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# Extraembryonic membrane development in a reproductively bimodal lizard, *Lacerta (Zootoca) vivipara*

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# Abstract

Reproductive mode has been remarkably labile among squamate reptiles and the evolutionary transition from oviparity to viviparity commonly has been accompanied by a shift in the pattern of embryonic nutrition. Structural specializations for placental transfer of nutrients during intrauterine gestation are highly diverse and many features of the extraembryonic membranes of viviparous species differ markedly from those of oviparous species. However, because of a high degree of evolutionary divergence between the species used for comparisons it is likely that the observed differences arose secondarily to the evolution of viviparity. We studied development of the extraembryonic membranes and placentation in the reproductively bimodal lizard Lacerta vivipara because the influence of reproductive mode on the structural/functional relationship between mothers and embryos can best be understood by studying the most recent evolutionary events. Lecithotrophic viviparity has evolved recently within this species and, although populations with different reproductive modes are allopatric, oviparous and viviparous forms interbreed in the laboratory and share many life history characteristics. In contrast to prior comparisons between oviparous and viviparous species, we found no differences in ontogeny or structure of the extraembryonic membranes between populations with different reproductive modes within L. vivipara. However, we did confirm conclusions from previous studies that the tertiary envelope of the egg, the eggshell, is much reduced in the viviparous population. These conclusions support a widely accepted model for the evolution of squamate placentation. We also found support for work published nearly 80 years ago that the pattern of development of the yolk sac of L. vivipara is unusual and that a function of a unique structure of squamate development, the yolk cleft, is hematopoiesis. The structure of the yolk sac splanchnopleure of L. vivipara is inconsistent with a commonly accepted model for amniote yolk sac function and we suggest that a long standing hypothesis that cells from the yolk cleft participate in yolk digestion requires further study. © 2004 Elsevier GmbH. All rights reserved.

Keywords: Chorioallantois; Yolk sac; Hematopoiesis; Placentation; Lacertilia

*Abbreviations:* ALL, allantois; BO, bilaminar omphalopleure; C, chorion; CA, chorioallantoic membrane; CV, choriovitelline membrane; EC, ectoderm; EM, embryo; EN, endoderm; EX, extraembryonic coelom; G, granulocytic cells; GL, uterine glands; IV, intravitelline cells; IY, isolated yolk mass; S, eggshell; SP, splanchnopleure; ST, sinus terminalis; UT, uterus; Y, yolk; YC, yolk cleft

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# Introduction

The large number of independent origins of viviparity is one of the notable characteristics of squamate reptiles (Blackburn 1982, 1985; Shine 1985) and many of these evolutionary transformations have occurred relatively recently (Camarillo 1990; Heulin et al. 1993, 1999;

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Blackburn 1998). As a result, squamates present an unrivaled opportunity to study evolutionary patterns through comparisons between oviparous and viviparous species. Extended intrauterine gestation increases the opportunity for exchange between mothers and embryos and studies of placental development, placental structure and patterns of embryonic nutrition have revealed impressive variation among viviparous species (Weekes 1935; Blackburn 1992, 1993, 2000; Stewart 1993; Stewart and Thompson 2000; Thompson et al. 2000; Flemming and Branch 2001; Jerez and Ramirez-Pinilla 2001; Blackburn and Vitt 2002; Flemming and Blackburn 2003). However, tests of hypotheses for the evolution of placentotrophic viviparity from lecithotrophic oviparity require comparisons between oviparous and viviparous species and these studies are rare (Hrabowski 1926; Stewart 1985; Stewart and Thompson 1996, 2003). Extraembryonic membrane ontogeny is known for only four oviparous lizard species, Lacerta agilis (Hrabowski, 1926), Elgaria multicarinata (Stewart, 1985), Bassiana duperreyi (Stewart and Thompson, 1996) and Eumeces fasciatus (Stewart and Florian, 2000) and one oviparous snake species, Elaphe guttata (Blackburn et al., 2003). Study of interpopulational variation in reproductively bimodal species in particular can reveal how extraembryonic membrane and uterine tissues vary in response to variation in the length of intrauterine gestation.

Lacerta vivipara is one of three squamate species known to be reproductively bimodal (Heulin et al. 1993, 1999; Smith and Shine 1997; Fairbairn et al. 1998; Smith et al. 2001; Odierna et al. 2001). Interpopulational variation in characters associated with reproductive mode has been studied extensively in L. vivipara. Viviparous populations are widely distributed geographically throughout Europe and northern Asia whereas oviparous populations have a more restricted allopatric distribution in northern Spain and southern France, Slovenia and northern Italy (Brana and Bea 1987; Heulin and Guillaume 1989; Heulin et al. 1993, 2000). Geographical barriers preclude gene flow between oviparous and viviparous populations, but karyological (Odierna et al. 2001), allozyme (Guillaume et al. 1997) and mtDNA (Heulin et al. 1999; Surget-Groba et al. 2001) data all support conspecific status of the populations and that viviparity has evolved from oviparity once within the species (Surget-Groba et al. 2001). Although there is no interbreeding between oviparous and viviparous populations in the wild, they produce viable hybrids in the laboratory (Heulin et al. 1989; Arrayago et al. 1996). Generally, life history characteristics of oviparous and viviparous populations are similar, but oviparous populations may have higher annual fecundity because of higher clutch frequency (Heulin et al. 1991, 1994, 1997).

Perhaps the most distinctive characteristic of viviparous *L. vivipara* is the thin eggshell membrane that persists throughout gestation (Hrabowski 1926; Jacobi 1936; Panigel 1956). Thickness of the eggshell is variable among populations of *L. vivipara* and is correlated with the length of intrauterine egg retention (Heulin 1990; Heulin et al. 1992, 2002). Viviparous populations have thinner eggshells than oviparous populations (Heulin 1990) and eggshell thickness varies also among oviparous populations (Heulin et al. 2002). Hybrid females produced by experimental interbreeding of oviparous and viviparous strains oviposit eggs with an eggshell thickness intermediate between the parent populations (Heulin et al. 1992).

Early workers established that development of the extraembryonic membranes and morphology of the oviduct of viviparous *L. vivipara* differ little from oviparous species (Hrabowski 1926; Jacobi 1936; Panigel 1956). These authors speculated that the sole function of the chorioallantoic membrane was respiratory and that all nutrition for development was supplied by yolk, i.e., the function of the extraembryonic membranes of viviparous *L. vivipara* did not differ from oviparous species. Experiments by Panigel (1956) supported this functional hypothesis by demonstrating limited transfer into the embryo of radioisotopes injected into the peritoneal cavity of the female.

The yolk sac is the primary tissue functioning in nutrient transfer in oviparous amniotes. Development of the yolk sac of squamates differs from that of other amniotes (Stewart 1993, 1997) and the yolk sac of viviparous L. vivipara is an unusual variation of the general pattern for squamates (Hrabowski 1926; Stewart and Thompson 2000). Hrabowski (1926) proposed that the extraordinary structure of the yolk sac in the vicinity of the abembryonic pole of L. vivipara was a specialization for yolk digestion and mobilization. The mechanism implicated by this hypothesis differs from models for volk mobilization that have been developed for other amniotes (Romanoff 1960; Lambson 1970). Thus, comparison of extraembryonic membrane development and structure between oviparous and viviparous L. vivipara offers insight into two important evolutionary patterns, the evolution of viviparity and the evolution of the amniote egg.

# Materials and methods

Oviparous females (n = 9) were collected from Louvie-Juzon, France and viviparous females (n = 11) from Lake of Paimpont, France in August and September, 1997 and were maintained under conditions suitable for hibernation at Station Biologique de Paimpont from 1 October 1997 to 3 February 1998. Females were paired

with males collected from the same populations and were maintained under identical conditions from 15 February to 8 March. Lizards were shipped via air cargo to East Tennessee State University on 1 April 1998 and were housed individually, or with males, in glass terraria with 40 W incandescent lights switched on for 7 hours a day. Cages were housed adjacent to windows that provided ambient daylength. Oviposited eggs were incubated at 25 °C in vermiculite:water (2:1), estimated to represent a water potential of -120+40 kPa as measured by thermocouple psychrometry using a Wescor C52 chamber and Wescor HR33T microvoltimeter. Eggs were fixed at intervals throughout development in formol-dioxane-picric acid (Griffiths and Carter, 1958). Viviparous females were sacrificed at intervals and oviducts and enclosed embryos fixed in the same fixative as oviparous eggs. Specimens were embedded in Paraplast, sectioned at 7 µm and stained with hematoxylin and eosin. Embryos were staged using the system of Dufaure and Hubert (1961) and included stages 31, 33, 34, 36, 37, 38, 39, 40 for oviparous eggs and stages 34, 36, 38, 39, 40 for viviparous eggs. Photomicrographs were taken with a Wild MPS 52 Photoautomat attached to a Leica DMLB using Ilford XP2-400 film. Scale of the figures was estimated by taking measurements of tissue from the microscope slides using a calibrated ocular micrometer.

Eggshell thickness was measured for 9 oviparous eggs and 10 viviparous eggs (stages 36, 38, 39, 40) using an ocular micrometer. Measurements were taken from 10 histological sections for each egg. Individual clutches are represented only once in the sample. Data were analyzed using a nested analysis of variance (BIOM-stat 3.2; Exeter Software, Setauket, NY) with reproductive mode and egg as levels.

#### Results

#### **Oviparous population**

#### **Embryonic stage 31**

Three eggs were fixed on the day of oviposition; a fourth was fixed one day after oviposition. The embryo, surrounded by the amnion, rests on its left side on top of the yolk and the allantois is positioned over the embryo and adjacent regions of the yolk sac. The position of the allantois relative to the yolk sac varies among the specimens, but the outer allantoic membrane is fused to the chorion to form the chorioallantoic membrane in all embryos. The allantois does not extend over the lateral surface of the yolk sac in two of the embryos. There is an extensive extraembryonic coelom located between the allantois and the choriovitelline membrane, or vascular trilaminar omphalopleure, in one of these specimens (Fig. 1a). The yolk sac splanchnopleure covers the yolk and the somatopleure forms the outer wall of the extraembryonic coelom. In the second specimen, the choriovitelline membrane is intact between the chorioallantoic membrane and the sinus terminalis, which is positioned near the equator of the egg. The allantois reaches the sinus terminalis in the remaining two specimens. The chorioallantoic and choriovitelline membranes of all four specimens are well vascularized and have similar squamous epithelia in contact with the eggshell (Figs. 1b and c). In all four specimens, the yolk sac below the sinus terminalis is a bilaminar omphalopleure (Fig. 1d). The bilaminar omphalopleure has an outer squamous epithelium similar to that of the chorioallantoic and choriovitelline membranes but lacks a supporting layer of mesoderm and is avascular. A double sheet of intravitelline mesoderm, which is aligned parallel to the bilaminar omphalopleure, extends into the yolk from the region of the sinus terminalis. This tissue encloses the yolk cleft and separates the isolated yolk mass from the main yolk mass (Fig. 1d). Intravitelline cells and the yolk cleft cover most of the abembryonic surface of the main yolk mass except for a small circular plug at the abembryonic pole.

#### Embryonic stage 33

The entire embryonic hemisphere of the egg, delimited by the sinus terminalis, is covered by the chorioallantoic membrane in stage 33 embryos (N=7; ages, oviposition to day 4). The histology of the chorioallantoic membrane does not differ from stage 31 embryos (Fig. 2a). The chorioallantoic membrane contacts the bilaminar omphalopleure at the upper margin of the isolated volk mass. The yolk cleft and isolated yolk mass extend across the abembryonic surface of the yolk (Fig. 2b), but the thickness and shape of the isolated yolk mass is variable among the seven specimens. It is relatively thin and overlies most of the surface area of the abembryonic hemisphere in four of the specimens (Fig. 2b). In the remaining three specimens, the isolated yolk mass is thicker and the surface area perpendicular to the long axis of the egg is less extensive. The inner wall of the isolated yolk mass is a squamous epithelium, derived from intravitelline mesoderm, that lines the yolk cleft (Fig. 2c). Neither the bilaminar omphalopleure, isolated yolk mass, nor the intravitelline cell membrane, is vascularized (Fig. 2c). The yolk sac splanchnopleure of the main yolk mass, which forms the inner wall of the yolk cleft, likewise lacks blood vessels. Omphalomesenteric blood vessels are present in the yolk sac splanchnopleure over the remainder of the surface of the yolk sac. The outermost of these vessels, i.e., farthest from the embryonic pole, is the sinus terminalis, which lies at the upper margin of the isolated yolk mass (Fig. 2c). Omphalomesenteric vessels do continue a short distance toward the abembryonic pole beyond the sinus



**Fig. 1.** *L. vivipara* (oviparous). Embryonic stage 31. (a) Components of the yolk sac. Scale  $bar = 70 \,\mu\text{m}$ . (b) Chorioallantoic membrane. Arrowheads indicate allantoic blood vessels. (c) Choriovitelline membrane. Arrowheads indicate omphalomesenteric blood vessels. (d) Bilaminar omphalopleure. Arrowheads indicate intravitelline mesoderm. Scale  $bars = 30 \,\mu\text{m}$ .



**Fig. 2.** *L. vivipara* (oviparous). Embryonic stage 33. (a) Chorioallantoic membrane. Arrowheads indicate allantoic blood vessels. Scale bar =  $30 \,\mu$ m. (b) Abembryonic hemisphere. Arrowheads highlight the yolk cleft. Scale bar =  $300 \,\mu$ m. (c) Margin of the isolated yolk mass. Arrows indicate omphalomesenteric blood vessels associated with a deep fold in the yolk sac splanchnopleure (arrowheads). Scale bar =  $35 \,\mu$ m.

terminalis, but these vessels lie within the yolk internal to the non-vascular splanchnopleure that forms the inner wall of the yolk cleft (Fig. 2c). These blood vessels are associated with a double sheet of cells that extends into the yolk from the region of the sinus terminalis parallel to the inner wall of the yolk cleft.

#### **Embryonic stage 34**

Six specimens were fixed between incubation day 3 and 7. The extraembryonic membranes of stage 34 embryos are similar to those of stage 33 embryos except that the intrusion of cells and blood vessels into the volk internal to the yolk cleft is located nearer to the midline of the egg. This pattern of growth produces a deep fold in the splanchnopleure that encircles the abembryonic pole leaving only a central plug of yolk connecting the main yolk sac to the isolated yolk mass/yolk cleft complex (Fig. 3a). This central region of yolk is well vascularized by vessels entering laterally from the fold in the yolk sac splanchnopleure and by a large omphalomesenteric vessel that lies in the middle of the main yolk mass and receives smaller vessels from the abembryonic region. Blood vessels course throughout the main yolk mass and also within the yolk internal to the yolk cleft, but do not enter the isolated yolk mass (Fig. 3a). The shape of the isolated yolk mass/yolk cleft complex is variable (Figs. 3a and b), but in all specimens the yolk cleft is a closed cavity. The wall of the yolk cleft contains patches of cuboidal epithelial cells that are conspicuous near the outer perimeter of the cavity (Fig. 3c) and the cleft contains large numbers of cells with small acentric nuclei that appear to float freely within this cavity (Figs. 3c and d).

The allantois surrounds the perimeter of the egg and contacts the margin of the isolated yolk mass/yolk cleft complex (Figs. 3a and b). Allantoic blood vessels vascularize the site of contact.

#### Embryonic stage 36

Nine specimens of embryonic stage 36 were examined (incubation days 6-12). The topology of the extraembryonic membranes is unchanged relative to stage 34 embryos. The allantois surrounds the embryo and yolk with the exception of a small area at the abembryonic pole over which the bilaminar omphalopleure of the isolated yolk mass persists (Fig. 4a). The deep fold in the yolk sac separates the isolated yolk mass/yolk cleft complex from the main yolk mass except for a narrow isthmus of yolk (Fig. 4a). Omphalomesenteric blood vessels supply the region via the connection with the main yolk mass (Fig. 4b), but the isolated yolk mass and bilaminar omphalopleure are not vascularized (Figs. 4b and c). The density of yolk platelets is reduced in the vascularized region compared to the isolated yolk mass (Fig. 4b). Blood vessels from the abembryonic pole drain into a large omphalomesenteric vein positioned

near the center of the yolk mass (Fig. 4b). This vessel also receives veins from throughout the yolk. The epithelium of the yolk cleft is cuboidal in some regions and the cleft houses a concentration of cells that are loosely organized and appear to be floating freely (Fig. 4d).

#### **Embryonic stage 37**

The isolated yolk mass/yolk cleft complex of stage 37 embryos (N=6; incubation days 8–12) is a distinctive feature of the abembryonic pole of the egg. As in earlier stages, the isolated yolk mass is variable in shape and the surface area of the bilaminar omphalopleure varies accordingly. If the isolated yolk mass is thick and rotund in cross-section, the bilaminar omphalopleure has a small surface area (Fig. 5a), whereas a thin isolated volk mass has a bilaminar omphalopleure with a larger surface area (Fig. 5b). The yolk cleft is a closed cavity and the isolated yolk mass/yolk cleft complex is attached to the main yolk mass by a narrow isthmus in both variants. The wall of the yolk cleft is in part a cuboidal epithelium and the cleft contains large numbers of cells (Figs. 5c and d). Yolk platelets are sparse in the isolated yolk mass and in the region immediately internal to the yolk cleft compared to the main yolk mass. The area of the abembryonic pole is well vascularized and vessels returning to the embryo connect to a large omphalomesenteric vein located within the main yolk mass (Fig. 6a). This vein contains clusters of basophilic cells, associated with the endothelium, that have a high nuclear/cytoplasm ratio and have the appearance of blood islands (Figs. 6b and c).

The structure of the yolk sac varies regionally. The bilaminar omphalopleure has a squamous epithelium and is avascular (Fig. 5d). The definitive yolk sac, the yolk sac splanchnopleure, is vascularized. The outer component of the yolk sac splanchnopleure is a squamous epithelium derived from mesoderm that is uniform over the surface of the main yolk mass, but the internal supportive structures, particularly in association with blood vessels, vary. The yolk sac underlying the embryo has an internal epithelium of large columnar endodermal cells with large densely staining basal nuclei (Fig. 7a). These cells contain vacuoles or large vesicles that are eosinophilic but other features of the cytoplasm stain weakly or not at all. In contrast, the lateral and abembryonic yolk sac splanchnopleure lacks a continuous epithelium of large endodermal cells and is primarily a thin stratified membrane composed of squamous cells (Figs. 7b and c). Localized concentrations of granulocytic cells with deeply basophilic granules and irregular-shaped nuclei are associated with blood vessels of the outer squamous epithelium of the splanchnopleure (Figs. 7b and c). Endodermal cells similar to those of the embryonic region of the yolk sac



**Fig. 3.** *L. vivipara* (oviparous). Embryonic stage 34. (a) Isolated yolk mass/yolk cleft complex. Branches of a large omphalomesenteric vein (arrowheads) are positioned medial to the complex. Scale bar =  $80 \,\mu\text{m}$ . (b) Isolated yolk mass/yolk cleft complex. A large omphalomesenteric vessel (arrowhead) lies medial to the complex. Scale bar =  $200 \,\mu\text{m}$ . (c) Yolk cleft. A cuboidal epithelium (arrowhead) forms part of the wall of the yolk cleft and the cleft contains a mass of cells. Scale bar =  $20 \,\mu\text{m}$ . (d) Cells within the yolk cleft. Scale bar =  $20 \,\mu\text{m}$ .



**Fig. 4.** *L. vivipara* (oviparous). Embryonic stage 36. (a) Isolated yolk mass/yolk cleft complex. The arrowhead indicates a large omphalomesenteric vessel coursing from the stalk suspending the isolated yolk mass. Scale bar =  $180 \,\mu\text{m}$ . (b) Isolated yolk mass/yolk cleft complex. Arrowheads mark the position of deep folds in the yolk sac splanchnopleure medial to the isolated yolk mass. Scale bar =  $85 \,\mu\text{m}$ . (c) Bilaminar omphalopleure. Scale bar =  $20 \,\mu\text{m}$ . (d) Cells within the yolk cleft. Scale bar =  $20 \,\mu\text{m}$ .



**Fig. 5.** *L. vivipara* (oviparous). Embryonic stage 37. (a) Isolated yolk mass/yolk cleft complex. Scale bar =  $80 \,\mu$ m. (b) Isolated yolk mass/yolk cleft complex. Scale bar =  $80 \,\mu$ m. (c) Detail of the cells within the yolk cleft of (a). Arrowheads indicate cuboidal epithelial cells of the wall of the yolk cleft. Scale bar =  $20 \,\mu$ m. (d) Higher magnification of one region of (b). Arrowheads indicate cells within the yolk cleft. Scale bar =  $40 \,\mu$ m.



**Fig. 6.** *L. vivipara* (oviparous). (a) Central region of the yolk of a stage 37 embryo. Arrowheads indicate presumptive blood islands in a large omphalomesenteric vein. Scale  $bar = 180 \,\mu\text{m}$ . (b) Higher magnification of the omphalomesenteric vein shown in (a). Arrowheads indicate position of the presumptive blood islands. Scale  $bar = 40 \,\mu\text{m}$ . (c) Detail of a presumptive blood island (arrowhead) shown in (b). Scale  $bar = 15 \,\mu\text{m}$ .

splanchnopleure do occur, but they are sparsely distributed. These cells have large, intensively staining basophilic nuclei and lightly staining cytoplasm with numerous vesicles (Fig. 7c). A third type of cell, with eosinophilic granules and a lobate nucleus, is also associated with the splanchnopleure (Fig. 7c). Like the endodermal cells, the eosinophilic granulocytes are less common than the basophilic granulocytes.

The distribution of the chorioallantoic membrane is similar to stage 36 embryos and the structure does not differ from any of the earlier stages in that the chorion has a thin, squamous epithelium in contact with the eggshell and is fused to a thin, highly vascularized outer allantoic membrane (Fig. 7d). The allantois surrounds all but a central region of the abembryonic pole occupied by the bilaminar omphalopleure of the isolated yolk mass and thus the surface area of the abembryonic pole covered by the chorioallantoic membrane depends on the shape of the isolated yolk mass (Figs. 5a and b). In contrast to the regionally diversified yolk sac splanchnopleure, the chorioallantoic membrane has a uniform morphology.

#### Embryonic stages 38-40

We examined 12 embryos of embryonic stage 38 (N=5, incubation days 13-19), embryonic stage 39 (N=6, incubation days 15-18), and embryonic stage 40 (N=1, incubation day 17). The isolated yolk mass/yolk cleft complex undergoes a gradual demise during later stages of embryonic development. As in stage 37 embryos, the allantois completely encircles the yolk and contacts the margin of the isolated yolk mass/yolk cleft complex at the abembryonic pole of the egg. The histology of the chorioallantoic membrane does not differ from earlier embryonic stages. The shape of the isolated yolk mass is variable and, as in earlier stages, its morphology influences the size and shape of the yolk cleft and the position of the allantois relative to the outer perimeter of the egg in the vicinity of the



**Fig. 7.** *L. vivipara* (oviparous). Embryonic stage 37. (a) Yolk sac splanchnopleure underlying the embryo. Scale bar =  $15 \mu m$ . (b) Yolk sac splanchnopleure along lateral margin of the yolk. Clusters of granulocytic cells are associated with omphalomesenteric vessels (arrowheads). Scale bar =  $20 \mu m$ . (c) Yolk sac splanchnopleure near the abembryonic pole of the yolk. Omphalomesenteric vessels (arrowheads) are surrounded by clusters of granulocytic cells and irregularly distributed large endodermal cells and eosinophilic cells (arrows). Scale bar =  $15 \mu m$ . (d) Chorioallantoic membrane. Arrowheads indicate allantoic blood vessels. Scale bar =  $20 \mu m$ .



**Fig. 8.** *L. vivipara* (oviparous). (a) Isolated yolk mass/yolk cleft complex of a stage 38 embryo. Scale bar =  $75 \,\mu$ m. (b) Isolated yolk mass/yolk cleft complex of a stage 38 embryo. Scale bar =  $40 \,\mu$ m. (c) Isolated yolk mass/yolk cleft complex of a stage 39 embryo. Scale bar =  $40 \,\mu$ m. (d) Yolk cleft of a stage 39 embryo. Scale bar =  $25 \,\mu$ m.

abembryonic pole (Figs. 8a and b). The density of blood vessels associated with the complex is greater than in earlier embryonic stages and the density of yolk platelets is reduced (Fig. 8c). The yolk cleft in specimens with a relatively thick isolated yolk mass is smaller than in earlier embryonic stages and the cleft does not contain the clusters of cells that were present in earlier embryonic stages (Figs. 8b and d).

#### **Viviparous population**

#### **Embryonic stage 34**

The earliest embryos that we examined from the viviparous population were stage 34 (N=3). The extraembryonic membranes of these embryos are similar to stage 34 embryos from the oviparous population in that the allantois lies over the embryo and contacts the isolated yolk mass below the equatorial plane of the egg (Fig. 9a). A thin chorioallantoic membrane covers the embryonic hemisphere of the egg (Fig. 9b). The chorionic epithelium is squamous and the allantois is densely vascularized. The uterine epithelium consists of a single layer of rounded cells with relatively large nuclei and the underlying lamina propria contains numerous simple glands with occluded lumina. Blood vessels are interspersed among the glands. A prominent shell membrane lies between the chorioallantoic membrane and the uterine epithelium (Fig. 9b) but this structure is thinner than the eggshell of oviparous eggs.

Two of the viviparous specimens differ from stage 34 oviparous embryos in that the yolk cleft is discontinuous in cross-section because it does not penetrate the yolk in the vicinity of the abembryonic pole, i.e., there is a central plug of yolk connecting the isolated yolk mass to the main yolk mass. The yolk cleft of the third specimen is a single cavity similar to that of oviparous embryos. The walls of the yolk cleft are not vascularized, but blood vessels do extend into the yolk internal to and parallel with the yolk cleft in a pattern similar to that of oviparous embryos (Fig. 9a). This tissue does not penetrate into the yolk the same distance as does the yolk cleft. The isolated yolk mass is thin and covers the abembryonic surface of the main yolk mass. The outer epithelium of the bilaminar omphalopleure is squamous and large irregular spaced endodermal cells form the inner layer (Fig. 9c). Endodermal cells are also dispersed throughout the isolated yolk mass. The uterine component of the omphaloplacenta is similar to that of the chorioallantoic placenta. The uterine epithelium consists of low, rounded cells and blood vessels and glands are present in the lamina propria. The shell membrane is also prominent and similar to that of the chorioallantoic placenta.

#### **Embryonic stage 36**

The topology of the extraembryonic membranes is similar to oviparous embryos in all specimens (N=12)that we examined. The yolk cleft is a single cavity and the isolated yolk mass covers a large area of the abembryonic pole (Fig. 10a). The pattern of vascularization of the yolk is similar to that of oviparous embryos. The isolated yolk mass is not vascularized but blood vessels are prominent in the deep fold that penetrates the yolk parallel to the isolated yolk mass (Fig. 10b). These vessels are closely associated with the inner wall of the yolk cleft. The fold and blood vessels turn upward toward the embryo near the central axis of the egg and vessels from this central region connect to large omphalomesenteric vessels in the center of the yolk mass (Fig. 10c). Blood islands, similar to structures seen in oviparous embryos, are present in large vessels in this region (Fig. 11a). The yolk cleft contains clusters of cells that are particularly prominent near its outer perimeter (Fig. 11b) and in some regions the cells are associated with patches of a simple cuboidal epithelium that lines the yolk cleft (Fig. 11c). With the exception of these patches, the epithelium lining the yolk cleft is squamous. The components of the omphaloplacenta are similar to stage 34 embryos. The outer epithelium of the isolated yolk mass, which contacts the shell membrane, is squamous (Fig. 11d). Cells of the simple uterine epithelium are larger than the apposing embryonic epithelium. The epithelium is supported by blood vessels of the lamina propria, which also contains simple, unbranched glands that lack lumina. A shell membrane separates the uterine and embryonic epithelia.

The structure of the yolk sac splanchnopleure, like that of oviparous embryos, varies regionally. The surface of the yolk sac facing the embryo contains areas where the outer squamous cell layer is underlain by a continuous layer of large endodermal cells (Fig. 12a). The endodermal cells, characterized by large nuclei and numerous large cytoplasmic vesicles, are commonly associated with blood vessels (Fig. 12b). Smaller cells with basophilic granules are also present in these regions (Fig. 12b). The sides and abembryonic regions of the yolk sac are covered by an outer squamous epithelium continuous with that of the abembryonic region (Fig. 12a), but large endodermal cells do not form a continuous layer. Where present, these cells occur singly and may be associated with the outer epithelium or scattered throughout the yolk mass. Cells with basophilic granules are also associated with the splanchnopleure and form dense aggregations adjacent to blood vessels (Fig. 12c).

The allantois fills the embryonic hemisphere over the embryo, surrounds the yolk sac and extends a short distance into the fold in the yolk sac (Figs. 10a and 11b). The chorioallantoic placenta is similar to stage 34



**Fig. 9.** *L. vivipara* (viviparous). Embryonic stage 34. (a) Margin of the isolated yolk mass. Arrows indicate omphalomesenteric blood vessels associated with a deep fold in the yolk sac splanchnopleure (arrowheads). Scale  $bar = 35 \,\mu m$ . (b) Chorioallantoic placenta. Arrowheads indicate allantoic and uterine blood vessels. Scale  $bar = 20 \,\mu m$ . (c) Bilaminar omphalopleure and isolated yolk mass. Scale  $bar = 20 \,\mu m$ .



**Fig. 10.** *L. vivipara* (viviparous). Embryonic stage 36. (a) Abembryonic pole. Scale  $bar = 180 \mu m$ . (b) Isolated yolk mass. The arrowhead indicates an omphalomesenteric vessel in a fold in the yolk sac splanchnopleure. Scale  $bar = 40 \mu m$ . (c) Embryonic stage 36. A large omphalomesenteric vein (arrow) drains the region medial to the isolated yolk mass/yolk cleft complex. Arrowheads show the position of deep folds in the yolk sac splanchnopleure internal to the isolated yolk mass/yolk cleft complex. Scale  $bar = 75 \mu m$ .



**Fig. 11.** *L. vivipara* (viviparous). Embryonic stage 36. (a) Detail of Fig. 10b. Presumptive blood island (arrowhead) in a large omphalomesenteric vein. Scale bar =  $15 \mu m$ . (b) Isolated yolk mass/yolk cleft complex. Asterisks highlight the yolk cleft which is filled with clusters of cells. Arrowheads indicate omphalomesenteric blood vessels along the inner wall of the yolk cleft. Scale bar =  $40 \mu m$ . (c) A cluster of cells within the yolk cleft adjacent to cuboidal epithelial cells (arrowheads) of the wall of the yolk cleft. Scale bar =  $20 \mu m$ . (d) Omphaloplacenta. Asterisks indicate cells within the yolk cleft. Omphalomesenteric vessels (arrowheads) are associated with a deep fold in the yolk sac splanchnopleure internal to the yolk cleft. Scale bar =  $20 \mu m$ .



**Fig. 12.** *L. vivipara* (viviparous). Embryonic stage 36. (a) Transitional region of the yolk sac splanchnopleure. The continuous layer of large endodermal cells located in the vicinity of the embryo does not continue (arrowhead) along the lateral and abembryonic regions of the splanchnopleure. Omphalomesenteric vessels are indicated by asterisks. Scale  $bar = 40 \,\mu m$ . (b) Endodermal and granulocytic cells surrounding an omphalomesenteric vessel in the yolk sac splanchnopleure. Scale  $bar = 15 \,\mu m$ . (c) Granulocytic cells associated with an omphalomesenteric blood vessel (arrowhead) in the yolk sac splanchnopleure of the abembryonic region of the egg. Scale  $bar = 20 \,\mu m$ . (d) Chorioallantoic placenta. Arrowheads indicate allantoic and uterine blood vessels. Scale  $bar = 20 \,\mu m$ .

embryos (Fig. 12d). The chorioallantoic membrane is thin with a squamous chorionic epithelium overlaying a network of allantoic blood vessels. The uterus of the chorioallantoic placenta is similar to that of the omphaloplacenta. A simple epithelium of low cuboidal cells contacts the shell membrane and is underlain by blood vessels and simple glands with occluded lumina. The prominent shell membrane has a similar appearance to that of the omphaloplacenta.

#### Embryonic stages 38-40

We examined 15 embryos: stage 38 (N=3), stage 39 (N=5), and stage 40 (N=7). As in oviparous embryos, the isolated yolk mass/yolk cleft complex of stage 38-40 viviparous embryos is reduced in size, yolk platelets are depleted within the isolated yolk mass and fewer cells occur within the yolk cleft. The isolated yolk mass of our stage 38 specimens has a large surface area comparable to that of stage 36 embryos, but is thinner in cross-section (Fig. 13a). The omphaloplacenta retains all components characteristic of earlier embryonic stages (Figs. 13b and c). The outer epithelium of the bilaminar omphalopleure is squamous and squamous intravitelline cells form a sheet that separates the isolated yolk mass from the volk cleft. Large endodermal cells span the width of the isolated yolk mass and contact both the outer epithelium and the intravitelline cells. Clusters of cells are scattered throughout the yolk cleft (Fig. 13c). The uterus is well supplied with blood vessels and has a simple, low epithelium similar to earlier stages. As in previous stages, a prominent shell membrane separates the uterine and embryonic epithelia. The surface area of the isolated yolk mass is much reduced in all of the stage 40 embryos and the chorioallantois surrounds all or nearly all of the perimeter of the egg (Fig. 14a). The components of the isolated yolk mass/yolk cleft complex are more difficult to discern but in some specimens remnants of the cuboidal epithelium and yolk cleft cells are still apparent (Fig. 14b).

The chorioallantoic placenta has the same morphology in the abembryonic hemisphere of the egg as it does in the region over the embryo (Figs. 14c and d). Squamous chorionic epithelial cells are in contact with the shell membrane and are fused to a highly vascular allantois. The uterine epithelium is composed of low cells with thin cytoplasmic extensions overlying blood vessels. The shell membrane is similar to that of earlier stages.

#### Variation in eggshell thickness

The eggshell of oviparous eggs was significantly thicker than that of viviparous eggs for later embryonic stages (36, 38–40) by nested analysis of variance (F=326, d.f.=1, 171, P<0.001) (Table 1). Variation among eggs contributed an insignificant component to total variance (F=36, d.f.=17, 171).

## Discussion

# Comparisons between oviparous and viviparous species

Comparisons of ontogeny of the extraembryonic membranes between oviparous and viviparous species of squamates are rare but include three families of lizards, Lacertidae (L. agilis and L. vivipara; Hrabowski, 1926), Anguidae (E. multicarinata and E. coerulea; Stewart, 1985), and Scincidae (B. duperreyi, E. fasciatus and Pseudemoia entrecasteauxii, P. spenceri; Stewart and Thompson, 1996, 2003). The general conclusions of these studies are that variation in timing of development and in topology and structure of the extraembryonic membranes are associated with differences in reproductive mode. Differences in the timing of developmental events are not consistent for all comparisons. Growth of the allantois is accelerated in viviparous L. vivipara compared to the oviparous congener L. agilis (Hrabowski, 1926) and in viviparous *Pseudemoia* sp. compared to confamilial oviparous species, B. duperreyi and E. fasciatus (Stewart and Thompson, 1996, 2003). However, development of the yolk cleft and isolated yolk mass occurs relatively later in viviparous L. vivipara, but earlier in Pseudemoia sp. compared to oviparous species. The topology or spatial relationship of the membranes also differs in association with reproductive mode. Generally, the growth of the allantois is more restricted in relation to the yolk sac in viviparous species of lizards and the bilaminar omphalopleure persists to later embryonic stages. The allantois enters the yolk cleft and surrounds the yolk sac in the oviparous anguid E. multicarinata, but not in viviparous E. coerulea (Stewart, 1985). The allantois encloses the yolk sac in the oviparous skinks B. duperreyi and E. fasciatus (Stewart and Thompson, 1996; Stewart and Florian, 2000), while in viviparous lizards, Pseudemoia sp., of the same species group, the allantois does not grow into the abembryonic hemisphere (Stewart and Thompson 1996, 2003).

Highly matrotrophic species have received most of the attention in the study of squamate placentation (Weekes 1935; Blackburn et al. 1984; Stewart and Thompson 1996; Jerez and Ramirez-Pinilla 2001, 2003; Blackburn and Vitt 2002) and there are pronounced differences in the structure of the epithelia of both the omphalopleure and chorioallantoic membrane of these species compared to confamilial oviparous species (Stewart and Thompson 1996, 2003; Stewart and Florian 2000; Blackburn and Vitt 2002). The principal differences





**Fig. 13.** *L. vivipara* (viviparous). Embryonic stage 38. (a) Isolated yolk mass/yolk cleft complex. Scale  $bar = 80 \,\mu m$ . (b) Omphaloplacenta. Scale  $bar = 20 \,\mu m$ . (c) Cells within the yolk cleft (arrowheads) of the omphaloplacenta. Scale  $bar = 20 \,\mu m$ .



**Fig. 14.** *L. vivipara* (viviparous). Embryonic stage 40. (a) Isolated yolk mass/yolk cleft complex. Scale bar =  $40 \,\mu$ m. (b) Yolk cleft with remnants of a cuboidal epithelium (arrowheads). Scale bar =  $20 \,\mu$ m. (c) Chorioallantoic placenta of the abembryonic hemisphere. Arrowheads indicate allantoic blood vessels. Scale bar =  $20 \,\mu$ m. (d) Chorioallantoic placenta of the embryonic hemisphere. Arrowheads indicate allantoic and uterine blood vessels. Scale bar =  $20 \,\mu$ m.

 Table 1.
 Eggshell thickness for embryonic stages 36, 38–40 of oviparous and viviparous L. vivipara

Reproductive mode	Ν	Eggshell thickness (µm)
Oviparous Viviparous	9 10	$49.1 \pm 2.4^{*} \\ 7.4 \pm 0.5^{*}$

Reported values are means  $\pm 1$  SE of the means of 10 measurements taken from 10 separate histological sections for each egg (N). Individual clutches are represented only once (N=19 clutches). \*P < 0.001.

associated with viviparity are an increase in height of the outer layer of epithelial cells of the omphalopleure (Stewart and Thompson 1996, 2000, 2003) and in some species regional differentiation of the chorioallantoic membrane (Weekes 1935; Blackburn 1992; Blackburn and Vitt 2002; Stewart and Thompson 1996, 2000, 2003). However, matrotrophic species are likely to exhibit secondary specializations that arise subsequent to the evolution of viviparity. For example, greater complexity in the structure of the epithelia of the chorioallantoic membrane and omphalopleure and developmental patterns that increase the prominence of the bilaminar omphalopleure likely enhance placental transfer but may reveal little about characteristics of the extraembryonic membranes during evolutionary transitions from oviparity to viviparity. Comparisons between predominantly lecithotrophic viviparous species and closely related oviparous outgroups provide better models for the early stages in the evolution of viviparity and these studies found differences in timing of development and topology of the extraembryonic membranes, but not in the structure of the epithelia (Hrabowski 1926; Stewart 1985).

We found no differences between oviparous and viviparous forms of L. vivipara in extraembryonic membrane topology or epithelial structure and only a single minor variation in timing of development of the yolk cleft. The yolk cleft was not fully formed in two of the three stage 34 viviparous specimens, whereas all six of the oviparous specimens of the same stage had a complete yolk cleft. No differences associated with reproductive mode were found in stage 36, or older, embryos. Formation of the yolk cleft/isolated yolk mass system and growth of the allantois do not differ in viviparous individuals compared to oviparous individuals and the chorioallantoic membrane of L. vivipara has a uniform morphology irrespective of reproductive mode. We conclude that the evolution of viviparity within this species was not accompanied by structural modification of the extraembryonic membranes.

However, our histological study does reveal a dramatic distinction between oviparous and viviparous eggs that corroborates conclusions based on more exhaustive research (Heulin 1990; Heulin et al. 1992,

2002; Arrayago et al. 1996). The eggshell of oviparous eggs of L. vivipara is significantly thicker than that of viviparous eggs (Heulin 1990; Table 1). The reduction in eggshell thickness and concomitant loss of specific nutrient components, e.g. calcium (Heulin 1990; Heulin et al. 1992) may be universally associated with the evolution of amniote viviparity (Packard et al. 1977; Guillette 1982, 1992; Qualls 1996; Andrews 1997; Heulin et al. 2002). At least one component of the eggshell, frequently termed the shell membrane (Packard and DeMarco 1991), may be present in the early development of all viviparous squamates (Blackburn 1993), but this structure is absent during middle to late developmental stages of many species (Guillette and Jones 1985; Blackburn 1993; Stewart and Thompson 1994, 1996, 1998, 2004; Blackburn and Callard 1997). The eggshell component of viviparous L. vivipara is present throughout gestation, as it is in many other viviparous species (Weekes 1927; Hoffman 1970; Stewart 1985, 1990; Baxter 1987; Stewart and Brasch 2003). How this structure acts functionally relative to the transfer of materials across the placenta is not known, but the expectation is that it limits exchange.

Our findings are consistent with a prominent model for the evolution of squamate viviparity that stipulates neither the structure nor the function of the chorioallantoic membrane is transformed in the transition to viviparity (Weekes 1935; Blackburn 1993) but that eggshell thickness is reduced to enhance gas exchange between the uterus and embryo (Packard et al. 1977; Guillette 1982). In this scenario, the chorioallantoic membrane of oviparous squamates has a uniform morphology consisting of a smooth stratified squamous epithelium vascularized by allantoic blood vessels that provide the sites for respiratory exchange for the embryo. This embryonic component contributes to the chorioallantoic placenta of viviparous descendents and the opposing uterine epithelium is also smooth and well vascularized. This squamate placental morphology has been termed a simple "type I" chorioallantoic placenta (Weekes 1935; Blackburn 1993).

#### Development of the yolk sac

The initial sequence of development of the yolk sac of *L. vivipara* is typical for squamates (Stewart 1997). A bilaminar omphalopleure grows outward from the embryo to cover the surface of the yolk and extraembryonic mesoderm extends between the layers of endoderm and ectoderm to form a trilaminar omphalopleure, the area opaca. Angiogenesis and hematopoiesis occur in the extraembryonic mesoderm resulting in a vascular network and the yolk sac is converted to a vascularized trilaminar omphalopleure, the choriovitel-line membrane or area vasculosa. The final stage of yolk

sac formation occurs when the internal sheet of mesoderm of the choriovitelline membrane divides resulting in a vascularized yolk sac splanchnopleure and an outer sheet of non-vascular somatopleure, the chorion. This sequence of development occurs over the upper or embryonic surface of the yolk mass. The mechanism that initiates division of the choriovitelline membrane to enclose an extraembryonic coelom is not known, but in the oviparous scincid lizard, E. fasciatus, the advancing edge of the allantois is in contact with the site of separation of the choriovitelline membrane (Stewart and Florian 2000). Thus, the allantois and the extraembryonic coelom expand in consort. In L. vivipara, the extraembryonic coelom can develop well in advance of the allantois (Fig. 1a) and expansion of the allantois is not restricted by the presence of the choriovitelline membrane.

Development of that portion of the yolk sac in the abembryonic hemisphere of the egg distinguishes squamate reptiles from other amniotes (Stewart 1997) and in addition, features of this general pattern are highly variable among squamates, reflecting both phylogenetic history and reproductive specialization (Stewart 1992, 1993; Stewart and Thompson 2000, 2003). Ontogeny of the abembryonic yolk sac of L. vivipara is unusual compared to other squamates, and in contrast to scincid lizards (Stewart and Thompson 1996, 2003), oviparous and viviparous forms are similar. The first detailed description of extraembryonic membrane development of a squamate reptile and the first description of the yolk cleft and isolated yolk mass, structures that distinguish squamates from other amniotes, was the work of Hanni Hrabowski (1926) on L. vivipara. Hrabowski (1926) noted that the cells, now termed intravitelline cells, that migrate into the volk from the vicinity of the sinus terminalis are derived from mesoderm and that clusters of these cells enclose small cavities that later coalesce into one large cleft space, termed distaler Dotterspalt (=yolk cleft). Our observations confirm that the yolk cleft (distaler Dotterspalt) is a closed cavity, formed by intravitelline cells, that extends across the abembryonic pole separating the isolated yolk mass from the main yolk mass. This developmental pattern is similar in all but a few squamates that have secondarily lost the isolated yolk mass (Stewart 1993; Flemming and Branch 2001; Jerez and Ramirez-Pinilla 2001, 2003; Flemming and Blackburn 2003). Hrabowski (1926) also observed that the intrusion of the vascularized splanchnopleure into the yolk in the abembryonic hemisphere was an additional component of a complex yolk cleft system. This feature of yolk sac development of L. vivipara is unusual. The circumferential fold in the yolk sac splanchnopleure partially subdivides the yolk mass into a large embryonic region and a much smaller abembryonic region (Fig. 15). The two regions remain confluent via a central plug of yolk. The mass of yolk



Fig. 15. Topology of the allantois and yolk sac of *L. vivipara*. Modified after Hrabowski (1926).

delimited by the splanchnopleuric fold was termed *Dottersacknabelblase* and the *distaler Dotterspalt* (=yolk cleft) was the lymph cleft of the *Dottersacknabelblase* (Fig. 15). The *Dottersacknabelblase* includes the isolated yolk mass, yolk cleft and a thin mass of yolk lying between the yolk cleft and the fold in the splanchnopleure (Hrabowski 1926) (Fig. 15). The fold in the splanchnopleure is confluent with the extraembryonic coelom, but the yolk cleft is a closed cavity. Thus, as the allantois expands around the yolk, it extends into the splanchnopleuric fold and not the yolk cleft. Previous interpretations of Hrabowski (1926) overlooked her description of the yolk cleft (=*distaler Dotterspalt*; Mossman 1974, 1987; Stewart and Thompson 2000).

## Function and evolution of the Dottersacknabelblase

Hrabowski (1926) speculated that the principal function of the *Dottersacknabelblase* was yolk digestion which was accomplished by *Lymphocyten* produced in the *distaler Dotterspalt* (=*Lymphspalt*). Yolk moved into the abembryonic region and was digested in situ. In addition, the *Lymphocyten* were distributed throughout the surface of the yolk sac in close proximity to blood vessels and at these sites transformed yolk into nutrients that were transported to the embryo in the blood vessels. The omphalomesenteric blood vessels extending from the periphery into the yolk above the *Dottersacknabelblase* provided arterial flow that was collected into a large omphalomesenteric vein, coursing through the center of the yolk mass that conveyed the products of yolk digestion directly to the embryo.

Hrabowski's (1926) hypothesis is unconventional, but some aspects are appealing. The volk cleft does fill with cells that appear to float freely within the cavity and the wall of the cleft contains patches of cuboidal cells that could be the germinal epithelium that produces the cells that aggregate in the yolk cleft. While it seems unlikely that yolk circulates into the Dottersacknabelblase from the yolk sac and is subsequently digested there as Hrabowski (1926) surmised, it is possible that derivatives of the cells of the volk cleft enter the main volk sac and contribute to yolk degradation there. This would be a surprising finding because the epithelium of the yolk cleft is mesodermal in origin and yolk digestion in Reptilia is thought to be accomplished by endodermal cells of the yolk sac splanchnopleure (Williams 1967; Lambson 1970; Ono and Tuan 1991). The models for yolk digestion are based on chickens and depict a yolk sac splanchnopleure with a uniform morphology (Lambson 1970). In contrast, the yolk sac splanchopleure of L. vivipara is regionally differentiated (Figs. 7a-c and 12a-c). The region immediately adjacent to the embryo contains vascular folds lined by large endodermal cells which contain numerous large eosinophilic vesicles and have large nuclei. Structurally, they are similar to endodermal cells that are implicated in yolk digestion and transport in domestic fowl (Lambson 1970). The remainder of the yolk sac splanchnopleure, i.e., lateral and abembryonic regions, lacks folds and, although large endodermal cells are not concentrated around blood vessels, aggregations of granulocytes do accompany blood vessels. The granulocytes are predominantly basophilic and distinctly smaller than the large endodermal cells with eosinophilic vesicles. Like the larger cells, the granulocytes are well positioned to mediate volk degradation and mobilization into the embryonic circulation. The derivation of these cells is unknown and there is no evidence to link them to the cells of the yolk cleft as Hrabowski (1926) suggested. The cells of the yolk cleft have a cytoplasm that stains uniformly eosinophilic and if they are progenitors of the granulocytes, transformation would have to occur after they leave the yolk cleft.

The isolated yolk mass/yolk cleft complex is a unique feature of squamate development. It is present in all oviparous and most viviparous species, but is reduced or absent in some highly matrotrophic species (Stewart and Thompson 1996, 1998; Flemming and Branch 2001; Jerez and Ramirez-Pinilla 2001, 2003; Flemming and Blackburn 2003). Developmental and structural similarities indicate that the yolk clefts of various squamate lineages are homologous (Stewart 1993, 1997). There are few detailed studies of oviparous species and, apart from speculation based on structural characteristics, nothing is known of the functional attributes of this system. However, characteristics of the yolk cleft system of the oviparous skink *E. fasciatus* suggest structural and

functional similarity with that of L. vivipara. The yolk cleft of *E. fasciatus* develops in an identical manner and contains small aggregations of cells similar to those of L. vivipara (Stewart and Florian, 2000). E. fasciatus differs from L. vivipara because an outgrowth of the yolk cleft, the yolk sinus, replaces the yolk cleft during later embryonic stages of E. fasciatus. Like the yolk cleft of L. vivipara, the yolk sinus is lined by a germinal epithelium and fills with cells. The yolk sinus cells have the capacity to digest yolk because they participate in the formation of the yolk sinus. The cells do not contribute to tissue within the yolk cleft or yolk sinus but apparently migrate from these cavities to participate in functions either in the yolk sac or embryo. The amniote yolk sac is a prominent hematopoietic organ, particularly in the production of erythrocytes during early embryonic development (Metcalf and Moore 1971). However, the volk cleft and yolk sinus do not appear to be erythropoietic because the cells do not have the high nuclear to cytoplasmic ratio nor are they basophilic like proerythrocytes from yolk sac blood islands (Sabin 1921; Edmonds 1966; Moore and Metcalf 1970). In addition, structures similar to avian blood islands are present in omphalomesenteric blood vessels of both E. fasciatus (Stewart and Florian, 2000) and L. vivipara (Figs. 6a-c and 11a). The presumptive blood islands of these lizards differ markedly from the yolk cleft or yolk sinus. Stewart and Florian (2000) speculated that the volk cleft and yolk sinus of E. fasciatus were sources of granulocyte progenitors for hatchlings because the yolk sac is drawn into the embryo prior to hatching. However, the yolk cleft of L. vivipara regresses prior to hatching and the presence of yolk cleft cells during all but the latest embryonic stages that we examined is more consistent with a role in yolk metabolism.

The hypothesis that the squamate yolk cleft system is a site of hematopoiesis and that cells derived from this tissue contribute to yolk digestion (Hrabowski 1926) is inconsistent with our present knowledge of the avian yolk sac but requires consideration because of the unique attributes of the squamate yolk sac. The yolk sac splanchnopleure of E. fasciatus (Stewart and Florian, 2000) and L. vivipara differs structurally from that of chickens and the mechanism of yolk catabolism and mobilization may also differ. The nearly universal distribution of the isolated yolk mass/yolk cleft system among squamates suggests that these tissues play a critical role in embryonic development and the histology of the yolk cleft systems in *E. fasciatus* and *L. vivipara* is consistent with a role in the production of hematopoietic stem cells. If the yolk cleft cells are associated with yolk degradation, functional characteristics of the squamate yolk sac, particularly features related to yolk metabolism, differ from that of birds and may be unlike any other amniote lineage. The evolutionary reduction or loss of the isolated yolk mass/yolk cleft system in

matrotrophic species of scincid lizards (Stewart and Thompson 1996, 1998, 2004; Flemming and Branch 2001; Jerez and Ramirez-Pinilla 2001, 2003; Flemming and Blackburn 2003) may reflect a growing dependence on placental, rather than yolk, nourishment. Understanding the functional characteristics of this system in *L. vivipara* may provide considerable insight because the yolk cleft is equally prominent in both modes of parity.

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