative humidity was relatively constant during any one measurement period but increased from about 80% to 90% over the course of the study. In preliminary trials eggs lost water rapidly and the change in mass was initially linear and remained linear for at least 1.5 h. These results indicate that the water content of eggshells did not appreciably influence rates of egg water loss. Eggs were weighed initially and then every 15 minutes over a period of 45 minutes (i.e. four weighings per egg). The water vapour pressure on the inner surface of the eggshell was calculated as the saturation vapour pressure at the temperature of the shell's outer surface. The temperature difference at the inner and outer surfaces of eggshells was negligible because shells of the species we examined are relatively thin ($\sim$25 μm, see Results).

To assess the rate of movement of water vapour across eggshells we first calculated the rate of water loss (MH_2O, mg^{-h}) for each egg using linear regression and then corrected these values to a standard barometric pressure of 101.3 kPa. We then adjusted MH_2O for the difference in water vapour pressure across the eggshell (ΔPH_2O, kPa) and the surface area of the egg (cm^2) using an analysis of covariance procedure (see Results). Surface area was calculated as above.

To permit comparison with other studies, we also computed the permeability of eggshells to water vapour (KH_2O) in the traditional manner using the equation

$$KH_2O = GH_2O/\text{egg surface area}$$

where $GH_2O = MH_2O/\Delta\ PH_2O$. The units and formula for egg surface area are the same as those above.

Statistical analyses

Analyses were conducted using the statistical packages Stat View 5.0.1 (SAS, 1998) and SuperANOVA, v1.11, (Abacus Concepts, 1991). Sample sizes for each variable measured are based on one egg per clutch. Differences among species in features of eggshells were evaluated using one-factor ANOVA. Differences among species in MH_2O were evaluated using one-factor ANCOVA with $\Delta\ PH_2O$ and egg surface area as the covariates. A homogeneity of slopes test was conducted and interaction terms, when nonsignificant, were sequentially dropped from the model (interaction terms with the largest *P*-values dropped first) and the model recalculated each time. Differences among means or least squares means were evaluated using Scheffe's test and *t*-tests, respectively. Means or least squares means are given ± 1 SD. All analyses were tested for statistical significance at the $P<0.05$ level.

RESULTS

Structure of eggshells

The general morphology of shells was similar to that of flexible-shelled eggs of other lizard species (Packard *et al.*, 1982; Schleich & Kästle, 1988; Packard & DeMarco, 1991) except for a thin 'cuticle' on the shells of the sceloporine species. As far as we know, a cuticle has not been reported for the eggshell of any squamate reptile.

The cuticle

The outer surface of eggshells of all sceloporine species was completely covered by a thin, dense, amorphous layer ($\sim 1.8\ \mu m$ thick) of an unknown organic material which we will refer to as the cuticle. This material was not visibly altered by exposure to hydrochloric acid other than its collapse down onto the shell membrane (Fig. 1C,F, 2C,F, 3F). The cuticle was most apparent on shells of *S. u. hyancinthinus*, *S. clarkii*, and *S. scalaris* where the outlines of the underlying blocks and spheroids of crystalline material were plainly visible beneath the cuticle (Figs 1A, D, 3A). The cuticle on shells of *S. u. consobrinus* and *S. virgatus*) was most easily discerned in radial views of these shells (Fig. 2B,E). In all species, there were no visible pathways or spaces through the cuticle. Eggshells of *U. ornatus* did not have a cuticle.

The crystalline layer

The structure and arrangement of crystalline material varied considerably among species and except for *S. u. consobrinus* and *S. virgatus*, it completely covered the entire

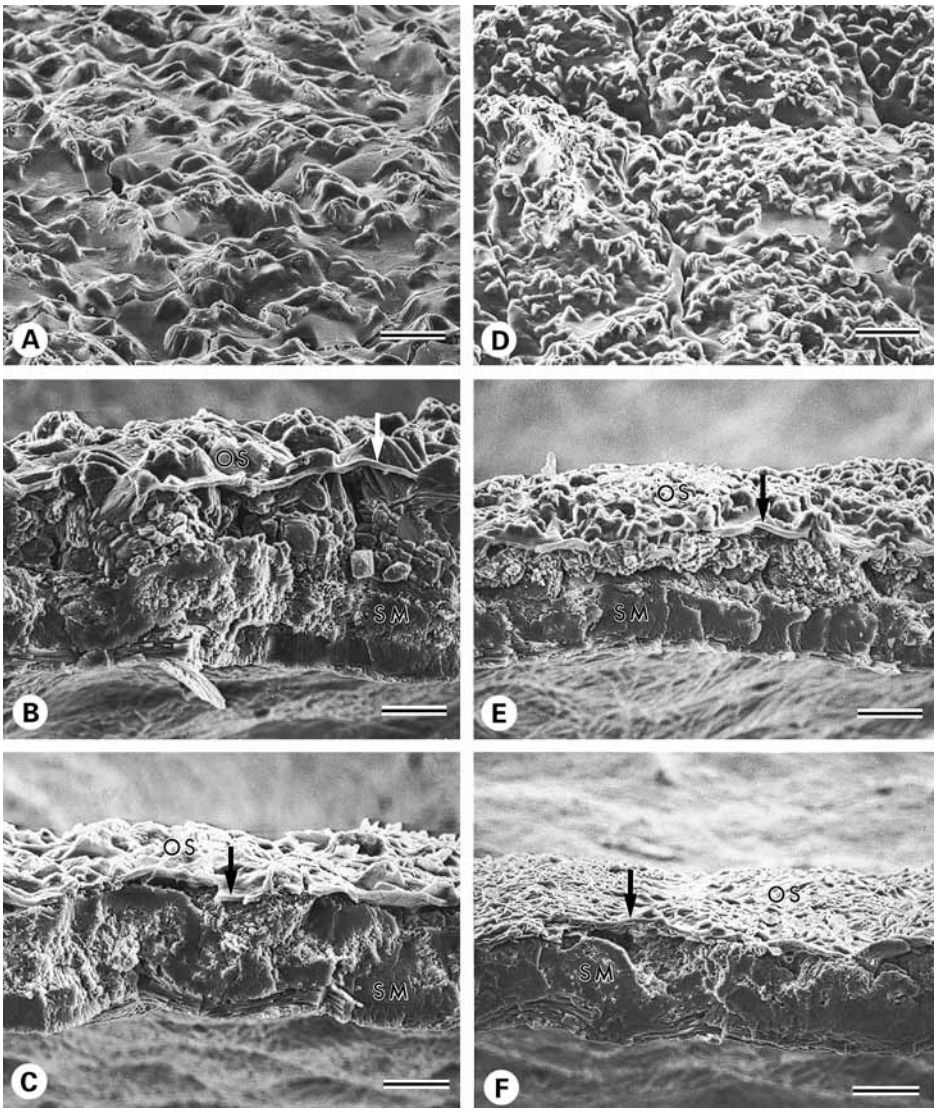

Figure 1. Scanning electron micrographs of eggshells of *Sceloporus undulatus hyancinthinus* (A–C) and *Sceloporus clarkii* (D–F). *S. u. hyancinthinus* and *S. clarkii* can support embryogenesis *in utero* up to embryo Stages 30 and 29.5, respectively. A, outer surface of eggshell showing the continuous coverage by the cuticle and underlying blocks of crystalline material. Cracks in the cuticle are presumably an artifact of shell preparation. B, radial section of the eggshell, with outer surface to the top of the picture, showing the cuticle (arrow) which overlies a layer of crystalline material, and the shell membrane. Note that the cuticle does not contact the shell membrane. C, radial section of the eggshell with the crystalline material removed. Note that the cuticle (arrow) is still present. Outer surface is towards the top of picture. D, outer surface of eggshell showing the continuous coverage by the cuticle and underlying blocks of crystalline material. E, radial section of the eggshell, with outer surface to the top of picture, showing the cuticle (arrow) which overlies a layer of crystalline material, and the shell membrane. F, radial section of the eggshell with the crystalline material removed. Outer surface is towards the top of picture. Note that the cuticle (arrow) is still present. OS, outer surface; SM, shell membrane. Scale bars = 30 μm.

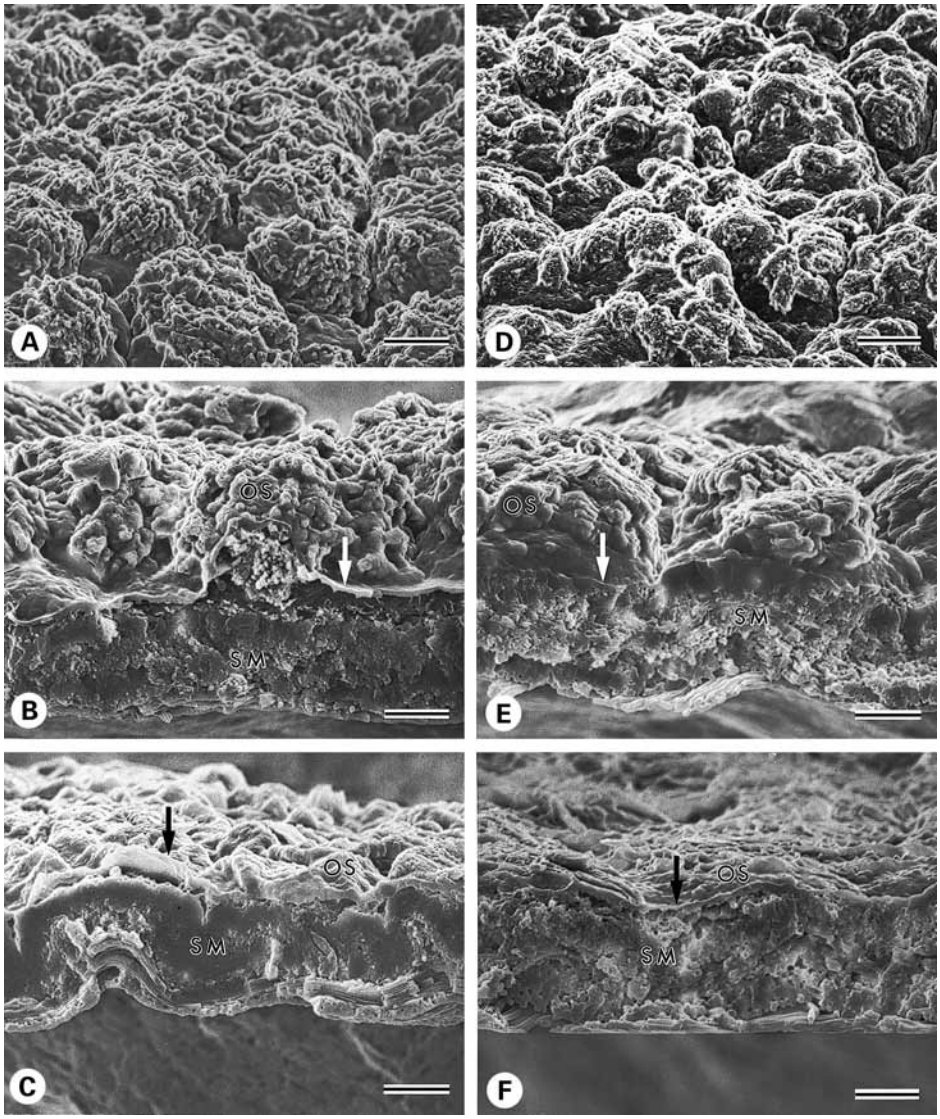

Figure 2. Scanning electron micrographs of eggshells of *Sceloporus undulatus* (A–C) *consobrinus* and *Sceloporus virgatus* (D–F). *S. u. consobrinus* and *S. virgatus* can support embryogenesis *in utero* up to embryo Stages 31 and 37, respectively. A outer surface of eggshell showing crystalline material in the form of nodules that are further organized into discrete clumps. Coverage of the crystalline material by the cuticle is continuous, although this is not obvious from surface views. B, radial section of the eggshell, with outer surface to the top of the picture, showing the cuticle (arrow), clumps of crystalline material, and the shell membrane. Note that the cuticle comes into contact with the shell membrane between the clumps crystalline material. C, radial section of the eggshell with the crystalline material removed. Outer surface is towards the top of picture. Note that the cuticle (arrow) is still present. D, outer surface of eggshell showing the coverage and organization of the crystalline material into clumps. Coverage of the crystalline material by the cuticle is continuous, although this is not obvious from surface views. E, radial section of the eggshell, with outer surface to the top of the picture, showing the clumps of crystalline material and the shell membrane. Only the outer surface of the cuticle is apparent in this view (arrow); its upper and lower edges are not discernible. Note that the cuticle comes into contact with the shell membrane between the clumps crystalline material. F, radial section of the eggshell with the crystalline material removed. Outer surface is towards the top of picture. Note that the cuticle (arrow) remains. OS, outer surface; SM, shell membrane. Scale bars: 30 μm (A, D), 15 μm (B, C, E, F).

underlying shell membrane (Figs 1–3). In all species there were numerous spaces and fissures between the units of crystalline material. The crystalline material on shells of *S. u. hyancinthinus* eggs was in the form of blocks that were irregular in shape and orientation (Fig. 1B). These blocks were distributed liberally and evenly over the shell membrane resulting in a crystalline layer that was considerably thicker than that of any of the other species. The structure and arrangement of crystalline material on shells of *S. clarkii* was very similar to that of *S. u. hyancinthinus* although the layer was much thinner than that of *S. u. hyancinthinus* (Fig. 1E). The structure and arrangement of crystalline material on shells of *S. u. consobrinus* and *S. virgatus* appeared identical (Fig. 2D,E). In both species the crystalline material was organized into nodules that were further organized into discrete clumps that were distributed regularly over the surface of the shell (Fig. 2D,E). Radial sections revealed that there was little or no crystalline material on the shell membrane between these clumps although the membrane in these areas was always covered by the cuticle. The structure of the crystalline material on shells of *S. scalaris* differed from that of all other species. Here, the crystalline material was in the shape of prolate spheroids that were distributed thinly and evenly over the shell membrane (Fig. 3A,B). The crystalline material on shells of *U. ornatus* was organized into irregular plaques which were pitted with holes and spaces (Fig. 3 D,E). However, these holes and spaces appeared to be blind ended and are thus unlikely to function as channels for gas diffusion.

The shell membrane

The arrangement of fibres in the shell membrane was similar in all species (Figs 1–3). The shell membrane was organized into a series of alternating hills and troughs (Packard & DeMarco, 1991) which usually, but not always, reflected the concerted undulations of underlying groups of large diameter fibres. These innermost, large diameter fibres were loosely packed with conspicuous empty spaces among fibres. In contrast, in most species the overlying smaller diameter fibres were tightly woven into a dense mat. Generally, these fibres were packed so tightly that this layer of the membrane appeared amorphous in the SEM (Figs 1C,E,F, 2B,C,E, 3E). The number and packing of the small diameter fibres in *S. scalaris* and *U. ornatus* differed somewhat from that of the shells of the other species. Shells of *S. scalaris* had relatively few small diameter fibres and these were packed fairly loosely. In contrast, shells of *U. ornatus* had many small diameter fibres and these were packed tightly. However, the overall thickness of this layer of the membrane was relatively thin in both species.

The shells of all species had an exceedingly thin inner boundary layer that covered the entire inner surface of the shell (Fig. 3C). The morphology of this layer did not appear to differ among species.

Thickness, density, and degree of calcification of shells

Eggshell thickness differed among species but was not negatively related to the degree of embryonic development as predicted (Table 2; one-way ANOVA: $F_{3,24} = 114.80$, $P<0.0001$). The thickness of eggshells of *S. virgatus*, a species with well developed embryos, did not differ from those of *S. u. consobrinus*, a species with relatively undeveloped embryos. Eggshells *U. ornatus*, a species with embryos even less developed than *S. u. consobrinus*, were thinner than those of any of the other

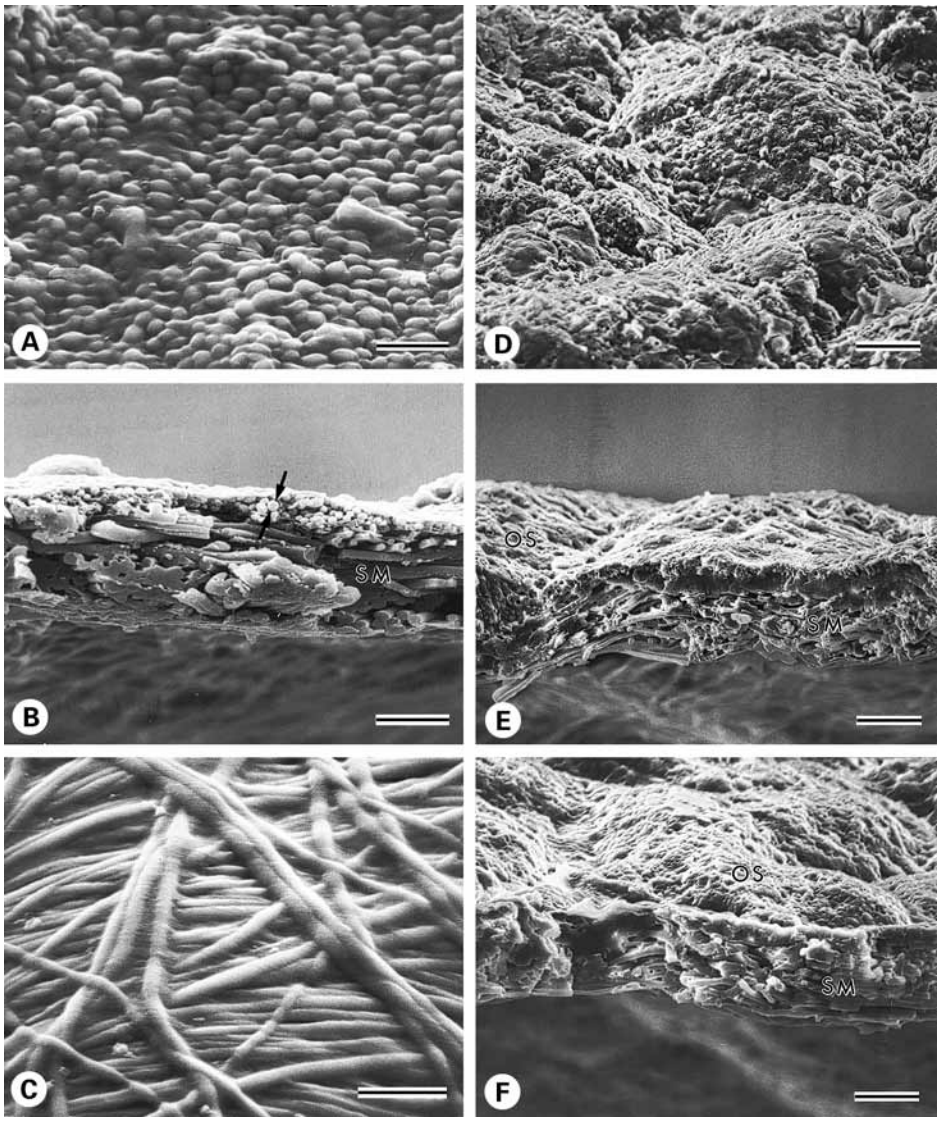

Figure 3. Scanning electron micrographs of eggshells of *Sceloporus scalaris* (A,B), *Sceloporus clarkii* (C), and *Urosaurus ornatus* (D–F). *S. scalaris* and *U. ornatus* can support embryogenesis *in utero* up to embryo Stages 40 and 30.5, respectively. A, outer surface of eggshell showing the continuous coverage by the cuticle and underlying crystalline material in the form of spheroids. B, radial section of the eggshell, with outer surface to the top of the picture, showing crystalline material in the form of spheroids (arrows), and the shell membrane. C, representative view of the inner surface of the inner boundary (*S. clarkii*) showing the characteristic indentations of the overlying fibres. The appearance of the inner boundary was similar among species. D, outer surface of eggshell showing a continuous coverage of crystalline material. A cuticle was not present. E, radial section of the eggshell, with outer surface to the top of the picture, showing the shell membrane and overlying crystalline material. F. Radial section of the eggshell with the crystalline material removed. Outer surface is towards the top of picture. The thin, but densely interwoven mat of small diameter fibres, is most apparent from the view of the outer surface of the shell. OS, outer surface; SM, shell membrane. Scale bars: 8 μm (A), 10 μm (B,C), 15 μm (D, E, F).

TABLE 2. Thickness (μm), mass (mg), density (mg/cm^3), and mineral layer coverage (mg/cm^2) of eggshells of six species of phrynosomatid lizards

Taxa	Shell thickness			Shell mass			Shell density			Mineral layer coverage		
	n	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD
Sceloporus undulatus hyacinthinus	8	86.29	± 11.36 A	8	26.63	±2.59	8	1183.62	± 99.54 A	8	5.57	0.44 A
Sceloporus undulatus consobrinus	7	35.61	± 5.65 B	6	9.48	±0.48	6	1103.69	±172.29 A	6	1.65	0.26 B
Sceloporus virgatus	6	31.92	± 5.10 B,C	6	8.35	±1.97	6	1093.37	±130.03 A	6	1.28	0.37 B
Sceloporus scalaris	8	26.6	...	...	...	...	...	...	...	...	...	...
Sceloporus clarkii	1	57.96	...	...	...	...	...	...	...	...	...	...
Urosaurus ornatus	7	18.94	±5.34 D	7	3.18	±0.47	7	1181.63	±124.35 A	7	0.76	0.24 B,C

Note. All values are means ±1 SD. Within a column, values followed by different letters are significantly different at the 0.05 or higher level (one-factor ANOVAs followed by Scheffe's tests).

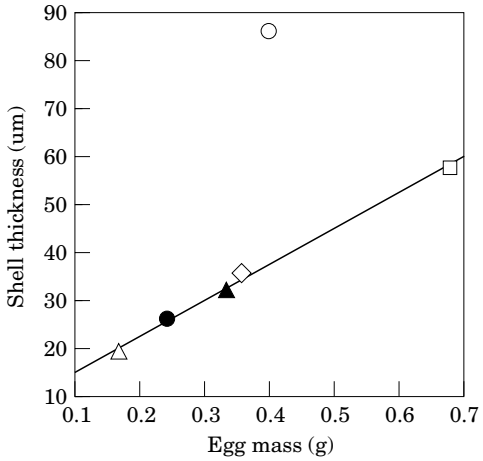

Figure 4. The relationship between mean eggshell thickness and mean egg mass at oviposition for six species of phrynosomatid lizards is described as follows: mean shell thickness = 7.56 + 75.1 (egg mass). The correlation is significant ($R^2 = 0.995$, $P = 0.0001$). (○) *Sceloporus undulatus hyancinthinus* (not included in regression); (□) *Sceloporus clarkii*; (◇) *Sceloporus undulatus consobrinus*; (▲) *Sceloporus virgatus*; (●) *Sceloporus scalaris*; (△) *Urosaurus ornatus*.

species. However, eggshells of *S. u. hyancinthinus*, the species with the least developed embryos, were substantially thicker than those of the other species.

Data was available for a sufficient number of species to permit examination of the relationship between mean shell thickness and mean egg mass using linear regression. When data for all species was included in the analysis, shell thickness was not related to egg mass ($F_{1,4} = 2.34$, $P = 0.20$). It was apparent, however, from visual inspection of the data that shells of *S. u. hyancinthinus* were considerably thicker than those of the other species (Fig. 4) and when this observation was dropped from the analysis, shell thickness was strongly related to egg mass ($F_{1,3} = 605.02$, $R^2 = 0.995$, $P = 0.0001$). Thus, nearly all of the variation in shell thickness among species from the western U.S. was explained by variation in egg mass. It is particularly notable that shells of *S. scalaris*, a species that can retain eggs nearly to hatching, are no thinner than expected given the mass of its eggs.

Shell density was not negatively related to the degree of embryonic development as predicted. To the contrary, shell density was remarkably similar among species (Table 2: one-way ANOVA: $F_{3,23} = 0.92$, $P = 0.44$).

The degree of calcification of the eggshell differed among species but not in the predicted order (Table 2: one-way ANOVA: $F_{3,23} = 309.48$, $P < 0.0001$). The mean mass of crystalline material/cm^2 on shells of *S. virgatus* did not differ from that of either *S. u. consobrinus* or *U. ornatus*. The mass of crystalline material on shells of *S. u. hyancinthinus*, however, was substantially greater than that of any of the other species.

Water vapour permeability of eggshells

There were no significant interactions between the covariates (ΔPH_2O and egg surface area) and factor (species) and these terms (P's > 0.05) were therefore dropped

TABLE 3. Egg surface area (cm^2), MH_2O (mg min^{-1}), and KH_2O (mg min^{-1} kPa^{-1} cm^{-2}) of eggshells of four species of phrynosomatid lizards

Taxa	Number of eggs	Surface area		MH_2O		KH_2O	
		Mean	SD	Mean	SD	Mean	SD
Sceloporus undulatus hyancinthinus	9	2.84	±0.12	0.25	±0.01 A	0.12	±0.04
Sceloporus undulatus consobrinus	9	2.49	±0.26	0.29	±0.01 B,C	0.17	±0.01
Sceloporus virgatus	6	2.62	±0.26	0.27	±0.01 A,B	0.14	±0.00
Urosaurus ornatus	6	1.56	±0.15	0.33	±0.02 C	0.18	±0.01

Note. Values for surface area and KH_2O are means ±1 SD. Values for MH_2O are least-square means ±1 SD. Within a column, values followed by different letters are significantly different at the 0.05 or higher level (one-factor ANCOVA followed by *t*-tests).

from the following analysis. MH_2O differed among species (one-factor ANCOVA; intercept test, $F=3.90$, df = 3,24, $P=0.02$; ΔPH_2O, $F=20.71$, df = 1, 24, $P=0.0001$; egg surface area, $F=45.32$, df = 1,24, $P=0.0001$, but again, not in the order predicted (Table 3). The MH_2O of eggs of *S. virgatus* did not differ from that of either *S. u. consobrinus* or *S. u. hyancinthinus*. Eggs of *U. ornatus* exhibited the highest mean MH_2O which differed significantly from that of *S. virgatus* and *S. u. hyancinthinus*. Values for eggshell permeability (KH_2O) varied in the same order as those of MH_2O (Table 3).

DISCUSSION

Eggshell structure, permeability, and the capacity to support embryonic development

Our prediction that structural features of eggshells associated with increased gas permeability as well as permeability per se, would vary systematically with the capacity to support embryonic development was not upheld. First, although there were substantial interspecific differences in the structure of shells, no features varied consistently in a way that would account for the observed differences in capacity to support embryonic development. For example, *S. u. consobrinus* and *S. virgatus* produce eggshells that are morphologically indistinguishable, yet embryogenesis proceeds much further in *S. virgatus* than *S. u. consobrinus*. Both *U. ornatus* and *S. scalaris* produce an eggshell consisting primarily of loosely packed large-diameter fibres overlaid by thin outer crystalline layer, yet development is arrested at an early developmental stage in *U. ornatus* whereas development proceeds almost to the stage at hatching in *S. scalaris*. Second, although mean eggshell thickness differed among species, it was not associated with the capacity to support embryogenesis. Those species that exhibit the greatest amount of embryonic development *in utero* (i.e. *S. scalaris* and *S. virgatus*) do not produce shells that are any thinner than expected on the basis of egg mass (Fig. 4). The strong relationship between shell thickness and egg mass suggests that interspecific differences in shell thickness are primarily the result of structural considerations, as is the case for eggs of avian species (Ar *et al.*, 1974). These results also illustrate the need to consider the possible allometry between shell thickness

and egg size when making inter and intraspecific comparisons of shell thickness. Lastly, adjusted MH_2O did not vary with embryo stage as predicted. Thus, the capacity to support uterine embryogenesis among species was not related in any systematic way to features of eggshells.

Our findings therefore support the view that thinning of the eggshell occurs after viviparity has evolved. Additional support for this view comes from studies on the skink *Saiphos equalis* where early reports indicated that females at some localities produce eggs that hatch within a few days of laying (Bustard, 1964; Greer, 1989), a condition that has recently been confirmed. Some forms of this species produce "relatively [to 'viviparous form'] thick-shelled calcareous eggs . . ." that hatch in less than one to nine days of oviposition (Smith & Shine, 1997). Thus, females in some populations are able to support embryonic development essentially to term within fully-shelled eggs within the oviducts. On the other hand, the alternate view that thinning occurs concurrently with egg retention is also supported. In three distinct reproductive forms ('normal' oviparous, intermediate, viviparous) of another Australian skink, *Lerista bougainvillii*, the thickness of the eggshell is negatively related to the degree of embryonic development at oviposition (Qualls, 1996). A similar relationship was observed in two oviparous forms of the lizard *Sceloporus scalaris* (Mathies & Andrews, 1995), a species with close viviparous relatives. The observation that all viviparous species examined to date produce shells that are extremely thin or absent has also been used to support this view (Blackburn, 1995), but this conclusion is weakened by the likely possibility that if thinning occurs after viviparity, the reduction would occur relatively rapidly on an evolutionary time scale. The limited information available to date therefore indicates that both patterns of shell thinning occur. The circumstances that would favor one route to viviparity over the other are unknown; this problem will be resolved only when more species that exhibit transitional reproductive features have been investigated.

Evolution of simple placentation

Because the oxygen demands of the embryos increase substantially during development, it has often been suggested, but never demonstrated, that shell thinning is necessary for viviparity to evolve (Packard *et al.*, 1977; Guillette, 1993; Blackburn, 1998). Although we agree that embryonic development in the oviducts of oviparous species probably becomes limited by poor gas exchange (Andrews & Mathies, 2000) and that modifications for alleviating this problem are prerequisite to evolving viviparity, the data presented here indicate, albeit indirectly, the presence of an alternate mechanism(s). The most plausible explanation is that gas exchange is mediated by one or both of the components that form the placenta of viviparous species, the oviduct and the extraembryonic membranes. Both structures are thought to play a major role in gas exchange (Guillette & Jones, 1985; Yaron, 1985; Masson & Guillette, 1987; Blackburn, 1993; Stewart & Thompson, 1993; Blackburn, 1998) and are present in all oviparous species. In support, in two closely related members of the *scalaris* species group, the vascular density of the oviduct is higher in the viviparous species. *Sceloporus bicanthalis*, than in the oviparous species, *Sceloporus aeneus* (Guillette & Jones, 1985). In addition, the chorioallantois of *S. scalaris* embryos covers a greater percentage of the inner surface area of the shell than that of *S. virgatus* embryos at similar stages of embryonic development, and development is less

retarded in *S. scalaris* than *S. virgatus* during extended egg retention. (Andrews, 1997). Thus, an incipient form of placentation may evolve concurrently with increases in the duration of egg retention through increases in the vascularity of the oviduct and chorioallantois, but without a thinning of the eggshell as generally envisioned. This morphological arrangement, while not considered a placenta *sensu stricto*, would certainly meet a functional definition for this structure (Mossman, 1937).

Blood properties could also contribute to the variation in the capacity to support embryonic development we observed. The fetal blood of various viviparous species has a higher oxygen affinity than maternal blood (Grigg & Harlow, 1981; Birchard *et al.*, 1984; Holland *et al.*, 1990; Ragsdale & Ingermann, 1991), but it is unknown whether the specific mechanisms involved were also present in their oviparous ancestors (Blackburn, 1993).

Comparative studies on the relationship between the capacity to support uterine embryonic development, oviductal and chorioallantoic vascularity, and functional properties of maternal and embryonic blood are needed to elucidate the proximate mechanisms that enable continued embryonic development within the oviducts. Such studies on closely related oviparous forms that exhibit similar shell structure, but differ in the capacity to support embryonic development (e.g. *S. virgatus* and *S. u. consobrinus*) could be particularly insightful.

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