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Development of the Uterine Shell Glands During the Preovulatory and Early Gestation Periods in Oviparous and Viviparous *Lacerta vivipara*

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ABSTRACT The evolutionary process leading to the emergence of viviparity in Squamata consists of lengthening the period of egg retention in utero coupled with marked reduction in the thickness of the eggshell. We used light microscopy and scanning electron microscopy to study uterine structure during the reproductive cycle of oviparous and viviparous females of the reproductively bimodal *Lacerta vivipara*. We compared the structure of the uterine shell glands, which secrete components of the eggshell, during preovulatory and early gestation phases of the reproductive cycle and also compared histochemistry of the eggshells. The uterine glands of both reproductive forms undergo considerable growth within a period of a few weeks during folliculogenesis and vitellogenesis preceding ovulation. The majority of the proteinaceous fibers of the shell membrane are secreted early in embryonic development and the uterine glands regress shortly thereafter. This supports previous observations indicating that, in Squamata, secretion of the shell membrane occurs very rapidly after ovulation. The most striking differences between reproductive modes were larger uterine glands at late vitellogenesis in oviparous females, 101 μm compared to 60 μm in viviparous females, and greater thickness of the shell membrane during early gestation in oviparous females (52–73 μm) compared to viviparous females (4–8 μm). Our intraspecific comparison supports the conclusions of previous studies that, prior to ovulation, the uterine glandular layer is less developed in viviparous than in oviparous species, and that this is the main factor accounting for differences in the thickness of the shell membrane of the two reproductive forms of squamates. J. Morphol. 266:80–93, 2005.

KEY WORDS: oviparity; viviparity; eggshell; uterus; shell glands

Evolutionary shifts from oviparity to viviparity have occurred more than 100 times in lineages of lizards and snakes (Blackburn, 1982, 1985, 1999; Shine, 1985). Whereas viviparous species give birth to live young, oviparous squamates are unusual among Reptilia in that most retain their eggs in utero for more than one-third of the embryonic developmental period prior to oviposition (Shine, 1983; Blackburn, 1995; Andrews and Matthies, 2000). Thus, intra-uterine egg retention is a common feature of squamate reproductive biology and viviparity evolves as an extension of oviparous egg retention. The eggs of most oviparous squamates are enclosed in a parchment-like eggshell that is mainly composed of a thick layer of proteinaceous fibers, the shell membrane, overlain by a thin calcified crust (Packard et al., 1982; Packard and Hirsch, 1986; Schleich and Kastle, 1988). Although some viviparous species of squamates have a shell membrane enveloping the embryo throughout development, this structure is not overlain by calcium carbonate deposits and is always much thinner than the shell membrane of oviparous species (Jacobi, 1936; Hoffman, 1970; Guillette and Jones, 1985; Stewart, 1985, 1990; Heulin, 1990; Guillette, 1993; Blackburn, 1993; Qualls, 1996). Hence, the evolutionary process leading to the emergence of viviparity in Squamata consists of a lengthening of the interval of egg retention in utero in conjunction with marked reduction in the thickness of the eggshell. Two nonexclusive evolutionary scenarios have been proposed for the correlation between thinner eggshells and prolonged intrauterine egg retention. Selection may favor thinner eggshells in egg-retaining species because they facilitate maternal–fetal gas exchange (Packard et al., 1977; Shine and Bull, 1979; Xavier and Gavaud, 1986; Qualls, 1996; Andrews and Mathies, 2000), and second, thin eggshells could influence the evolution of viviparity by enhancing the diffusion of chemical signals from the embryo to

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the mother, allowing maternal recognition of pregnancy and delaying the time of oviposition (Guillette, 1991, 1993).

Whatever the selective advantage, one of the critical steps in the evolution of viviparity is reduction in thickness of the eggshell, and thus, understanding the timing and regulation of eggshell secretion is critical to developing a model for the sequence of events in the transition from oviparity to viviparity. The proteinaceous fibers of the eggshell of oviparous species are secreted by uterine glands and deposited around the egg after ovulation (Palmer and Guillette, 1991; Packard and Demarco, 1991; Guillette, 1992; Palmer et al., 1993). Differentiation and growth of the uterine shell glands occurs during the period of follicular growth and vitellogenesis preceding ovulation (Guillette et al., 1989; Guillette, 1993; Perkins and Palmer, 1996; Girling, 2002). Hence, either preovulatory development of the uterine shell glands may be less pronounced in viviparous than in oviparous squamates or the postovulatory secretory activity of these glands may be reduced in viviparous females. A thorough test of these hypotheses requires comparison of closely related oviparous and viviparous taxa in order to minimize the confounding effect of phylogenetic differences. The lizard *Lacerta vivipara*, which is one of three species of squamates that are reproductively bimodal (i.e., with conspecific oviparous and viviparous populations) is an ideal model for such a comparative study.

Viviparous populations of *Lacerta vivipara* are widely distributed from the British Isles and central France into Scandinavia and eastern Russia, whereas two distinct groups of oviparous populations (one in southern France/northern Spain, the other in northern Italy-Slovenia) are restricted to the southern margin of the range (Heulin et al., 1993, 2000; Ghielmi et al., 2001; Surget-Groba et al., 2001). Viviparous females of *L. vivipara* give birth to fully formed offspring (Stage 40 of Dufaure and Hubert, 1961), whereas oviparous females oviposit eggs containing embryos of Stages 30–35 (Brana et al., 1991; Heulin et al., 1991, 2000, 2002). The embryos of viviparous females are lecithotrophic (yolk nutrition) and remain enveloped in a thin shell membrane during the entire gestation period (Panigel, 1956; Heulin, 1990). This viviparous shell membrane is composed of fibers and is much thinner (6–10 μm) than the corresponding fibrous layer (40–65 μm) of the oviparous eggshell (Panigel, 1956; Heulin, 1990; Heulin et al., 2002; Stewart et al., 2004).

The uterine shell glands of both oviparous (*Lacerta agilis*) and viviparous (*L. vivipara*) lacertid lizards undergo seasonal hypertrophy, with the greatest increase in size during folliculogenesis and vitellogenesis (Jacobi, 1936; Panigel, 1956). The histology of the uterine cycle of oviparous *L. vivipara* has not been studied.

The present study was undertaken to compare intraspecific variation in uterine shell glands during the reproductive cycle of oviparous and viviparous populations of *L. vivipara* to determine if previously identified interspecific variation (Jacobi, 1936) is correlated with reproductive mode.

MATERIALS AND METHODS

Animals

Lacerta vivipara (Jacquin) is a small-sized (adults of 45–75 mm snout–vent length) ground-dwelling lacertid, generally living in moist habitats. Detailed information on the reproductive cycle, life-history, and geographic distribution of its oviparous and viviparous populations have been published elsewhere (Heulin et al., 1991, 1997, 2000). The data presented here were obtained from males and females that were caught in September 1998 in the oviparous population of Louvie (43° 06′ N, 0° 23′ W, Alt. 370 m) in southwestern France and in the viviparous populations of Paimpont (48° N, 2° W, Alt. 150 m) in northwestern France. On the first of October the lizards were placed in small boxes containing damp sand and wet mosses that were kept in the dark in a hibernation chamber. The temperature in the hibernation chamber was progressively cooled from 10–4°C during the first week and afterwards maintained constant at 4°C. The lizards were removed from this chamber and placed in terraria after 3 months (males) or 4 months (females) of hibernation. Such hibernation conditions allow normal vitellogenesis of females and normal copulatory activity of males in the month following hibernation (Gavaud, 1983; Heulin, unpubl. obs.). In the present study the females were allowed to copulate with males for 2 or 3 days during the third week following hibernation. The lizards were kept in our laboratory until they copulated (males) or were dissected (females). During the activity period (before and after hibernation) the lizards were reared separately in plastic terraria. Each terrarium (30 × 20 × 20 cm) was equipped with a shelter, dishes of food and water, and a 40W bulb that provided heat for 6 h/day. Under the rearing conditions described above, completion of vitellogenesis and the onset of ovulation occur about 1 month after the end of hibernation (Gavaud, 1983; Heulin, unpubl. obs.). Therefore, in the present study the majority of the females (17 oviparous, 24 viviparous) were dissected 20–30 days after the end of hibernation to observe both fully developed uterine shell glands (in preovulatory vitellogenic females) and the decrease in size of these glands during eggshell formation (in females with recently ovulated eggs). For comparative purposes, we also dissected some females during the 2 days following the removal from hibernation (n = 6 oviparous and n = 6 viviparous) and 45 days after the end of hibernation (n = 2 viviparous and n = 3 viviparous) before the egg-laying of oviparous females. The females were chilled to 3°C for 20 min before decapitation and were dissected in Ringer's solution. Females without oviductal eggs were assigned either to the nonvitellogenic category (NV) (small white-translucent follicles in their ovaries) or to the vitellogenic category (V) (when presenting large yellow follicles). We measured to the nearest 0.1mm the diameters, d (and calculated the corresponding spherical volumes as $\pi d^3/6$) of the 3 or 4 largest follicles. For females with oviductal eggs (category E), we measured the length L and width W (and calculated the corresponding ellipsoidal volume as $\pi LW^2/6$) of each egg. The left oviducts were fixed in Bouin's fluid (24 h), dehydrated in 95° ethanol (3 × 6 h), and stored in butanol until they were processed for light microscopy. The right oviducts were fixed in 10% formalin (24 h) and stored in 75% ethanol. Some of these right oviducts were subsequently prepared for SEM microscopy or embryo staging. For each of the E females, we dissected one egg (fixed in formalin and stored in 75% ethanol) to determine the embryonic stage of development according to the nomenclature of Dufaure and Hubert (1961).

TABLE 1. Biometric characteristics of the wall of the uterus and volume of the ovarian follicles of oviparous and viviparous Lacerta vivipara *during the preovulatory period*

Category	Age (days)	N	Muscle (μm)	Glands (μm)	Epithelium (μm)	Follicle (mm^3)
NV oviparous	1–2	6	22.6 ± 5.3	34.0 ± 6.1	8.1 ± 2.0	2.0 ± 0.3
NV viviparous	1–2	6	21.7 ± 6.2	32.3 ± 5.2	9.9 ± 1.8	2.6 ± 0.8
V1 oviparous	20–30	14	10.3 ± 5.1**	69.4 ± 17.3**	6.8 ± 2.3***	32.9 ± 18.7
V1 viviparous	20–25	11	16.4 ± 5.4	49.6 ± 12.5	9.6 ± 1.4	25.9 ± 14.4
V2 oviparous	22–30	9	8.2 ± 1.6***	100.7 ± 16.7***	6.3 ± 1.3**	93.4 ± 18.7
V2 viviparous	20–25	12	18.8 ± 5.3	63.0 ± 7.6	10.1 ± 2.4	84.7 ± 14.3

Categories of females: NV, nonvitellogenic; V1, vitellogenic with follicles 5–65 mm^3; V2, vitellogenic with follicles 65–125 mm^3.
Age: days after removal from hibernation.
Significant differences at $^{**}P < 0.01$ or $^{***}P < 0.001$ between oviparous and viviparous values, Mann-Whitney *U*-test.

Light Microscopy

Segments from the uterine part of the right oviduct were embedded in paraffin and sectioned at 3–5 μm. For females with oviductal eggs, we examined sections of uterine incubation chambers (containing eggs) and sections between uterine chambers (interembryonic region). The sections were mounted on glass slides and treated with variety of stains: hematoxylin-eosin (general histology), Masson trichrome (general histology and connective tissues), periodic acid-Schiff (PAS, for a variety of carbohydrates), and Alcian blue 8GX at pH 2.5 (for primarily carboxylated acidic mucosubstances) counterstained with nuclear fast red. Additionally, we treated sections with Barnett and Seligman's DDD (dihydroxy-6,6′disulfide-dinapthyl) which stains the disulfide S-S and sulfhydryl S-H groups of proteins (Barnett and Seligman, 1952; Martoja and Martoja, 1967). We used this stain because it was previously shown that the shell membrane of another lacertid lizard (*Lacerta sicula*) is mainly composed of S-H- and S-S-rich protein (Botte, 1973).

Scanning Electron Microscopy (SEM)

We examined by SEM the surface of the luminal epithelium of the left uterus for a subset of nonvitellogenic (six oviparous, five viviparous), vitellogenic (eight oviparous, eight viviparous), and postovulatory (eight oviparous, eight viviparous) females. For postovulatory females, we excised one uterine chamber and carefully separated the uterine tissues and the egg. We dissected this egg for embryo staging and for SEM examination of its shell membrane. For each individual the uterine tissue sample and the shell membrane for females with eggs was cut into several pieces using microsurgery scissors. These pieces were dehydrated in a graded series of ethanol (from 75% to 100%), CO_2 critical point-dried, mounted with double face scotch tape on a brass tub, coated with gold with a JEOL JFC 1000 sputter-coater, and examined with a JEOL JSM 6301F scanning electron microscope.

Morphometrics and Statistics

Measurements were performed on digital photos of histological sections of the uterine wall using the software Image-pro-plus v. 3.0. For each individual we estimated the mean thickness (by averaging 10–20 measures of individual sections separated by at least 30 μm) of the muscular layer, of the glandular layer (the height of a gland is the distance from its base to its luminal extremity), and of the luminal epithelium of the uterus and, for females with eggs, of the shell membrane. In addition, we also estimated the thickness of the shell membrane (average of 10 measures) on SEM digital photos of cross sections. All averages are given ± 1 SD. The values reported in our tables correspond to the average of the averages for each individual (i.e., the sample size is the number of individuals). Because of the small number of individuals in each category, we exclusively used nonparametric statistics (Mann-Whitney *U*-tests) to compare the oviparous and viviparous values. The Minitab 11.11 program was used for all statistics.

RESULTS

Reproductive Status

All of the oviparous and viviparous females autopsied 1 or 2 days after the end of hibernation had small (1.5–1.9 mm diameter) nonvitellogenic follicles (NV). The females autopsied 20–30 days after the end of hibernation either had vitellogenic follicles (V) or recently ovulated eggs (E) containing embryos in early development (segmentation phase, between Stages 1–4 of Dufaure and Hubert, 1961) (Tables 1, 2). We distinguished two groups of vitellogenic females corresponding to two follicular size

TABLE 2. Biometric characteristics of the wall of the uterine incubation chambers, volume of oviductal eggs, and thickness of the shell membrane of postovulatory oviparous and viviparous females

Category	Age (days)	N	Muscle (μm)	Glands (μm)	Epithelium (μm)	Egg volume (mm^3)	Shell-LM (μm)	Shell-SEM (μm)
E1 oviparous	25	1	5.7	100.3	8.4	92.9	<1 μm	<1 μm
E1 viviparous	30	1	6.1	40.8	5.4	113.3	<1μm	<1 μm
E2 oviparous	22–29	4	3.9 ± 0.2*	23.3 ± 4.8	2.9 ± 1.0	110.9 ± 14.9	63.1 ± 8.3*	52.0 ± 6.4*
E2 viviparous	23–29	6	6.9 ± 3.1	24.4 ± 13.0	4.3 ± 2.1	106.7 ± 13.2	6.3 ± 2.6	4.2 ± 2.3
E3 oviparous	45	3	3.0 ± 2.7	8.9 ± 1.1	1.4 ± 0.5	205.0 ± 47.0	72.9 ± 6.7*	59.0 ± 3.6*
E3 viviparous	45	2	3.9 ± 0.9	10.1 ± 3.4	1.7 ± 0.4	174.7 ± 27.5	7.7 ± 0.3	5.5 ± 0.7

Categories of females: E1, with oviductal eggs containing stage 1 embryos of Dufaure and Hubert (1961); E2, with oviductal eggs containing stage 3 or 4 embryos; E3, with oviductal eggs containing embryos of stages 28–31. Thickness of the shell membrane measured from histological sections (Shell-LM) or from scanning electron microscopy pictures (Shell-SEM). Age: days after removal from hibernation. $^{*}P < 0.05$ between oviparous and viviparous values, Mann-Whitney *U*-test.

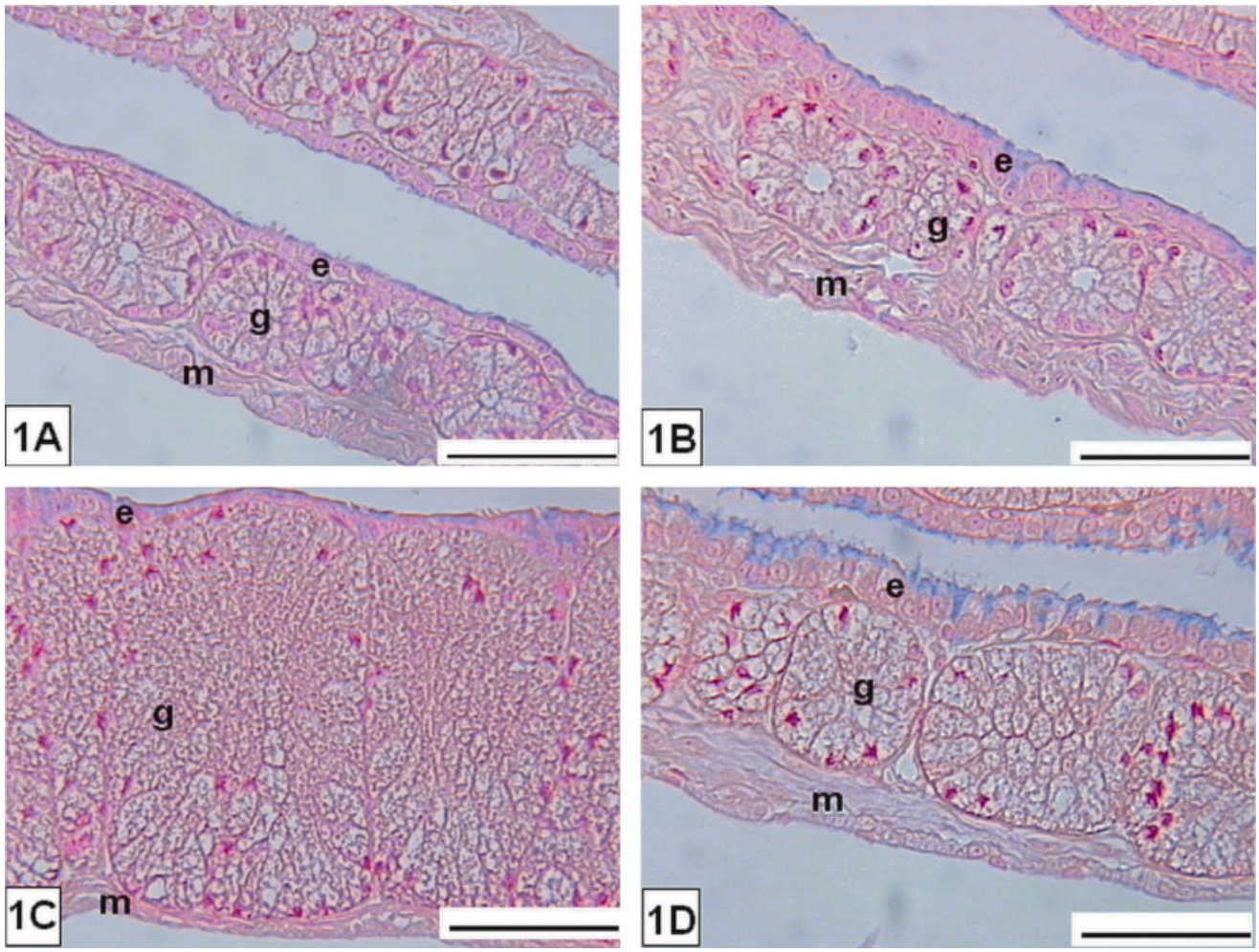

Fig. 1. Histology of the uterine glands in preovulatory *Lacerta vivipara*. Alcian blue + nuclear fast red. **A:** Nonvitellogenic, oviparous. **B:** Nonvitellogenic, viviparous. **C:** Late vitellogenic, oviparous. **D:** Late vitellogenic, viviparous. Scale bars = 50 μm. e, epithelial layer; g, uterine glands; m, muscular layer.

categories: 1) V1 females (early vitellogenesis) with follicles between 5 and 65 mm^3 (i.e., diameter 2.1–4.9 mm), and 2) V2 females (late vitellogenesis) with follicles between 65 and 125 mm^3 (i.e., diameter 5–6.2 mm). Among females with recently ovulated eggs, two (Category E1) had embryos in early segmentation (Stage 1) and an extremely thin shell membrane (less than 1 μm thick), whereas all others (Category E2) had embryos in late segmentation (Stage 3–4) and thicker shell membranes (Table 2). The females dissected 45 days after hibernation (Category E3) had oviductal eggs containing Stage 28–31 embryos and a well-developed shell membrane.

Uterine Shell Glands

The uterine wall of both oviparous and viviparous females is composed of an external muscular layer, an intermediate layer, the lamina propria, containing blood vessels and glands and an inner luminal epithelium (Figs. 1–4). The lamina propria of the uterine wall is composed of a layer of glands interspersed with irregular connective tissue. The uterine glands of oviparous and viviparous females are histologically very similar. At the end of hibernation, the uterine glands of nonvitellogenic females are ovoid, often show an obvious central lumen, and do not stain with PAS, Alcian blue, or DDD (Figs. 1A,B, 4A,B). When the glands are fully developed, by late vitellogenesis, the cytoplasm of the epithelial cells is granular and DDD-positive (Fig. 4C,D) and the lumen is not visible (Fig. 1C,D). Soon after ovulation and eggshell formation, the depleted glands are no longer DDD-positive (Fig. 4E,F), and some have a central lumen (Figs. 2C, 3C,D, 4E). Also following eggshell formation, the glands located between incubation chambers are reduced in size but retain an ovoid shape (Fig. 3E,F), whereas those located in incubation chambers are considerably

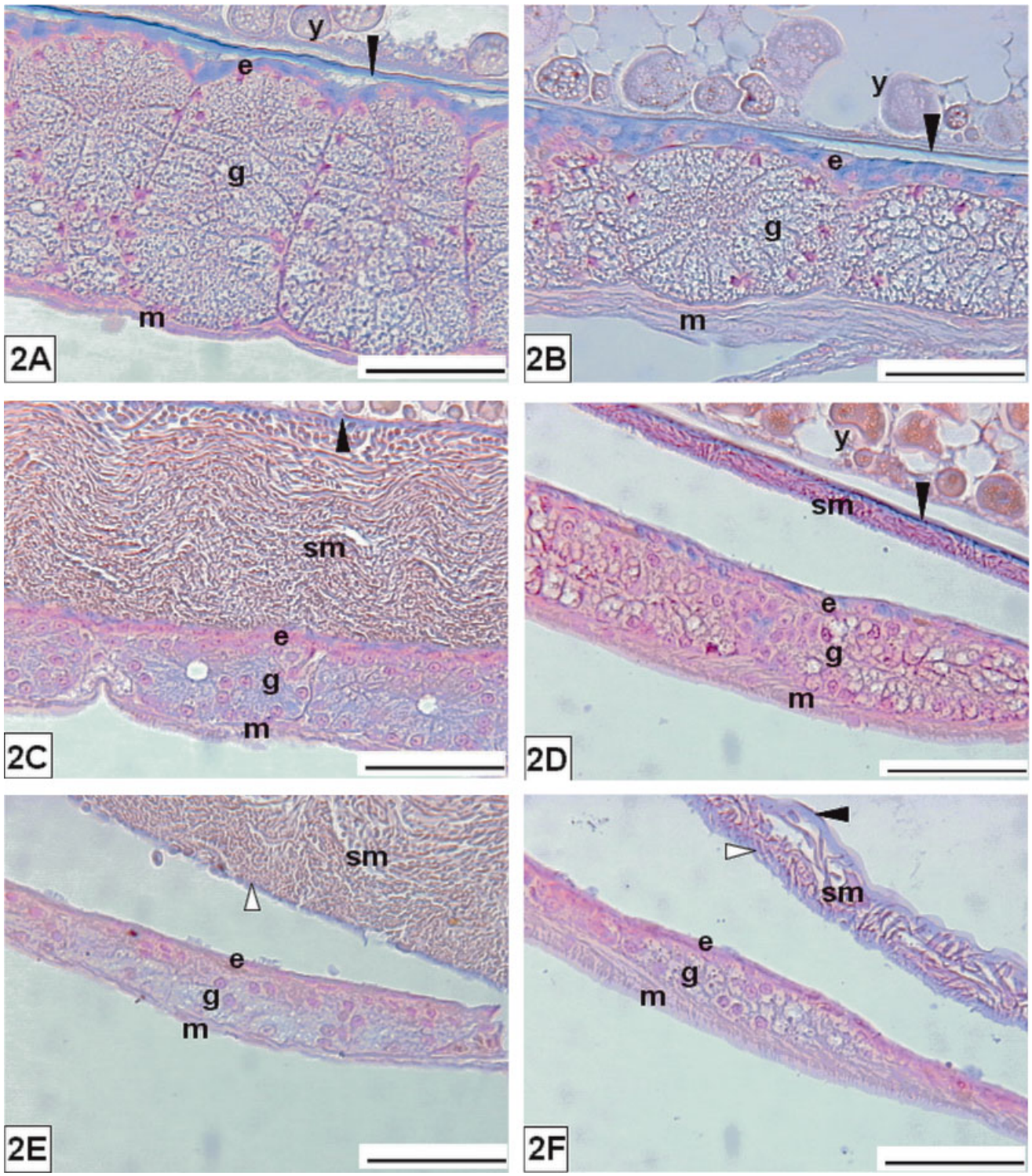

Fig. 2. Histology of the uterine glands in incubation chambers of postovulatory *Lacerta vivipara*. Alcian blue + nuclear fast red. **A:** Oviparous females with Stage 1 embryos (Category E1). **B:** Viviparous females with Stage 1 embryos (Category E1). **C:** Oviparous females with embryos of Stages 3–4 (Category E2). **D:** Viviparous females with embryos of Stages 3–4 (Category E2). **E:** Oviparous females with embryos of Stages 28–31 (Category E3). **F:** Viviparous females with embryos of Stages 28–31 (Category E3). Scale bars = 50 μm. e, epithelial layer; g, uterine glands; m, muscular layer; sm, shell membrane; y, yolk; black arrowhead, inner boundary; white arrowhead, light coating of Alcian blue-positive material on the outer surface of the shell membrane.

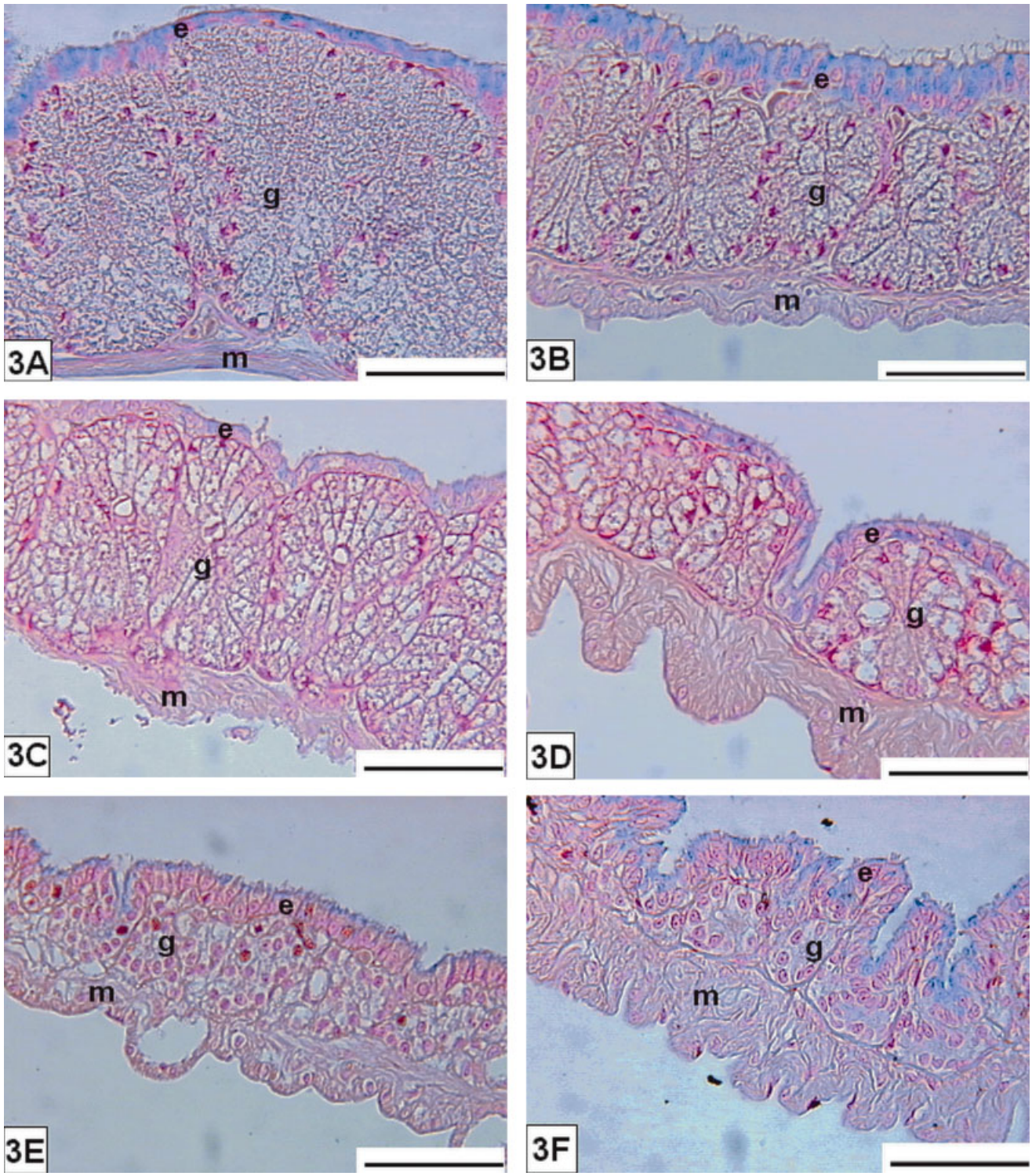

Fig. 3. Histology of the uterine glands between incubation chambers of postovulatory *Lacerta vivipara*. Alcian blue + nuclear fast red. **A:** Oviparous females with embryos of Stage 1 (Category E1). **B:** Viviparous females with embryos of Stage 1 (Category E1). **C:** Oviparous females with embryos of Stages 3–4 (Category E2). **D:** Viviparous females with embryos of Stages 3–4 (Category E2). **E:** Oviparous females with embryos of Stages 28-31 (Category E3). **F:** Viviparous females with embryos of Stages 28–31 (Category E3). Scale bars = 50 μm. e, epithelial layer; g, uterine glands; m, muscular layer.

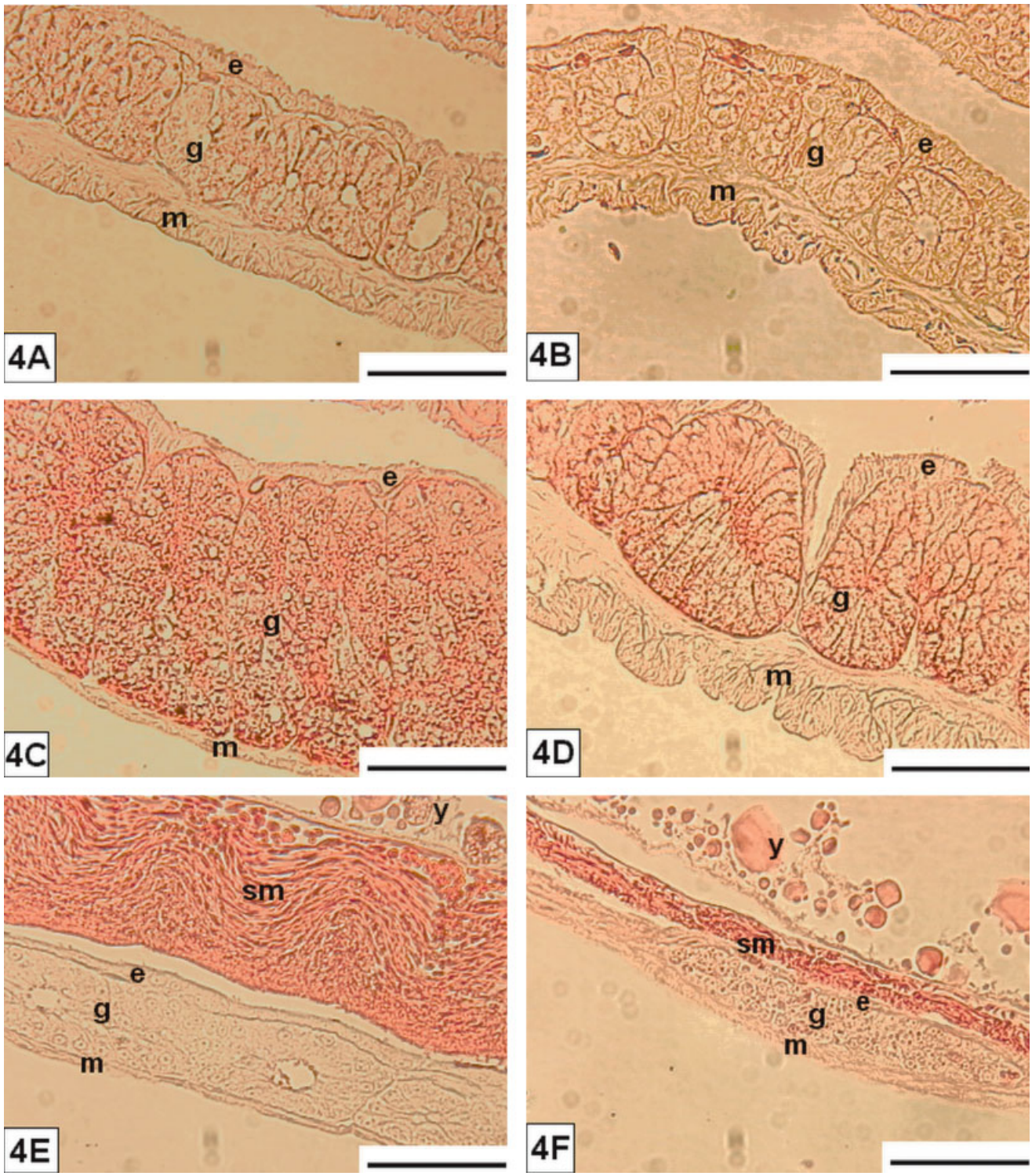

Fig. 4. The uterine glands and eggshell membrane of *Lacerta vivipara*. Dihydroxy-6,6′Disulfide-Dinaphtyl (DDD). **A:** Nonvitellogenic, oviparous. **B:** Nonvitellogenic, viviparous. **C:** Late vitellogenic, oviparous. **D:** Late vitellogenic, viviparous. **E:** Oviparous postovulatory females with embryos of Stage 3–4 (Category E2). **F:** Viviparous postovulatory females with embryos of Stage 3–4 (Category E2). Scale bars = 50 μm. e, epithelial layer; g, uterine glands; m, muscular layer; sm, shell membrane; y, yolk.

stretched and only remain as elongated groups of cells (Fig. 2E,F). In both oviparous and viviparous females, the thickness (height) of the glands increases from the end of hibernation to late vitellogenesis (Table 1, Fig. 1), then decreases after ovulation while the shell membrane is being secreted

TABLE 3. Biometric characteristics of the wall of the uterus between incubation chambers of postovulatory oviparous and viviparous females

Category	N	Muscle (μm)	Glands (μm)	Epithelium (μm)
E1 oviparous	1	8	127.6	7.6
E1 viviparous	1	17.8	68.2	11.5
E2 oviparous	4	13.9 ± 1.9*	60.1 ± 12.5	9.5 ± 2.0
E2 viviparous	6	21.8 ± 5.4	54.0 ± 12.4	10.7 ± 1.7
E3 oviparous	3	21.6 ± 7.1	34.8 ± 10.5	11.1 ± 3.1
E3 viviparous	2	23.9 ± 1.2	29.8 ± 1.9	11.6 ± 0.7

Same categories and same individuals as in Table 2.
*$P < 0.05$ between oviparous and viviparous values, Mann-Whitney *U*-test.

(Table 2, Fig. 2). The postovulatory decrease in the thickness of the glands occurs both in the uterine incubation chambers (containing eggs) and in uterine segments between chambers (with no egg). However, this decrease is much more pronounced in the uterine chambers (Table 2, Fig. 2) than between them (Table 3, Fig. 3). Hence, it is likely that the thinning of the glands observed in the incubation chambers of E3 females (Fig. 2E,F) is both due to the complete depletion of these glands (i.e., after secretion of the shell membrane) and to the stretching of the uterine wall by the developing egg.

We did not find significant differences in the thickness of the uterine glands of oviparous and viviparous females before vitellogenesis. In contrast, the height of the uterine glands is significantly greater in oviparous than in viviparous females during vitellogenesis (Table 1). By late vitellogenesis the uterine glands are very strongly compressed laterally and have a mean height of 101 μm in the oviparous females, whereas they are more oval in cross-section and have a mean height of only 63 μm in the viviparous females (Figs. 1C,D, 4C,D).

Muscular Layer

At the end of hibernation the muscular layer of the uterus is about 22 μm thick, both in oviparous and viviparous females. During vitellogenesis it is significantly thinner in oviparous than in viviparous females (Table 1). After ovulation there is an obvious distention of the muscular layer in the incubation chambers (Fig. 2). The mean thickness of this layer decreases to 3 or 4 μm in the incubation chambers of E3 females, but remains thicker (22–24 μm) between the chambers (Tables 2, 3).

Luminal Epithelial Layer

The luminal epithelium undergoes variation in thickness that parallels the muscular layer. It is 8–10-μm-thick in nonvitellogenic oviparous and viviparous females, significantly thinner in oviparous compared to viviparous females during vitellogenesis, and thins considerably (1–4 μm) in the incubation chambers of all the females during early pregnancy (Tables 1, 2).

The luminal epithelium contains both ciliated and nonciliated cells (Fig. 5). These cells are generally columnar or cuboidal, except in the incubation chambers of the uterus, where they are squamous. The apical part of the nonciliated cells stains with Alcian blue and PAS in all categories of females. However, a more intense staining reaction to Alcian blue and PAS is observed immediately after ovulation (i.e., in the category E1 females, Figs. 2A,B, 3A,B). The nonciliated cells of the luminal epithelium bear low, irregular microvilli, which are relatively rare in nonvitellogenic females but more abundant in vitellogenic females and in females with ovulated eggs (Fig. 5).

Shell Membrane

The shell membrane is composed of a very thin inner boundary layer overlain by fibers (Fig. 6). In E1–E2 females we observed such fibers extruding from ducts opening into the lumen of the uterus (Fig. 5E,F). During shelling the fibers are wrapped in different directions around the egg, onto the inner boundary layer (Fig. 6B).

In females with very recently ovulated eggs (Category E1), there are only a few fibers overlying the inner boundary layer and the thickness of the shell membrane is less than 1 μm (Fig. 6A,B). For females with later-stage embryos (Categories E2, E3), the shell membranes have multiple layers of fibers (Fig. 6C,D) and have a thickness of 4–8 μm in viviparous females and 50–70 μm in oviparous females (Table 2). Values of thickness estimated from measurements performed on Bouin-fixed paraffin-embedded eggs are higher than those obtained from measurements performed on SEM pictures (Table 2). In both estimates (from histological slides or from SEM) the shell membrane of Category E2 and E3 females is significantly thicker in oviparous than in viviparous females (Table 2). The inner boundary membrane is PAS-positive and Alcian blue-positive, whereas the fibrous layer is not (Fig. 2). We also observed a light coating of PAS-positive and Alcian blue-positive material on the external surface of the shell membrane of E3 females (Fig. 2E,F). The fibrous layer of the eggshell stains intensely when treated with DDD, whereas the inner membrane does not (Fig. 4).

DISCUSSION

Uterine Histology

Lacerta vivipara has long been a model for research on the reproductive system of reptiles. This species is featured in some of the earliest accounts of structure and function of the reptilian oviduct (Giacomini, 1893, 1894; Giersberg, 1922; Jacobi, 1936) and more recently in a comprehensive study of in-

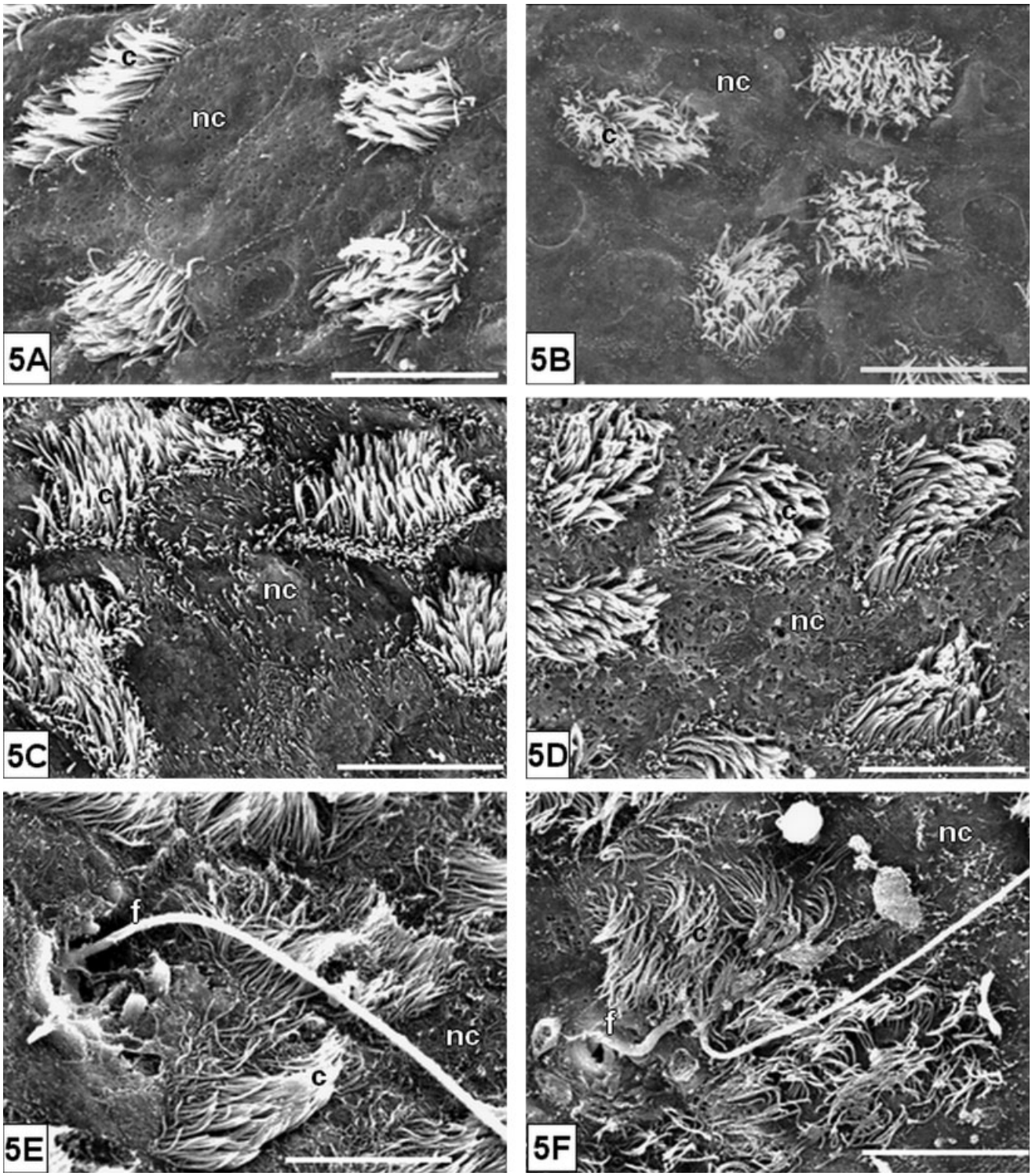

Fig. 5. The surface of the luminal epithelium of the uterus of *Lacerta vivipara*. SEM. **A:** Nonvitellogenic, oviparous. **B:** Nonvitellogenic, viviparous. **C:** Late vitellogenic, oviparous. **D:** Late vitellogenic, viviparous. **E:** Postovulatory, oviparous. **F:** Postovulatory, viviparous. Scale bars = 10 μm. c, ciliated cell; nc, nonciliated cell; f, fiber extruding from duct opening into the lumen of the uterus.

trauterine gestation (Panigel, 1951, 1956). Indeed, one of the most detailed descriptions of the histology of the squamate oviduct is a comparison of *L. agilis*, an oviparous species, and viviparous *L. vivipara* throughout the reproductive cycle (Jacobi, 1936). Jacobi (1936) studied seasonal variation in each of four regions of the oviduct with the aim of discovering characteristics associated with the evolution of viv-

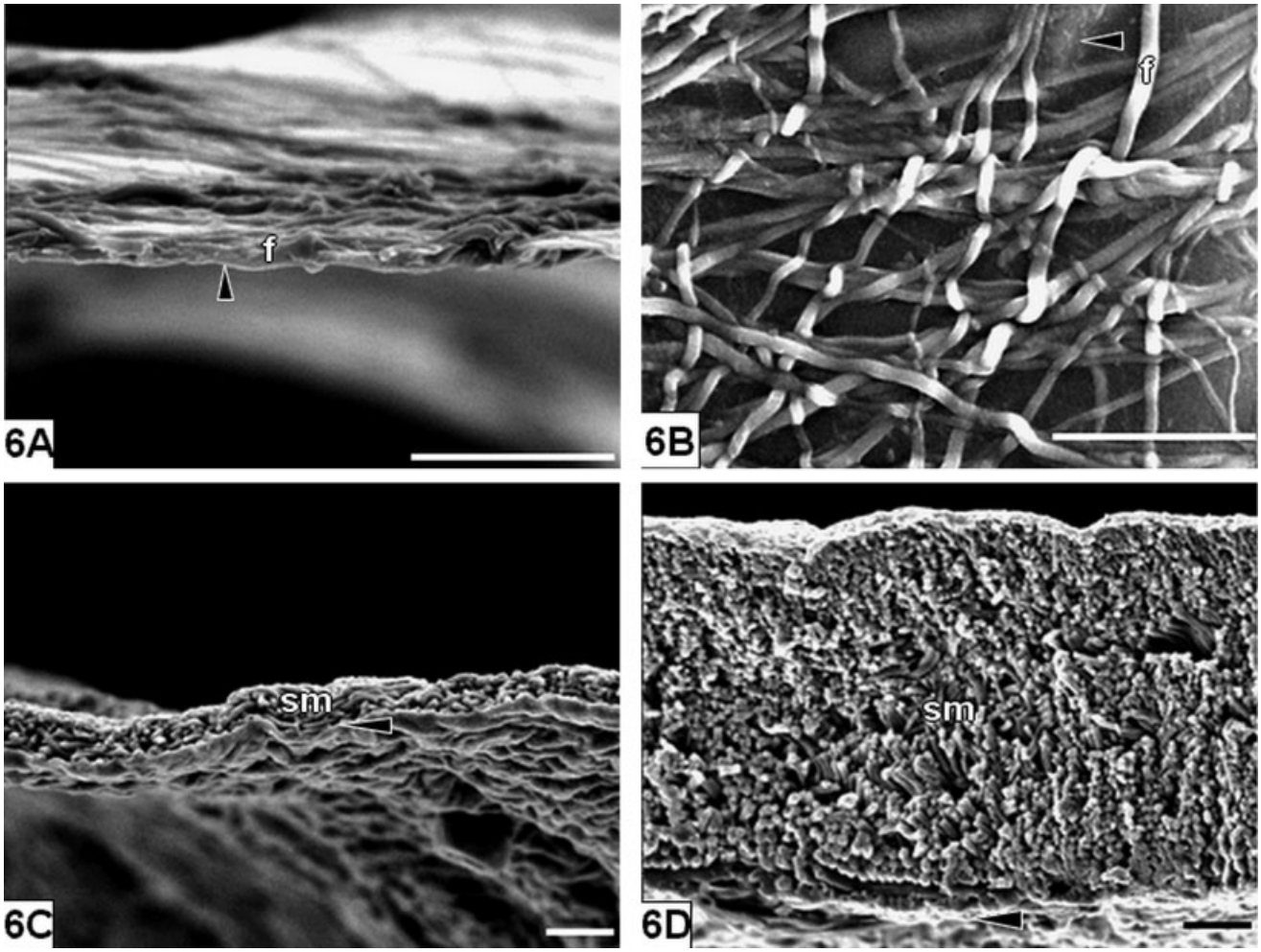

Fig. 6. The shell membrane of *Lacerta vivipara*. SEM. **A:** Cross section showing the inner boundary and a few fibrils during early shelling in an oviparous female with Stage 1 embryos. **B:** Outer surface of the eggshell shown in **A**. **C:** Cross section of the shell membrane of an egg from a viviparous female with Stage 30 embryos. **D:** Cross section of the shell membrane of an egg from an oviparous female with Stage 30 embryos. Scale bars = 10 μm. f, fiber; arrowhead, inner boundary membrane; sm, fibrous shell membrane.

iparity. Our observations on uterine histology for the oviparous population of *L. vivipara* are similar to those on *L. agilis* and likely indicate features common to oviparous *Lacerta*. Tissue layers of the squamate uterus include an inner epithelium facing the uterine lumen, a middle layer, the lamina propria, containing blood vessels, glands, and loose connective tissue, and an outer muscular layer (Blackburn, 1998). The most distinctive feature of the uterus of *L. agilis* and oviparous *L. vivipara* is the presence of glands that occupy the lamina propria. In these oviparous lizards the glands undergo a three-fold increase in height during late stages of vitellogenesis compared to nonvitellogenic females (Jacobi, 1936; Table 1). The increase is so great that adjacent glands develop an extensive area of contact and the height of the glands, i.e., the distance between the muscular layer and the luminal epithelium of the uterus, exceeds the width (Fig. 1C). The size of the glands along the longitudinal axis of the uterus appears to be restricted by their density and the dramatic increase in the size of the glands appears to stretch the uterus and thus influence the width of the muscular and epithelial layers (Jacobi, 1936; Table 1). The uterine epithelium consists of both ciliated and nonciliated cells throughout the reproductive cycle of oviparous *Lacerta*. These cells are columnar or cuboidal in nongravid females but epithelial cells overlying the incubation chambers of gravid females are squamous (Jacobi, 1936; Fig. 2A,C,E). The reduction in height of the epithelium is likely a response to stretching of the wall of the uterus by both the large size of the glands and the size of the egg filling the uterine lumen.

Our observations on seasonal variation in size of the uterine glands of viviparous *Lacerta vivipara* are consistent with those of Jacobi (1936). The height of the glands increases during late vitellogen-

esis but less so than the glands of the oviparous *L. agilis* or oviparous *L. vivipara* (Jacobi, 1936; Table 1). In addition, the glands of viviparous *L. vivipara* are not densely packed in the lamina propria, as occurs in *L. agilis* and oviparous *L. vivipara*. The considerable increase in size of the uterine glands in vitellogenic oviparous females causes distension of the uterus and a reduction in the thickness of the epithelial and muscular layers of the uterine wall. The smaller glands of viviparous females have less of an effect on these layers and as a result both the luminal epithelium and the muscular layer are significantly thicker in viviparous females compared to the situation in oviparous females during vitellogenesis. The size of the uterine glands regresses following eggshell deposition in E3 females (with embryonic Stage 28–31 oviductal eggs) and there is no difference between oviparous and viviparous females in the thickness of the epithelial and muscular layers in interembryonic segments of the uterus (Table 3, Fig. 3E,F). The uterine incubation chambers of gravid viviparous females that we examined were covered by a squamous epithelium (Fig. 2), but we did not observe the extreme thinning of the epithelium or the formation of ridges associated with blood vessels reported by Jacobi (1936). In a study of placental ontogeny, Stewart et al. (2004) found that the uterine epithelium of viviparous *L. vivipara* was thin, but with a smooth surface facing the uterine lumen. The composition of the uterine epithelium of viviparous *L. vivipara* is similar to oviparous *Lacerta* in consisting of both ciliated and nonciliated cells throughout the reproductive cycle (Jacobi, 1936; Fig. 5).

Eggshell Deposition

Under the rearing conditions used in our study the timing of follicular growth, vitellogenesis, ovulation, and early embryonic development (from segmentation to Stage 30) were similar in oviparous and viviparous females. The formation of the eggshell membrane and the staining properties of the eggshell and uterine tissues of oviparous and viviparous females were also similar. Both reproductive forms have a thin inner boundary layer of the eggshell membrane that stains positively with Alcian blue. Positive Alcian blue staining of the inner boundary layer of four other species of lizards has been interpreted to indicate that this membrane is composed of glycosaminoglycans (GAGs) (Guillette et al., 1989; Guillette, 1992; Corso et al., 2000). However, Alcian blue binds to carboxyl and sulfate-ester groups of a wide diversity of substances (polysaccharides, proteoglycans, glycoproteins) (Kiernan, 1981), and there is no good evidence for the GAG specificity of Alcian blue in oviductal tissues (Blackburn, 1998). The origin of the inner boundary layer of the shell has not been determined, but both the oviductal infundibulum (Guillette et al., 1989) and luminal epithelium of the uterus (Corso et al., 2000) have been suggested as possible sources based on positive Alcian blue staining properties of secretory cells in these regions. The uterine shell glands have also been considered as a possible source of the secretion that is incorporated into the inner boundary layer (Cree et al., 1996), but this is unlikely for *Lacerta vivipara* because we never observed a positive reaction to Alcian blue in the uterine shell glands.

In addition to the very thin inner boundary layer, we observed a thicker outer layer of fibers in the shell membranes of both oviparous and viviparous *Lacerta vivipara*. The estimates of the shell membrane thickness from histological slides were always higher than those from SEM pictures (Table 2), indicating that the fibrous layer may be differentially sensitive to the method of preparation of the tissue. Our histological analysis indicates that the fibers contain a DDD-positive material (i.e., S-S- and S-H-rich protein). The uterine shell glands are the most likely source for these fibers because the glandular epithelial cells stain positively with DDD in late vitellogenic females but do not stain with DDD following eggshell formation. The eggshell membrane and preovulatory uterine shell glands of *L. sicula* also stain positively with DDD (Botte, 1973). In addition to histochemical evidence, our SEM analysis is consistent with the observations of Palmer et al. (1993) indicating that, in the lizard *Sceloporus woodi*, the secretory products of the uterine glands coalesce into fibers that are extruded from ducts opening into the lumen of the uterus, and that multiple layers of these fibers are then wrapped around the egg. Palmer et al. (1993) also showed that the majority of the fibers of the shell membrane were deposited within 1 day after oviposition. As we did not determine the ovulation date for each female in the present study, it was not possible to infer the exact duration of the shell membrane deposition from our data. Nevertheless, we observed that deposition of fibers occurred rapidly during early development of the embryo (segmentation phase), whereas there was only a small increase in shell thickness afterwards (between segmentation and embryonic Stage 30) (Table 2).

Although the staining properties of the uterus and eggshell membrane of oviparous and viviparous *Lacerta vivipara* were similar, we found important differences in eggshell thickness and size of the shell glands. Deposition of the eggshell membrane occurred soon after ovulation (during the embryonic segmentation phase) in both reproductive modes but the fibrous layer of the shell membrane was significantly thicker in oviparous females. This is correlated with differences in the preovulatory development of the uterine shell glands in the two groups. The shell glands of oviparous and viviparous females are similar in size and structure at the end of hibernation, and although the glands of both repro-

ductive modes increased considerably in size during vitellogenesis, the preovulatory growth of the uterine shell glands is much more pronounced in oviparous females. After ovulation the uterine shell glands of oviparous and viviparous females are depleted and there is no difference in the thickness of these glands between the two groups. Because the uterine shell glands are the likely source of the fibrous layer of the shell membrane (Botte, 1973; Guillette et al., 1989; Guillette, 1992; Palmer et al., 1993; Figs. 4C–F, 5E,F) and shell membrane deposition occurs in a relatively short period of time, the difference in shell membrane thickness of oviparous and viviparous *L. vivipara* results from differences in preovulatory development of the uterine glandular layer and not because of differences in the length of time the glands are active after ovulation. Secretion of the eggshell membrane commonly occurs shortly after ovulation in both oviparous and viviparous squamates (Hoffman, 1970; Botte, 1973; Ortiz and Morales, 1974; Guillette and Jones, 1985; Guillette et al., 1989; Packard and Demarco, 1991; Guillette, 1992; Palmer et al., 1993; Perkins and Palmer, 1996; Girling et al., 1997). Our intraspecific comparison of *L. vivipara*, as well as previous interspecific comparisons (Guillete and Jones, 1985; Guillete, 1992, 1993; Girling et al., 1998) reveal that preovulatory development of the glandular layer of the uterus is more pronounced in oviparous than in viviparous females and that this accounts for the differences in shell membrane thickness between the two reproductive modes of squamates. Hypertrophy of the glandular area of the uterus during folliculogenesis and vitellogenesis is regulated by ovarian estrogen secretion (reviewed in Girling, 2002) and variation in this regulatory mechanism may be subject to selection during early stages in the evolution of viviparity.

Eggshell Reduction and the Evolution of Viviparity

The eggshell of squamate reptiles consists of several inner layers of organic fibers and an outer inorganic layer composed of calcium carbonate (Packard and Demarco, 1991). The organic portion has a thin inner boundary overlain by multiple layers of fibers. The eggshell of viviparous species is much thinner (less than 10 μm) than that of oviparous species (range 20–500 μm) (Jacobi, 1936; Hoffman, 1970; Guillette and Jones, 1985; Schleich and Kastle, 1988; Heulin, 1990; Guillette, 1991; Qualls, 1996; Mathies and Andrews, 2000). In some viviparous species, including *Lacerta vivipara*, the embryo remains encased in a thin shell membrane throughout pregnancy (Heulin, 1990; Stewart, 1990; Guillette, 1992; Qualls, 1996), whereas in other viviparous species the shell membrane is present only during early embryonic development and is disrupted in later development (Guillette and Jones, 1985; Yaron, 1985; Blackburn, 1993; Stewart and Thompson, 1994; Girling et al., 1997; Corso et al., 2000; Blackburn and Lorenz, 2003). The shell membrane of viviparous species varies in thickness and in composition. In the snakes *Thamnophis sirtalis* and *Virginia striatula* the shell membrane is extremely thin, but consists of distinct layers or zones as seen with TEM (Hoffman, 1970; Stewart and Brasch, 2003). The homolog of this structure in oviparous species is uncertain but it may be derived from the inner boundary layer. The shell membrane of viviparous *L. vivipara* is more complex than that of the two snakes because it consists of an inner boundary layer that stains with Alcian blue and a thin fibrous layer that stains with DDD. This morphology is similar to that of the shell membrane of the scincid lizard *Sphenomorphus fragilis*, and the inner boundary layer in this species also stains with Alcian blue (Guillette, 1992). The organic layer of the eggshell of oviparous *L. vivipara* contains the same two layers as in viviparous forms and these layers have the same staining properties. Thus, the evolution of viviparity in this species is associated with reduction in the thickness of the organic components of the eggshell, not in total loss of one of the major constituents. In contrast, some viviparous natricine snakes have apparently lost the entire outer proteinaceous layer of the eggshell (Hoffman, 1970; Stewart and Brasch, 2003).

In addition to a thicker organic component to the eggshell, oviparous species commonly have a thin outer crust of calcium carbonate overlying the shell membrane (Packard et al., 1982; Packard and Hirsch, 1986; Schleich and Kastle, 1988; Packard and Demarco, 1991; Mathies and Andrews, 2000), whereas an outer layer of calcium does not occur in viviparous eggs. Previous SEM observations (of shell membranes fixed with ethanol at oviposition) revealed the presence of a relatively thin (about 5 μm thick) calcite crust on the external surface of the shell membrane of oviparous *Lacerta vivipara*, whereas no calcium crystals (or very rare traces) were observed on the shell membrane of the viviparous form of this species (Heulin, 1990). In contrast, the SEM observations performed in the present study (on formaldehyde-fixed shell membranes) never revealed the presence of calcium crystals on the shell membranes (or on the uterine luminal epithelium), even for oviparous females close to oviposition (containing Stage 30 embryos). This discrepancy likely indicates that the fixation procedures used in the present study were not appropriate to investigate the secretion and deposition of calcium carbonate because formaldehyde (even neutral buffered) and Bouin's fluid are known to be decalcifying agents (Humason, 1972). Although our methods were inappropriate to verify earlier studies, oviparous females of *L. vivipara* do oviposit eggs with an outer calcareous layer (Heulin, 1990; Heulin et al., 2002), whereas the eggshell membrane of viviparous females is not calcified (Heulin, 1990). Thus, reduction of eggshell thickness in viviparous *L. vivipara* involves complete loss of the inorganic component in addition to reduction in

thickness of the outer organic layers. Reduction in the thickness of the fibrous layer in addition to the calcified layer may contribute importantly to enhanced respiratory exchange during intrauterine gestation because both layers are barriers to oxygen diffusion (Feder et al., 1982).

Reduction in thickness of the eggshell is universally associated with the evolution of viviparity, but neither the mechanism regulating eggshell reduction nor the proximate selective advantages of a thinner eggshell to intrauterine gestation are known. Certainly, the shell membrane is a barrier that alters maternal–fetal exchange. The most commonly proposed model for a selective advantage to reduction in eggshell thickness is that it facilitates maternal–fetal gas exchange during prolonged intrauterine egg retention, particularly during the final embryonic growth phase when oxygen requirements increase dramatically (Packard et al., 1977; Shine and Bull, 1979; Guillette, 1982; Birchard et al., 1984; Xavier and Gavaud, 1986; Qualls, 1996; Andrews and Mathies, 2000). This hypothesis has never been tested empirically. A decrease in eggshell thickness is not the only mechanism available to avoid embryonic hypoxia during a prolonged retention in the uterus (e.g., increased vascularization of the extraembryonic membranes and/or of the oviduct, enhanced oxygen affinity of embryonic blood) (Guillette and Jones, 1985; Masson and Guillette, 1987; Blackburn, 2000; Matthies and Andrews, 2000; Andrews, 2002) and variations that enhance gas exchange may be selected on both maternal and embryonic compartments of the placenta. Reduction in eggshell thickness would also facilitate the exchange of other substances. For example, enhanced exchange of recognition factors between the mother and the embryo could stimulate endocrinological pathways (e.g., stimulation of progesterone secretion by corpora lutea, inhibition of prostaglandin secretion in the oviducts) that would delay oviposition and drive the evolution of viviparity (Guillette, 1991, 1993). Whatever the proximate cause, the length of intra-uterine retention of eggs is correlated inversely with thickness of the eggshell in some lineages of oviparous squamates (Mathies and Andrews, 1995; Qualls, 1996; Heulin et al., 2002).

Thinning of the eggshell is a key event in the evolution of viviparity in Squamata. Our study of oviparous and viviparous *Lacerta vivipara* supports conclusions of interspecific comparisons that preovulatory development of the uterine glands is a critical factor that influences the thickness of the shell membrane. Future studies should focus on the endocrinological factors (in particular, levels of circulating estrogen and uterine estrogen receptors) that are known to strongly influence seasonal development of the uterine glands. There is also a need for comparative studies to clarify the mechanisms that regulate secretion and deposition of the calcified layer of the shell.

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LITERATURE CITED

Andrews RM. 2002. Low oxygen: a constraint on the evolution of viviparity in reptiles. Physiol Biochem Zool 75:145–154.

Andrews RM, Mathies T. 2000. Natural history of reptilian development: constraints on the evolution of viviparity. Bioscience 50:227–238.

Barnett RJ, Seligman AM. 1952. Demonstration of protein bound sulfhydryl and disulfide groups by two new histochemical methods. J Natl Cancer Inst 13:215–216.

Birchard GF, Black CP, Schuett VW, Black V. 1984. Influence of pregnancy on oxygen consumption, heart rate and haematology in the garter snake: implications for the costs of reproduction in live-bearing reptiles. Comp Biochem Physiol 77:519–523.

Blackburn DG. 1982. Evolutionary origins of viviparity in the Reptilia. I. Sauria. Amphib Reptil 3:185–205.

Blackburn DG. 1985. Evolutionary origins of viviparity in the Reptilia. II. Serpentes, Amphisbaesnia and Ichthyosaura. Amphib Reptil 6:259–291.

Blackburn DG. 1993. Chorioallantoic placentation in squamate reptiles: structure, function and evolution. J Exp Zool 266:414–430.

Blackburn DG. 1995. Saltationist and punctuated equilibrium models for the evolution of viviparity and placentation. J Theor Biol 174:199–216.

Blackburn DG. 1998. Structure, function, and evolution of the oviducts of squamate reptiles, with special reference to viviparity and placentation. J Exp Zool 282:560–617.

Blackburn DG. 1999. Are viviparity and egg-guarding evolutionary labile in squamates? Herpetologica 55:556–573.

Blackburn DG. 2000. Reptilian viviparity: past research, future directions, and appropriate models. Comp Biochem Physiol A 12:391–409.

Blackburn DG, Lorenz RL. 2003. Placentation in garter snakes. II. Transmission EM of the chorioallantoic placenta of *Thamnophis radix* and *T. sirtalis*. J Morphol 256:171–186.

Botte V. 1973. Morphology and histochemistry of the oviduct in the lizard *Lacerta sicula*: the annual cycle. Boll Zool 40:305–314.

Brana F, Bea A, Arrayago MJ. 1991. Egg retention in lacertid lizards: relationships with reproductive ecology and the evolution of viviparity. Herpetologica 47:218–226.

Corso G, Delitala GM. 2000. Uterine morphology during the annual cycle in *Chalcides ocellatus tiligugu* (Gmelin) (Squamata: Scincidae). J Morphol 243:153–165.

Cree A, Guillette LJ, Reader K. 1996. Eggshell formation during prolonged gravidity of the tuatara *Sphenodon punctatus*. J Morphol 230:129–144.

Dufaure JP, Hubert J. 1961. Table de développement du lézard vivipare: *Lacerta (Zootoca) vivipara*. Arch Anat Microscop Exp 50:309–328.

Feder ME, Satel SL, Gibbs AG. 1982. Resistance of the shell membrane and the mineral layer to diffusion of water and oxygen in flexible-shelled eggs of the snapping turtle (*Chelydra serpentina*). Respir Physiol 49:279–291.

Gavaud G. 1983. Obligatory hibernation for completion of vitellogenesis in the lizard *Lacerta vivipara*. J Exp Zool 225:397–405.

Ghielmi S, Heulin B, Surget-Groba Y, Guillaume C. 2001. Identification de populations ovipares de *Lacerta (Zootoca) vivipara* en Italie. Bull Soc Herpetol Fr 98:19–29.

Giacomini E. 1893. Sull' ovidutto del sauropsidi. Monit Zool Ital 4:202–265.

Giacomini E. 1894. Sur l'oviducte des sauropsides. Arch Ital Biol 21:147–151.

Giersberg H. 1922. Untersuchungen uber physiologie und histologie des eileiters der reptilien und vogel: nebst einem beitrag zue fasergenese. Zeit Wiss Zool 70:1–97.

Girling JE. 2002. The reptilian oviduct: a review of structure and function and directions for future research. J Exp Zool 293:141–170.

Girling JE, Cree A, Guillette LJ. 1997. Oviductal structure in a viviparous New Zealand gecko *Hoplodactylus maculatus*. J Morphol 234:51–68.

Girling JE, Cree A, Guillette LJ. 1998. Oviducal structure in four species of gekkonid lizard differing in parity mode and egshell structure. Reprod Fertil Dev 10:139–154.

Guillette LJ. 1982. Effects of gravidity on the metabolism of the reproductively bimodal species *Sceloporus aeneus*. J Exp Zool 223:33–36.

Guillette LJ. 1991. The evolution of viviparity in amniote vertebrates: new insights, new questions. J Zool Lond 223:521–526.

Guillette LJ. 1992. Morphology of the reproductive tract in a lizard exhibiting incipient viviparity (*Sphenomorphus fragilis*) and its implication for the evolution of the reptilian placenta. J Morphol 212:163–173.

Guillette LJ. 1993. The evolution of viviparity in lizards. Bioscience 43:742–751.

Guillette LJ, Jones RE. 1985. Ovarian, oviductal and placental morphology of the reproductively bimodal lizard *Sceloporus aeneus*. J Morphol 184:85–98.

Guillette, LJ, Fox SL, Palmer BD. 1989. Oviductal morphology and egg shelling in the oviparous lizards *Crotaphytus collaris* and *Eumeces obsoletus*. J Morphol 20:145–159.

Heulin B. 1990. Etude comparative de la membrane coquillière chez les souches ovipares et vivipares de *Lacerta vivipara*. Can J Zool 68:1015–1019.

Heulin B, Osenegg K, Lebouvier M. 1991. Timing of embryonic development and birth dates in oviparous and viviparous strains of *Lacerta vivipara*: testing the predictions of an evolutionary hypothesis. Acta Oecol 12:517–528.

Heulin B, Guillaume CP, Bea A, Arrayago MJ. 1993. Interprétation biogéographique de la bimodalité de reproduction du lézard *Lacerta vivipara*: un modèle pour l'étude de l'évolution de la viviparité. Biogeographica 69:1–11.

Heulin B, Osenegg K, Michel D. 1997. Demography of a bimodal reproductive species of lizard (*Lacerta vivipara*): survival and density characteristics of oviparous populations. Herpetologica 53:432–444.

Heulin B, Guillaume CP, Vogrin N, Surget-Groba Y, Tadic Z. 2000. Further evidence of the existence of oviparous populations of *Lacerta vivipara* in the NW of the Balkan Peninsula. C R Acad Sci Paris 323:1–8.

Heulin B, Ghielmi S, Vogrin N, Surget-Groba Y, Guillaume CP. 2002. Variation in eggshell characteristics and in intra-uterine egg retention between two oviparous clades of the lizard *Lacerta vivipara*: insight into the oviparity-viviparity continuum in squamates. J Morphol 252:255–262.

Hoffman LH. 1970. Placentation in the garter snake: *Thamnophis sirtalis*. J Morphol 131:57–88.

Humason GI. 1972. Animal tissue techniques. San Francisco: WH Freeman.

Jacobi L. 1936. Ovoviviparie bei einheimischen Eidechsen. Zeit Wiss Zool 148:401–464.

Kiernan JA. 1981. Histological and histochemical methods: theory and practice. Oxford: Pergamon Press.

Martoja R, Martoja M. 1967. Initiation aux techniques de l'histologie animale. Paris: Masson.

Masson G, Guillette LJ. 1987. Changes in the oviducal vascularity during the reproductive cycle of three oviparous lizards. J Reprod Fertil 80:361–371.

Mathies T, Andrews RM. 1995. Thermal and reproductive biology of high and low elevation populations of the lizard *Sceloporus scalaris*: implications for the evolution of viviparity. Oecologia 104:101–111.

Mathies T, Andrews RM. 2000. Does reduction of the eggshell occur concurrently with or subsequent to the evolution of viviparity in phrynosomatid lizards? Biol J Linn Soc 71:719–736.

Ortiz E, Morales MH. 1974. Development and function of the female reproductive tract of the tropical lizard *Anolis pulchellus*. Physiol Zool 47:207–217.

Packard GC, Tracy C, Roth JJ. 1977. The physiological ecology of reptilian eggs and embryos and the evolution of viviparity within the class Reptilia. Biol Rev 52:71–105.

Packard MJ, DeMarco VG. 1991. Eggshell structure and formation in eggs of oviparous reptiles. In: Deeming DC, Ferguson MWJ, editors. Egg incubation: its effects on embryonic development in birds and reptiles. Cambridge, UK: Cambridge University Press. p 53–69.

Packard MJ, Hirsch KF. 1986. Scanning electron microscopy of eggshells of contemporary reptiles. Scan Electron Microsc 198: 1581–1590.

Packard MJ, Packard GC, Boardman TJ. 1982. Structure of eggshells and water relations of reptilian eggs. Herpetologica 38:136–155.

Palmer BD, Guillette LJ. 1991. Oviductal proteins and their influence on embryonic development in birds and reptiles. In: Deeming DC, Ferguson MWJ, editors. Egg incubation: its effects on embryonic development in birds and reptiles. Cambridge, UK: Cambridge University Press. p 29–47.

Palmer BD, Demarco VG, Guillette LJ. 1993. Oviductal morphology and eggshell formation in the lizard *Sceloporus woodi*. J Morphol 217:205–217.

Panigel M. 1951. Rapports anatomo-histologique établis au cours de la gestation entre l'œuf et l'oviducte maternel chez le lézard ovovivipare *Zootoca vivipara* W. (*Lacerta vivipara* J.). Bull Soc Zool Fr 76:163–170.

Panigel M. 1956. Contribution à l'étude de l'ovoviviparité chez les reptiles: gestation et parturition chez le lézard vivipare *Zootoca vivipara*. Ann Sci Nat Zool 18:569–668.

Perkins MJ, Palmer BD. 1996. Histology and functional morphology of the oviduct of an oviparous snake, *Diadophis punctatus*. J Morphol 227:67–79.

Qualls CP. 1996. Influence of the evolution of viviparity on eggshell morphology in the lizard *Lerista bougainvilli*. J Morphol 228:119–125.

Schleich HH, Kastle W. 1988. Reptile egg-shells SEM atlas. Stuttgart: Gustav Fischer.

Shine R. 1983. Reptilian reproductive modes: the oviparity-viviparity continuum. Herpetologica 39:1–8.

Shine R. 1985. The evolution of viviparity in reptiles: an ecological analysis. In: Gans C, Billet F, editors. Biology of the Reptilia, vol. 15. New York: John Wiley & Sons. p 605–694.

Shine R, Bull JJ. 1979. The evolution of live-bearing in lizards and snakes. Am Nat 113:905–923.

Stewart JR. 1985. Placentation in the lizard *Gerrhonotus coeruleus* with a comparison to the extraembryonic membranes of the oviparous *Gerrhonotus multicarinatus* (Sauria: Anguidae). J Morphol 185:101–114.

Stewart JR. 1990. Development of the extraembryonic membranes and histology of the placentae in *Virginia striatula* (Squamata: Serpentes). J Morphol 205:33–43.

Stewart JR, Brasch KR. 2003. Ultrastructure of the placentae of the natricine snake, *Virginia striatula* (Reptilia: Squamata). J Morphol 255:177–201.

Stewart JR, Thompson MB. 1994. Placental structure of the Australian lizard, *Niveoscincus metallicus* (Squamata: Scincidae). J Morphol 220:223–236.

Stewart JR, Ecay TW, Blackburn DG. 2004. Source and timing of calcium mobilization during embryonic development of the corn snake, *Pantherophis guttattus*. Comp Biochem Physiol A 139:335–341.

Surget-Groba Y, Heulin B, Guillaume CP, Thorpe RS, Kupriyanova LMS, Vogrin N, Maslak R, Mazzotti S, Venczel M, Ghira I, Odierna G, Leontyeva O, Monney JC, Smith ND. 2001. Intraspecific phylogeography of *Lacerta vivipara* and the evolution of viviparity. Mol Phyl Evol 18:449–459.

Xavier F, Gavaud J. 1986. Oviparity-viviparity continuum in reptiles; physiological characteristics and relation with environment. In: Assenmacher I, Boissin J, editors. Endocrine regulation as adaptive mechanisms to the environment. Paris: CNRS Press. p 79–93.

Yaron Z. 1985. Reptilian placentation and gestation: structure, function, and endocrine control. In: Gans C, Billet F, editors. Biology of the Reptilia, vol. 15. New York: John Wiley & Sons. p 527–603.