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UTERINE ANGIOGENESIS IN SQUAMATE REPTILES: IMPLICATIONS FOR THE EVOLUTION OF VIVIPARITY

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***Abstract.*—Proliferation of the uterine vascular bed is one of the essential events required to sustain embryonic development during prolonged gestation. We tested the hypothesis that density of uterine blood vessels increases as embryonic mass, and therefore metabolic rate, increase during pregnancy/gravidity. We used an antibody generated against human von Willebrand Factor, immunofluorescence and laser-scanning confocal microscopy to quantify uterine microvascular density during gravidity/pregnancy in the skinks *Ctenotus taeniolatus* (oviparous) and *Saiphos equalis* (viviparous). The anti-human von Willebrand Factor antibody cross reacted with both *C. taeniolatus* and *S. equalis* and resulted in strong positive staining of both major vessels as well as microvasculature. Overall uterine vascular density was approximately 17% less in *C. taeniolauts* than in *S. equalis,* and vascular density did not increase significantly during the slow growth phase of the first half of gestation in either species. For *S. equalis,* however, overall vessel density increased by 48% during the exponential growth phase of late gestation. The increase in vascular density coincides with the period of rapid embryonic growth in mass and associated increase in embryonic metabolic oxygen consumption. The capacity of the uterine vascular bed to increase in surface area in response to embryonic oxygen demand may be a key transitional event in the evolution of viviparity.**

Key Words.— blood vessels; confocal microscopy; *Ctenotus taeniolatus*; embryonic development; placentation; *Saiphos equalis*; Skinks

INTRODUCTION

The evolutionary transition from oviparity to viviparity is one of the most fundamental, yet least understood processes in evolutionary physiology and has been the focus of numerous studies for nearly a century (e.g. Weeks 1935; Packard et al. 1977; Shine 1985; Thompson et al. 2002). At least three essential steps are necessary for viviparity to evolve from oviparity: 1) increased length of retention of the embryo *in utero* during gestation, 2) reduction and/or loss of the eggshell, and 3) elaboration of the vascular bed of the uterus and extra-embryonic membranes (Packard et al. 1977; Shine 1985; Andrews and Mathies 2000). These major changes in reproductive physiology require modifications to a suite of integrated physiological features to both maintain the gravid state (i.e. increase egg retention) as well as support differentiation and growth of embryos retained *in utero*.

Proliferation of blood vessels (angiogenesis) in the uterus and extra-embryonic membranes is necessary for support of embryonic development during prolonged gestation because the vascular system is responsible for both delivery and uptake of oxygen to embryonic tissues. Embryonic growth in mass and metabolic oxygen consumption increases throughout gestation becoming highest during the latter half of development (Thompson and Stewart 1997). In lizards, and presumably most reptiles, embryonic development is reduced or arrested when embryonic oxygen demand equals or exceeds oxygen availability in the uterus (Andrews 2002; Parker et al. 2004; Parker and Andrews 2006). The capacity to support embryonic development *in utero* during prolonged gestation is thus largely determined by the capacity of the uterus to accommodate increasing embryonic oxygen demand as development progresses.

The role of angiogenesis in the evolution of viviparity has been inferred primarily from comparative histological studies examining the ontogeny of placental membranes during gestation (e.g. Weeks 1935; Stewart and Thompson 1996). In contrast, only a few studies have examined blood vessels in oviparous and viviparous species directly (Guillette and Jones 1985; Masson and Guillette 1987). For example, the viviparous species (*Sceloporus bicanthalis*) shows a higher vascular density than the closely related oviparous species (*Sceloporus aeneus*), although no information on the ontogeny of vascular proliferation associated with embryonic growth *in utero* is provided for either species (Guillette and Jones 1985). The ability to visualize vascular morphology using standard techniques, such as ink and vascular casts, is constrained by the small size of appropriate model reptile species, is time consuming to execute, and poses several technical

drawbacks such as incomplete perfusion of small vascular structures. Using modern imaging technology and techniques from immunofluorescence microscopy, however, we can now build on these initial studies to obtain detailed images of changes in vascular morphology associated with the evolution of viviparity. These techniques provide the best opportunity to identify and understand the fundamental role of angiogenesis in supporting embryonic development during prolonged gestation.

The purpose of our study is to quantify uterine microvascular architecture in oviparous and viviparous lizards and test the hypothesis that density of blood vessels in the uterine epithelium increases in concert with embryonic mass, and, therefore, metabolic oxygen demand during gravidity/pregnancy. We used indirect immunofluorescence and laser-scanning confocal microscopy to quantify uterine microvascular density and morphology during the reproductive cycle in two species of Australian *Sphenomorphous*-group skinks-*Ctenotus taeniolatus* (Copper-tailed Skink) and *Saiphos equalis* (Three-toed Skink). The species used in the study where chosen on the basis of their differing capacities to support embryonic development during extended egg retention. *Ctenotus taeniolatus* is a typical oviparous species, which oviposits at a maximum Dufaure and Hubert (1961) embryo stage of 31 (S.L. Parker, unpubl. data). *Saiphos equalis* has the capacity to retain eggs until embryonic development is complete (i.e. stage 40; Smith and Shine 1997). Moreover, *S. equalis* has a simple placenta and therefore analyses of the vascular bed required for gas exchange are not confounded by substantial placentotrophy.

MATERIALS AND METHODS

We collected gravid female *C. taeniolatus* (n=10) from The Royal National Park (34° 7' S, 151° 03' E) and *Saiphos equalis* (n=17) from populations in the vicinity of Sydney (33°, 59' S, 150°, 03' E) and from Riamukka State Forest (31°, 20' S, 151°, 39 E), New South Wales, Australia. We allocated gravid female *C. taeniolatus* to three reproductive stages (non-gravid: n=3; post-ovulatory: < embryonic stage 25; n=5, and mid: embryonic stages 26–30; n=5) for sampling. Because *C. taeniolatus* typically lays eggs at a maximum embryonic stage of approximately 31 (S. L. Parker, unpubl. data), it was not possible to obtain uterine tissues at embryo stages beyond stage 31. We allocated gravid female *S. equalis* to five reproductive stages (non-gravid: n=3, postovulatory: < stage 25; n=3, mid: stages 26–34; n=7, late: stages 35–38; n=3, and near term: stages 39–40; n=4 females) for sampling. At the appropriate reproductive stage, we anesthetized females with an intraperitoneal injection of 0.6mg/L sodium pentabarbitone and we euthanized them by cervical dislocation. We removed uteri with eggs from females and immediately fixed uteri with eggs intact in 10% neutral buffered formalin for 24 h and then stored them in 70% ethanol at 4° C.

We visualized vascular structures using indirect immunofluorescence and laser-scanning confocal microscopy on whole-mount tissues preparations. We dissected eggs removed from each uterus and we removed embryos and extra-embryonic membranes. We determined the stage of embryos according to the Dufaure and Hubert (1961) staging system to determine the developmental stage achieved at the time of sampling. After determining stage, we dried embryos to a constant mass at 40° C and weighed them to the nearest 0.001 g. We immunohistochemically stained vascular tissues using an antibody generated against human von Willebrand Factor (Dako, Carpinteria, California USA). Von Willebrand Factor is a glycoprotein that is constitutively expressed in endothelial cells and functions in vertebrate blood-clotting processes (Sadler 1998). We used an Olympus Fluroview 1000 laser scanning confocal microscope with 488 nm laser excitation and 525-555 nm emission filter to obtain stacks of images from stained tissues. Six fields of view were obtained along linear transects covering the surface each uterine egg chamber (635 x 635 μm per field of view at 200x magnification). We measured an index of vessel density (number vessels*0.4 mm^{-2}) using ImageJ image analysis software (National Institute of Health, Bethesda, Maryland, USA). We measured vessel density by superimposing a grid (72 x 72 μm grid squares) over each field of view and counting the number of vessel segments that touched each vertical line on the grid. Statistical analyses were conducted using SAS Statistical Package version 9.1.2 (SAS Institute, 2004). We evaluated contrasts of microvascular density between species and among embryonic stages using a two-factor analysis of variance (ANOVA). We analyzed the effect of embryonic stage on uterine vessel density using a single factor ANOVA. Analyses of the effect of embryonic stage on uterine vascular proliferation were based upon the average of data obtained from six fields of view for each uterine egg chamber. We analysed microvascular density and morphology were analysed using ImageJ image analysis software (National Institute of Health, Bethesda, Maryland, USA). For all tests, $\alpha = 0.05$.

RESULTS

Immunofluorescent labeling using anti-human von Willebrand Factor antibody resulted in strong positive

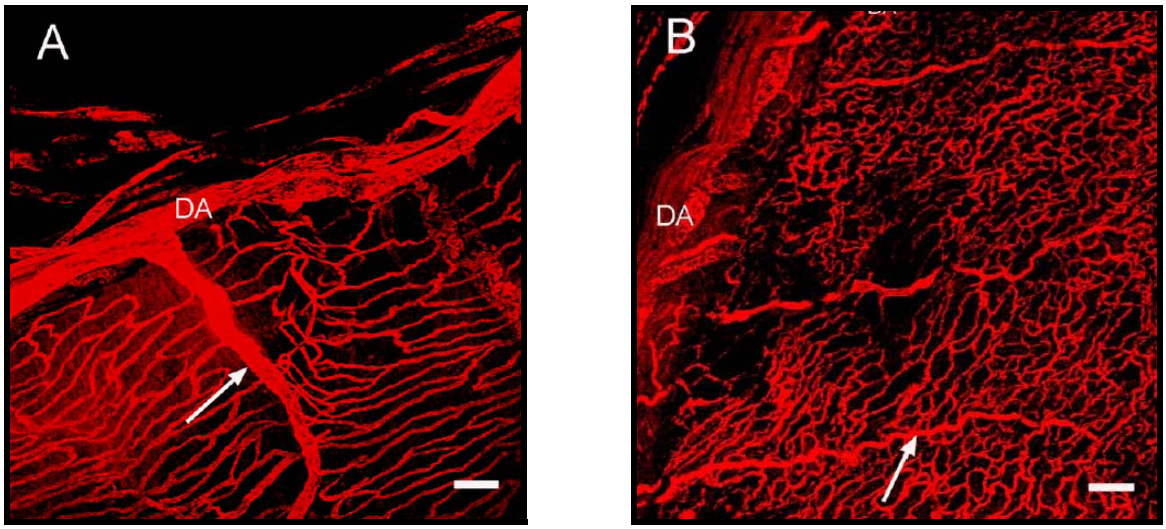

FIGURE 1. Immunofluorescent confocal micrographs of uterine microvasculature from non-gravid female *Ctenotus taeniolatus* (A) and *Saiphos equalis* (B). Arrows indicate primary lateral blood vessels; *DA*-dorsal artery. Scale bar is 100 μm.

staining of uterine vascular endothelial cells in both *C. taeniolatus* and *S. equalis* (Fig. 1A and 1B). We observed staining in vessels of all size classes ranging from primary vessels (50–75 μm diameter) to capillaries (3–10 μm diameter). The uterine vascular bed in both species consists of a reticulate network of capillaries that extend between lateral arteries that circle the uterus.

Overall vessel density at early and mid reproductive stages was 17% lower in *C. taeniolatus* than in *S. equalis* ($F_{1,16}$ = 5.72, P = 003; Table 1). For *C. taeniolatus*, uterine vascular density did not change significantly throughout the period of gravidity ($F_{1,8}$ = 2.5, P = 0.15). Vessel density in *S. equalis* also remained stable during the first half of pregnancy (i.e. the slow growth phase, prior to stage 31) but increased by approximately 48% at near term embryo stages ($F_{3,13}$ = 5.15, P = 0.014; Table 1). The increase in vessel density was also accompanied by a marked increase in tortuosity and vessel diameter (data not shown). In both species, mass of embryos changes relatively little during the first half of gestation (i.e. prior to approximately stage 31; Table 1). In *S. equalis*, however, embryo mass increased exponentially after embryo stage 35. For example, embryo dry mass increased by an average of 14 mg between stages 37–40 compared to only approximately 4 mg between stages 33–37.

DISCUSSION

Embryonic growth and vessel proliferation.—The rapid increase in embryonic oxygen demand late in development creates a physiological challenge for supporting embryonic growth during prolonged gestation. In the absence of adequate oxygen supply, embryonic development becomes retarded and may eventually result in death of the conceptus *in utero* (Black and Snyder 1980; Kam 1992; Parker and Andrews 2006). While vessel surface area is lower in *C. taeniolatus* compared to *S. equalis,* overall vessel density remained stable during the slow growth phase of devolpment (i.e. prior to Dufaure and Hubert [1961] embryo stage 31). During this period, embryos remain relatively small and embryonic oxygen consumption is also relatively low. Prior to the formation of the

TABLE 1. Mean values and standard errors for uterine vessel density and embryo dry mass sampled at early and mid embryonic stages for *Ctenotus taeniolatus*, and early, mid, late and near-term embryonic stages for *Saiphos equalis*. Number in bracket is the sample size. Within each row, values with different letters are significantly different from one another ($P < 0.05$).

	Early	Mid	Late	Near term
C. taeniolatus				
Nv (vessels * 0.4 mm $^{-2}$)	117.3 ± 4.4 (5) [a]	126.5 ± 3.7 (5) [a]	---	---
Embryo dry mass (mg)	0.028 ± 0.008 (5)	0.985 ± 0.067 (5)		
S. equalis				
Nv (vessels * 0.4 mm^{-2})	147.2 ± 22.1 (3) [a]	144.4 ± 9.3 (7) [a]	157.6 ± 4.2 (3) [a]	214.8 ± 16.5 (4) [b]
Embryo dry mass (mg)	0.018 ± 0.01 (3)	0.937 ± 0.25 (7)	5.60 ± 0.144 (3)	20.0 ± 4.8 (4)

respiratory chorioallantoic membrane at embryo stage 30, most, if not all of the embryo's oxygen demands are likely to be supplied by simple diffusion of oxygen from the maternal vascular bed directly to embryonic tissues. Because oxygen consumption of embryos prior to stage 30 is relatively low, the uterine vascular bed is likely to be able to accommodate embryonic oxygen demand relatively easily. In contrast, the latter half of development (beyond stage 35) is characterized by rapid growth in embryo mass and increased metabolic rate. For example, the metabolic rate of embryos of the oviparous North American skink *Plestiodon fasciatus* is approximately 25 ul O_2 h^{-1} at oviposition compared to approximately 160 ul O_2 h^{-1} late in development near stage 40 (Stewart and Thompson 1997). The marked increase in uterine surface area late in gestation observed in *S. equalis* therefore presumably occurs in response to elevated embryonic oxygen demand. Similarly, in oviparous species such as *P. fasciatus*, the chorioallantoic membrane also increases in size over the course of development, eventually covering the majority of the inner surface of the eggshell by late in development.

As in the uterus, the increase in surface area of the chorioallantoic membrane presumably occurs in response to the increasing oxygen requirements of late stage embryos. If this is true, the capacity of the uterine vasculature to increase in concert with the embryo size and metabolism is likely to be an important transitional step during the evolution of reptilian viviparity. Further comparative studies on the ontogeny of placental vascular development in squamate reptiles with diverse reproductive natural histories and among closely related taxa within families are required to test this hypothesis.

Angiogenesis is required to support embryonic development during gestation in species where eggs are retained, such as for *Saiphos equalis*, but the underlying mechanisms regulating uterine blood vessel proliferation in oviparous and viviparous reptiles is not well understood. Recent advances in molecular biology and bioinformatics, for the first time, provide the opportunity to identify fundamental molecular mechanisms associated with placental angiogenesis during the evolution of viviparity. For example, techniques from quantitative proteomics such as 2-dimensional gel electrophoresis and protein bioinformatics can be used to quantify global differences in uterine protein expression between oviparous and viviparous species during gestation. The ultimate goal of these studies is to identify fundamental genetic changes that are associated with the capacity to support prolonged gestation during the evolution of viviparity.

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