Evolution of Viviparity in Sceloporine Lizards: In Utero Po_2 as a Developmental Constraint during Egg Retention

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Accepted 11/1/2005; Electronically Published 4/5/2006

ABSTRACT

Reptilian viviparity evolves through selection for increasingly prolonged egg retention within the oviduct. In the majority of sceloporine lizard species, however, egg retention past the normal time of oviposition results in retarded or arrested embryonic development. In this study, we tested the hypothesis that the amount of embryonic development normally attained in utero is directly related to in utero oxygen partial pressure (Po_2). The three species of sceloporine lizards we used are characterized by developmental arrest (*Urosaurus ornatus*), retarded development (*Sceloporus virgatus*), and normal development (*Sceloporus scalaris*) when eggs are retained. We incubated eggs of these species for 10 d under conditions that simulated retention in the oviduct at a range of experimental oxygen partial pressures (Po_2). We estimated in utero Po_2 from a standard curve generated from the stage and dry mass of experimental embryos incubated for 10 d at known Po_2. The standard curve was then used to predict the Po_2 associated with the observed rate of development of embryos retained in utero. The results of this study showed that the degree of embryonic development attained in utero during egg retention was positively associated with in utero Po_2. The results indicate that oxygen availability in utero is associated with interspecific differences in the capacity to support intrauterine development in sceloporine lizards.

Introduction

Reptilian viviparity putatively evolves through selection for increasingly prolonged retention of eggs within the oviduct (Packard et al. 1977; Shine 1983, 1985). Recent studies in which gravid female lizards have been experimentally induced to retain eggs past the normal time of oviposition, however, have shown that embryonic development is often retarded or arrested during retention within the oviduct (Andrews and Rose 1994; Andrews 1997; Mathies 1998; Mathies and Andrews 1999, 2000). Moreover, even if environmental conditions are not suitable for nesting, oviposition may still occur at the normal time. More generally, the majority of oviparous lizard species lay eggs at developmental stages 26–31 of Dufaure and Hubert's (1961) staging system, where stage 0 is fertilization and stage 40 is hatching (Shine 1983; Blackburn 1995; Andrews and Mathies 2000). Eggs oviposited with embryos at stage 30 have completed approximately 25%–40% of their total development (development time in utero plus development time in the nest) at the time of oviposition (Shine 1983; DeMarco 1993). The observations that few lizard species appear capable of retaining eggs much past stage 31, coupled with the fact that embryonic development is often retarded or arrested during extended egg retention, suggests that the capacity to support embryonic development much past stage 31 is constrained (Mathies and Andrews 1999, 2000). Selection for prolonged egg retention will thus not lead to viviparity unless the physiological and morphological features necessary to support embryogenesis evolve concurrently with egg retention (Mathies and Andrews 1999, 2000; Andrews 2002).

Oxygen availability for the developing embryo has been implicated as an important constraint on the evolution of reptilian viviparity (Packard et al. 1977; Guillette 1982; Shine 1985). The latter half of embryonic development is characterized by substantial growth in mass (Xavier and Gavaud 1986) and a concomitant large increase in metabolic oxygen demand by the embryo (Dmi'el 1970; Birchard et al. 1984; Vleck and Hoyt 1991). During the later stages of development, the uterine environment becomes increasingly hypoxic as a result of the metabolic demands of the growing embryo (Webb and Brent 1972). Embryonic development should thus become arrested when embryonic oxygen demand exceeds intrauterine oxygen availability. The maximum embryonic stage at which squamate eggs are laid thus may be determined by the oxygen supply of the oviduct. Accordingly, species capable of supporting embryonic development during prolonged egg retention should possess physiological and morphological features to enhance oxygen availability to developing embryos during gestation.

Studies on the oviparous lizard *Sceloporus undulatus* provide direct evidence that oxygen is the proximate factor that con-

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Physiological and Biochemical Zoology 79(3):581–592. 2006. © 2006 by The University of Chicago. All rights reserved. 1522-2152/2006/7903-5094$15.00

strains the degree of embryonic development in utero (Andrews 2002; Parker et al. 2004). For example, Andrews (2002) demonstrated that embryonic growth in mass and morphological differentiation were retarded by incubation at low (7 kPa Po_2) oxygen partial pressure under simulated nest conditions. In a subsequent study, Parker et al. (2004) incubated eggs of *S. undulatus* in a range of Po_2's under conditions that simulated retention in the oviduct. In this latter study, the rate of growth and differentiation of *S. undulatus* embryos incubated at 8.6 kPa Po_2 under simulated in utero conditions was similar to that of the reduced rate of growth and differentiation of *S. undulatus* embryos retained in the oviduct for 10 d past the normal time of oviposition. The results of these studies suggest that the rate of embryonic development in utero is directly related to oxygen availability.

Comparative studies of species that differ in capacity to support embryonic development during egg retention have the potential to provide further insights regarding the influence of oxygen availability as a developmental constraint during egg retention. Sceloporine lizards are an ideal model taxon for comparative studies on the physiology of reptilian egg retention because the stage at which embryos are oviposited varies widely among species (Andrews 1997; Méndez-de la Cruz 1998; Mathies and Andrews 2000). Most species lay eggs with embryos at stages 28–30 (Mathies and Andrews 2000). A few species of *Sceloporus*, however, have the capacity to retain eggs for extended periods, with some species (e.g., *Sceloporus scalaris*) capable of retaining eggs until development is nearly complete (Andrews 1997; Mathies and Andrews 2000).

The objective of this study was to test the hypothesis that the degree of embryonic development attained by reptilian embryos in utero is directly related to in utero oxygen availability. To meet this objective, we evaluated responses of embryos (survival, growth in mass, and morphological differentiation) of three species of sceloporine lizards in response to incubation at a range of Po_2's under conditions that simulated retention in the oviduct. These data plus an assessment of actual embryonic development in utero were used to estimate the in utero Po_2 experienced by embryos during egg retention. The lizard species chosen for the study were selected on the basis of their differing capacities to support embryonic development during extended egg retention. *Urosaurus ornatus* (Baird) (sister genus of *Sceloporus*) is capable of retaining eggs facultatively for nearly 1 mo past the normal time of oviposition (Mathies and Andrews 1999). During egg retention, however, development of embryos is arrested at stages 30–30.5. *Urosaurus ornatus* embryos resume normal development once oviposition occurs. *Sceloporus virgatus* (Smith), a member of the *undulatus* species group, has the capacity to retain eggs to stages 36–37; however, development of retained embryos is retarded compared with embryos laid at the normal time of oviposition (Andrews and Rose 1994; Andrews 1997). Finally, *S. scalaris* (Smith), a member of the *scalaris* species group, has the capacity to retain eggs until development is nearly complete (i.e., stage 39). Moreover, during egg retention *S. scalaris* embryos develop at nearly the same rate as those laid at the normal time of oviposition (Mathies and Andrews 1995, 1996). On the basis of our knowledge of the developmental stage attained by embryos of the three species when retained past the normal time of oviposition, we predicted that the in utero Po_2 increases in the order of *U. ornatus* < *S. virgatus* < *S. scalaris*. We also predicted that survival and development of embryos would increase in the same order as a function of in utero Po_2.

Material and Methods

Collection and Maintenance of Gravid Females

Gravid females of *Urosaurus ornatus* ($n = 20$), *Sceloporus virgatus* ($n = 20$), and *Sceloporus scalaris* ($n = 2$) were captured at the Chiricahua Mountains, Cochise County, Arizona, during June 30–July 5, 2003. Gravid females of *U. ornatus* and *S. virgatus* were captured along canyons and dry washes approximately 2 km northwest of the American Museum of Natural History's Southwestern Research Station (SWRS). Gravid females of *S. scalaris* ($n = 2$) were captured in the vicinity of Barfoot and Rustler Park. On the day of capture, females were measured for snout-vent length and weighed to the nearest 0.1 g. Females were maintained in glass terraria supplied with bark and branches in an animal facility at SWRS. The open-air animal facility was shaded and screened and thus provided a natural photoperiod and diel temperature range. Females were watered by misting and fed with a variety of insects captured in the vicinity of SWRS daily. On July 6, females were placed singly in cloth bags for transport by car to Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, Virginia. From July 8, 2003, through the experiments, females were maintained in an animal facility at Virginia Tech. *Sceloporus virgatus* and *S. scalaris* were housed in plastic containers (73 cm × 48 cm × 22 cm, two or three females per container), and females of *U. ornatus* were housed in 94 × 51 × 50-cm plastic containers (10 females per container). Daily photoperiod was provided by ambient light from animal room windows and also from full spectrum Vita-lites (0800–1900 hours). A 100-W spotlight suspended at one end of each container (0900–1900 hours) provided a temperature gradient that allowed females to thermoregulate. Containers were provided with rocks and boards for basking. Females were fed crickets and mealworms daily. Water was provided daily by misting rocks, boards, and sides of terraria. Shallow plastic dishes were also placed in each container to collect water. Lizards were thus able to drink water accumulated on surfaces within the terraria while the shallow sand substrate remained dry. Experimental and animal care protocols were approved by the Virginia Tech Animal Care Committee (proposal 02-101-Biol).

Experimental Design: Urosaurus ornatus *and* Sceloporus virgatus

Urosaurus ornatus and *S. virgatus* females were assigned to one of two groups. Individuals were selected so that size classes of females were uniformly distributed between the two groups. Gravid females of one group (*U. ornatus*: $n = 10$, *S. virgatus*: $n = 10$) were obtained at the normal time of oviposition of *U. ornatus* and *S. virgatus* populations in Arizona. Oviposition of both species occurs with the onset of summer monsoon rains (Andrews and Rose 1994). In 2003, summer rains began during the second week of July. Accordingly, female *U. ornatus* and *S. virgatus* were killed by decapitation and eggs and oviducts surgically removed on July 12 and 14, respectively. Eggs were used to determine the rate of embryonic development over 10 d at a range of Po_2's (see "Manipulation of Po_2" below) under simulated in utero conditions (hereafter referred to as the "experimental group").

A second group was used to determine developmental rate of embryos retained in utero. Females from this group (*U. ornatus*: $n = 10$, *S. virgatus*: $n = 10$) were induced to retain eggs for 10 d past the normal time of oviposition by maintaining them on a thin, dry sand substrate to inhibit oviposition (hereafter referred to as the "retained group"). A dry substrate simulates drought conditions, and females facultatively respond by retaining eggs (Andrews and Rose 1994). Females from the retained group were killed by decapitation on July 22 and 24, respectively, and eggs and oviducts removed.

Developmental rates of embryos in the experimental and retained groups are comparable only if they are incubated at the same temperature. Therefore, body temperatures of gravid females of both species were measured during their activity period (0900–1900 hours) before oviposition. To minimize disturbance, body temperatures were measured using a Raytek Ranger ST 60 infrared temperature sensor. For a subset of measurements, body temperatures were also measured cloacally with a thermocouple thermometer (Physi-Temp Bat-12); on average, the two sets of measurements differed by less than 0.3°C. The body temperature of each female was measured one to four times, and no female was measured twice within the same day. Overall, average activity body temperatures were calculated for each species on the basis of the mean body temperatures of each individual. Activity body temperatures averaged 33.0° ± 0.42°C and 32.7° ± 0.39°C for *U. ornatus* and *S. virgatus*, respectively. We assumed that body temperature declined to that of ambient temperature when females were inactive and unable to thermoregulate. Mean body temperatures during inactivity (1900–0900 hours) were estimated by placing temperature probes in the substrate at the bottom of the enclosures and recording substrate temperatures at hourly intervals. The mean temperatures inside the enclosures during inactivity averaged 24.4° ± 0.01°C (*U. ornatus*) and 24.5° ± 0.05°C (*S. virgatus*). Overall body temperatures of female *U. ornatus* and *S. virgatus* during egg retention were calculated based on daily mean body temperatures, 27.8° ± 0.10°C and 27.6° ± 0.09°C, respectively.

Experimental Design: Sceloporus scalaris

Female *S. scalaris* were maintained on a dry substrate to inhibit oviposition, as described above. Because only two females were captured, we did not assign females to experimental and retained groups. Both females were therefore induced to retain eggs on a dry substrate until July 26. At this time, water was added to the substrate of their enclosures. Both females constructed nests; one oviposited on July 26 and the other on July 29. Eggs were thus laid approximately 2 wk after the time when oviposition would have occurred in Arizona. One egg from each clutch was sampled for embryo stage, and the remainder were allocated among experimental treatments.

Sampling of Eggs and Embryos

Eggs were weighed to the nearest 0.01 g within 2 h of removal from the female and numbered consecutively within each clutch. Eggs were maintained in contact with moist Kimwipes to ensure that the eggshells remained wet until placed under experimental conditions. A single egg from each clutch was used to measure the area of the chorioallantoic membrane (CAM) at oviposition. The extent of the CAM is visible through the moist eggshell and was therefore easily measured. The major and minor axes of each CAM were measured to the nearest 0.1 mm using dial calipers, and the surface area of the CAM was estimated using the formula for an ellipse. If the CAM covered more than 50% of the eggshell surface, the major and minor axes of the region of eggshell not covered by the CAM were measured, and the area not covered by the CAM was subtracted from the total surface area of the egg. The surface area of the egg was estimated from egg mass using the formula $\text{area} = 4.835\ \text{mass}^{0.662}$ (Panganelli et al. 1974), where area is in square centimeters (cm^2) and mass is in grams. The size of the CAM was expressed as absolute area and as a percentage of the surface area of the egg.

After completion of CAM measurements, one or two eggs were dissected to determine the embryonic stage at oviposition according to Dufaure and Hubert (1961), where stage 0 is fertilization and stage 40 is hatching. Half-stages were assigned for embryos exhibiting intermediate suites of traits. After staging, embryos were dried to a constant mass at 40°C and weighed.

Manipulation of Po_2

Growth and differentiation of experimental embryos were determined under conditions that simulated retention on the oviduct. To simulate oviductal conditions, eggs were incubated in

contact with a moist substrate so the channels of the eggshell remained fluid filled, as would normally occur within the oviduct. One or two eggs were placed in 70-mL glass specimen jars lined with Whatman filter paper moistened with distilled water (pH adjusted to 7.4). The filter paper was remoistened at least every 3 d to ensure that the channels of the eggshell remained fluid filled throughout incubation (see also Seymour et al. 1991 for similar experimental approaches). This procedure may best simulate in utero conditions because during egg retention eggs are pressed against the walls of the oviduct and are thus in close apposition to the maternal blood supply (R. S. Seymour, personal communication). Eggs from each clutch were allocated among four oxygen treatments (target values: 5%, 9%, 15%, and 21%; Table 1). As a control (simulating nest conditions), one or two eggs from each clutch were placed in specimen jars partially filled with vermiculite moistened with distilled water (0.7 : 1.0 g H_2O : vermiculite), corresponding to a water potential of −200 kPa (determined by thermocouple psychrometry, 21% O_2 only). Experimental and control eggs in the 21% O_2 treatment were thus treated identically, except that the shells of experimental eggs remained fluid filled during incubation, whereas the shells of control eggs remained dry and filled with air.

Experimental and control eggs were placed, according to treatment, into one of four airtight metal boxes, and the boxes were flushed with the appropriate gas mixture (O_2 and N_2) using a Cameron Instruments model GF-3/MP gas-mixing flowmeter. Air inside the boxes was humidified by bubbling the gas mixture through distilled water. Boxes were flushed at least every 3 d, and every time the boxes were flushed the oxygen concentration inside the boxes was measured using an Applied Electrochemistry S-3A/II oxygen analyzer. Mean oxygen concentrations (in dry air) over the course of the study period were 5.8%, 8.7%, 15.3%, and 20.4%. On the basis of a mean air pressure at Blacksburg (625 m) of 94.5 kPa (711 mmHg), a mean incubation temperature of 28°C, and a P_{H_2O} of 3.8 kPa of water vapor in air, the actual values of Po_2 for the four oxygen treatments during the study period were 5.3, 7.9, 13.8, and 18.5 kPa Po_2.

The boxes were placed in a single programmable temperature chamber and incubated for 10 d under a fluctuating temperature regime. The temperature regime was selected to match the body temperatures of females in the retained group. Temperatures inside the boxes varied linearly for 4 h between daily maximum and minimum temperatures (mean daily maximum: 32.8°C, mean daily minimum: 23.1°C, overall mean: 27.9°C). Incubation temperatures were measured inside the boxes where the eggs were maintained: for simulated oviductal treatments, the temperature probe was placed at the bottom of the specimen jar in contact with the moistened filter paper. For simulated nest treatments, the temperature probe was placed in the center of the specimen jar and covered with vermiculite. The boxes were rotated within the chamber every 3–5 d to minimize position effects on embryonic development.

Table 1: Allocation of eggs from each clutch into treatments

Egg No.	Po_2 (kPa)	Treatment
1	…	Sampled at oviposition
2	5.3	Simulated oviduct
3	7.9	Simulated oviduct
4	13.8	Simulated oviduct
5	18.5	Simulated oviduct
6	18.5	Simulated nest

Note. Eggs 2–5 of each clutch were incubated under simulated in utero conditions, and egg 6 was incubated in vermiculite, simulating nest conditions. When clutches had more than six eggs, eggs were systematically allocated among simulated oviductal treatments.

Estimation of In Utero Po_2

In utero Po_2 was estimated by comparing the developmental rate of embryos retained in utero (where Po_2 is unknown) with the developmental rate of embryos incubated under simulated in utero conditions at known Po_2's. After 10 d of incubation/retention, eggs from the experimental and retained groups were dissected to determine CAM area, embryo stage, and embryo dry mass ("Sampling of Eggs and Embryos" above). To estimate Po_2 in utero, a standard curve was generated from the stage and dry mass of experimental embryos incubated for 10 d under known Po_2. The standard curve was then used to predict the Po_2 associated with the observed in utero rate of embryonic differentiation (stage) and growth (dry mass).

Data Manipulation and Statistical Analyses

Statistical analyses were conducted using the SAS statistical package (SAS Institute 1996). Because no embryos of *U. ornatus* survived in the 5.3 and 7.9 kPa Po_2 treatments, the effects of oxygen treatment on survival of embryos in the 13.8 and 18.5 kPa Po_2 treatments were analyzed using a Fisher's exact test. For *S. virgatus*, the effect of oxygen treatment on egg survival in the 5.3, 7.9, 13.8, and 18.5 kPa treatments was analyzed using a χ^2 test of independence (FREQ procedure). The effect of oxygen treatment on embryo survival in the 18.5 kPa Po_2 treatment and control (simulated nest) was analyzed using a Fisher's exact test. Contrasts of embryonic features and water uptake by eggs for oviposited (day 0) and retained groups (day 10) were analyzed using a Student's *t*-test (*t*-test procedure). The effect of oxygen treatment on embryonic features and water uptake by eggs at 10 d was evaluated using analysis of covariance (ANCOVA; GLM procedure) with values at oviposition of em-

bryo dry mass (when dry mass was the dependent variable), stage (when stage was the dependent variable), or wet egg mass (when CAM area was the dependent variable) as covariates. Analyses of treatment effects were on the basis of clutch means. No *U. ornatus* embryos survived in the 7.9 kPa treatment, although embryos increased substantially in dry mass and stage, suggesting that embryos survived until late in the 10-d observation period. Dead *U. ornatus* embryos from this treatment were thus included in our analyses on the effect of oxygen treatment on embryonic growth and differentiation, which otherwise included only live embryos. To assess the consequences of using dead embryos in analyses, we also conducted separate analyses of treatment effects using both live and dead *U. ornatus* and *S. virgatus* embryos (stage only; mass could not be determined reliably for embryos that died early in the 10-d period). Observations of relative area of CAM were arcsine transformed before analysis. When the covariate was not significant, single-factor ANOVA was used to evaluate treatment effects. Before ANCOVA, the assumption of homogeneity of slopes was tested. For all ANCOVAs, post hoc pair-wise comparisons were made using a least significant difference test on least squared means. For all ANOVAs, post hoc pair-wise comparisons were made using a Tukey's honestly significant difference test. Because only two *S. scalaris* clutches were available, sample sizes were too low for statistical analysis. Data from *S. scalaris* eggs and embryos, however, were reported for comparative purposes. Data are reported as the mean ± SEM unless otherwise reported, and probability values less than 0.05 were considered significant.

Results

Survival of Embryos Retained in Utero

Survival of embryos differed among species (Table 2). After 10 d of retention, survival by *Urosaurus ornatus* embryos was lower than that of *Sceloporus virgatus* embryos when assessed as the number of clutches containing dead embryos (Fisher's exact test: $n = 20$, $P = 0.033$; data not shown in Table 2) and as total embryo survival (Fisher's exact test: $n = 38$, $P = 0.007$). Fifty percent of retained *U. ornatus* clutches contained dead embryos, and overall survival of retained embryos was 67%. In contrast, all retained embryos of *S. virgatus* survived. All embryos of *Sceloporus scalaris* survived 14 or 17 d of retention in utero before they were transferred to the experimental treatments.

Survival of Experimental Embryos

Survival of embryos varied as a function of both treatment and species (Table 2). Survival increased with Po_2 and was lowest for *U. ornatus* and highest for *S. scalaris*. Survival of *U. ornatus* and *S. virgatus* embryos varied among oxygen treatments (*U. ornatus*: $\chi^2 = 33.1$, $n = 62$, df = 3, $P < 0.001$; *S. virgatus*: $\chi^2 = 39.6$, $n = 65$, df = 3, $P < 0.001$). Survival of *U. ornatus* embryos was lower than that of *S. virgatus* embryos in the 7.9 kPa Po_2 treatment (Fisher's exact test: $n = 33$, $P = 0.018$). Survival of *U. ornatus* and *S. virgatus* embryos did not differ in either the 13.8 (Fisher's exact test: $n = 31$, $P = 0.066$) or the 18.5 kPa Po_2 treatments (Fisher's exact test: $n = 31$, $P = 0.10$). For *S. scalaris*, survival of embryos was 50% in the 5.3 kPa treatment and 100% in the 7.9, 13.8, and 18.5 kPa treatments. Because of low survival of *U. ornatus* and *S. virgatus* embryos in the 5.3 kPa treatment, observations on embryonic differentiation and growth in this treatment were not included in subsequent analyses.

Table 2: Percentage survival of *Urosaurus ornatus*, *Sceloporus virgatus*, and *Sceloporus scalaris* embryos (experimental and retained groups)

	U. ornatus	*S. virgatus*	*S. scalaris*
Experimental group:			
Number of clutches	10	10	2
5.3 (simulated oviduct)	0 (16)	0 (16)	50 (4)
7.9 (simulated oviduct)	0 (16)	35 (17)	100 (4)
13.8 (simulated oviduct)	47 (15)	81 (16)	100 (4)
18.5 (simulated oviduct)	80 (15)	100 (16)	100 (4)
18.5 (simulated nest)	100 (10)	100 (11)	100 (3)
Retained group	67 (18)	100 (20)	100 (19)

Note. The number of embryos is given in parentheses. Embryos from experimental clutches were incubated for 10 d past the normal time of oviposition at 5.3, 7.9, 13.8, and 18.5 kPa Po_2. Embryos from the retained group were retained in utero for 10 d (10 *U. ornatus* females and 10 *S. virgatus* females) and about 2 wk (*S. scalaris*) past the normal time of oviposition.

Effects of Retention in Utero on Eggs and Embryos

The effect of retention in utero on embryonic development varied among species (Table 3). After 10 d of retention in utero, embryos of *U. ornatus* did not differ in stage or dry mass from that at the normal time of oviposition (day 0). Embryos at day 0 and at day 10 had a mean stage of about 30 and a mean dry mass of about 0.6 mg. In contrast, after 10 d of retention in utero, *S. virgatus* embryos had advanced from a mean stage of 32 to a mean stage of 33.8 and had nearly doubled in dry mass (2.3 to 4.2 mg, respectively). We could not compare the amount of embryonic growth and differentiation between oviposition and retention for embryos of *S. scalaris* because they were not sampled at the normal time of oviposition. Both females laid eggs with embryos at stage 39; that is, embryos were almost fully developed. Observations by Mathies and Andrews (1995) on embryos of *S. scalaris* at the time of normal oviposition suggest that embryos would have advanced from stages 34–36 to stage 39 during the roughly 2 wk of retention that we observed.

In contrast to embryonic development during egg retention,

Table 3: Comparisons of stage, embryo dry mass, wet egg mass, and absolute and relative area of chorioallantoic membrane (CAM) at day 0 and after 10 d of retention for *Urosaurus ornatus* and *Sceloporus virgatus*

	Day 0	Day 10	*P*
U. ornatus:			
Stage	29.6 ± .41 (10)	30.2 ± .164 (10)	.156
Embryo dry mass (mg)	.55 ± .064 (10)	.61 ± .061 (10)	.497
Egg mass (g)	.18 ± .008 (10)	.19 ± .007 (9)	.479
Absolute area of CAM (mm^2)	33.2 ± 2.42 (9)	37.9 ± 2.28 (9)	.169
Relative area of CAM (mm^2)	21 ± .013 (9)	24 ± .015 (9)	.229
S. virgatus:			
Stage	32.1 ± .208 (10)	33.8 ± .316 (10)	<.001
Embryo dry mass (mg)	2.33 ± .187 (10)	4.19 ± .313 (10)	<.001
Egg mass (g)	.35 ± .010 (10)	.36 ± .009 (10)	.611
Absolute area of CAM (mm^2)	115.7 ± 2.80 (10)	125.6 ± 4.74 (10)	.079
Relative area of CAM (%)	48 ± .007 (10)	50 ± .004 (10)	.067

Note. Values are clutch means ± SEM (number of clutches). Statistical tests of egg and embryonic features were made using *t*-tests.

egg wet mass and absolute and relative area of CAM did not differ between embryos at day 0 and day 10 for either *U. ornatus* or *S. virgatus*. Eggs therefore did not take up water, and the CAM did not increase in size during the 10 d of retention.

Growth and Differentiation of Experimental Embryos

Development of embryos varied as a function of treatment (Tables 4, 5; Figs. 1, 2). Growth in mass of embryos of all species increased with oxygen availability (Table 5; Figs. 1, 2). For both *U. ornatus* and *S. virgatus*, growth of experimental embryos incubated at 18.5 kPa Po_2 was reduced compared with the growth of embryos incubated under simulated nest conditions at 18.5 kPa. In contrast, growth of experimental *S. scalaris* embryos incubated at 18.5 kPa Po_2 was similar to that of embryos incubated at 18.5 kPa Po_2 under simulated nest conditions (Fig. 2). Treatment effects on differentiation (stage) paralleled those of growth in mass (Table 5; Fig. 1). ANOVAs using both live and dead *U. ornatus* and *S. virgatus* embryos gave the same significance levels for treatment contrasts as those presented in Table 5.

Of the three species, *S. scalaris* embryos were the least affected by incubation at low Po_2. For example, three of four embryos in the 5.3 kPa Po_2 treatment attained stage 40, as did all experimental embryos incubated at 7.9, 13.8, and 18.5 kPa and control embryos incubated at 18.5 kPa Po_2 during the 10-d experimental period. Seven (of the eight) *S. scalaris* eggs that remained under experimental conditions, after the 10-d sample, hatched after a total of 12–16 d of incubation. There was a trend of increasing incubation period with decreasing Po_2. On average, the incubation period was shortest in the 18.5 kPa Po_2 simulated oviductal treatment (mean = 12.5 ± 0.5 d) and longest in the 5.3 kPa Po_2 treatment (mean = 15.5 ± 0.5 d). Hatchling masses increased as a function of Po_2. Mean hatchling masses in the simulated oviductal treatments after 12–16 d of incubation were 36.2 ± 0, 49.9 ± 8.5, 59.7 ± 4.8, and 66.5 ± 4.3 mg (5.3, 7.9, 13.8, and 18.5 kPa Po_2 treatments, respectively) and 63.1 ± 0 mg in the 18.5 kPa Po_2 simulated nest treatment.

Mass and Chorioallantois of Experimental Eggs

Eggs of all three species exhibited similar patterns of water uptake as judged by the wet mass of eggs after 10 d of incubation (Tables 4, 5). Mass of eggs increased in parallel with oxygen availability, and eggs in the simulated nest treatment took up less water than eggs in any of the experimental treatments.

The size of the chorioallantois paralleled that of wet egg mass. In general, the absolute and relative areas of the CAM increased with increasing oxygen. The absolute area of the CAM of eggs incubated in vermiculite was lower than that of eggs incubated in water at 18.5 kPa Po_2. Because CAM coverage was essentially complete, eggs that took up less water would have had lower surface areas and thus absolutely smaller CAMs. This is why, for example, the relative areas of the CAM in the 18.5 kPa treatment were almost identical (about 100%) for all species at the end of the 10-d period.

In Utero Po_2

Estimates of in utero Po_2 projected from growth in dry mass and stage differed slightly (1–2 kPa Po_2) for both *U. ornatus* and *S. virgatus*. Similarly, estimates of in utero Po_2 based on embryo stage differed when both live and dead embryos were used to generate the standard curve. Nonetheless, lower esti-

Table 4: Comparisons of wet egg mass and absolute and relative area of chorioallanotoic membrane (CAM) at 10 d of incubation for *Urosaurus ornatus*, *Sceloporus virgatus*, and *Sceloporus scalaris*

Variable	*U. ornatus*	*S. virgatus*	*S. scalaris*
Egg wet mass (g):			
5.3	…	…	1.12 ± .014 (2)
7.9	…	.86 ± .022 (4)	1.67 ± .229 (2)
13.8	.46 ± .050 (5)	.98 ± .049 (9)	1.62 ± .146 (2)
18.5	.50 ± .038 (8)	1.02 ± .051 (10)	1.65 ± .295 (2)
18.5 (simulated nest)	.32 ± .031 (10)	.65 ± .024 (6)	1.06 ± .007 (2)
Absolute area of CAM (mm^2):			
5.3	…	…	521.2 ± 5.78 (2)
7.9	48.6 ± 4.70 (5)	353.8 ± 9.08 (4)	676.1 ± 61.87 (2)
13.8	213.1 ± 35.53 (5)	431.7 ± 20.54 (9)	665.9 ± 39.64 (2)
18.5	285.9 ± 19.53 (8)	465.8 ± 20.85 (10)	670.7 ± 80.11 (2)
18.5 (simulated nest)	223.9 ± 11.05 (10)	363.4 ± 9.14 (6)	503.4 ± 22.02 (2)
Relative area of CAM (%):			
5.3	…	…	100 ± 0 (2)
7.9	33 ± .124 (5)	81 ± .009 (4)	100 ± 0 (2)
13.8	86 ± .047 (5)	90 ± .018 (9)	100 ± 0 (2)
18.5	92 ± .031 (7)	95 ± .020 (10)	100 ± 0 (2)
18.5 (simulated nest)	99 ± .0002 (10)	100 ± 0 (6)	100 ± 0 (2)

Note. Values are clutch means ± SEM (number of clutches). Simulated nest indicates clutches incubated in vermiculite. Values for embryo stage and dry mass are presented in Figures 1 and 2.

mates for *U. ornatus* than *S. virgatus* supported our initial prediction (Fig. 1). Embryos of *U. ornatus* retained in utero for 10 d had a mean dry mass of 0.6 mg and a mean stage of 30 (Table 3). These values correspond most closely to those of *U. ornatus* embryos incubated under simulated oviductal conditions at 5–6 kPa Po_2 (6%–7% Po_2). In contrast, embryos of *S. virgatus* retained in utero for 10 d had a mean dry mass of 4 mg and a mean stage of 34 (Table 3). These values correspond most closely to those of *S. virgatus* embryos incubated at 9–11 kPa (10%–12% Po_2) under simulated oviductal conditions.

We suspect that development of *S. scalaris* embryos is not limited by in utero oxygen availability during extended egg retention. Our conclusion is based on four main observations. First, gravid female *S. scalaris* retained eggs until embryonic development was nearly complete (i.e., stage 39). Second, we did not observe differences in growth between experimental embryos and embryos incubated under simulated nest conditions at 18.5 kPa Po_2. Third, eggs hatched in all treatments while under experimental conditions. Finally, in previous studies, *S. scalaris* embryos retained in utero developed at the same or nearly the same rate as embryos incubated under standard conditions (Mathies and Andrews 1996; Andrews 1997).

Discussion

Interspecific Comparisons of Po_2 in Utero

The embryonic stages at which eggs of the three species were oviposited in the laboratory represent the normal range of stages for eggs oviposited in the field. Because development of *Urosaurus ornatus* embryos is arrested at about stage 30, gravid females oviposit eggs with embryos at stages no greater than 30 (Mathies and Andrews 1999). Females of *Sceloporus virgatus* normally oviposit at stages 31–33 (Andrews and Mathies 2000), and *Sceloporus scalaris* from high-elevation populations (where we also collected females) oviposit at stages 35–37 (Mathies and Andrews 1995). As a consequence, we cannot directly compare developmental rates among the three species. Nonetheless, our results provide comparative estimates of in utero Po_2.

As predicted, *U. ornatus* embryos had the lowest in utero Po_2 as well as the lowest survival at low Po_2, of the three species (Table 2). *Urosaurus ornatus* embryos did not develop at 5.3 kPa Po_2. Some development occurred at 7.9 kPa Po_2, although no embryos survived the 10-d experimental period. Development of *U. ornatus* embryos thus became arrested at an in utero Po_2 of about 6 kPa (Fig. 1) when embryos reached stage 30 (Table 3). Developmental arrest presumably occurred when oxygen demands of the embryos exceeded oviductal oxygen availability. Embryo survival was reduced at 13.8 kPa Po_2 compared with the 18.5 kPa Po_2 simulated oviductal treatment, although there was no difference in growth and differentiation between the two treatments.

In contrast, *S. virgatus* embryos developed at Po_2's as low as 7.9 kPa, although survival, growth, and differentiation were much reduced compared with the 13.8 and 18.5 kPa Po_2 treatments (Table 2; Fig. 1). Moreover, two *S. virgatus* eggs sampled

Table 5: Statistical tests of responses of *Urosaurus ornatus* and *Sceloporus virgatus* eggs and embryos to incubation at 7.9, 13.8, and 18.5 kPa Po_2 for 10 d

	In Utero Contrasts	Results	In Utero versus Nest Contrasts (18.5 kPa Po_2)	Results
U. ornatus:				
Stage	$F_{2, 16} = 36.6$, $P \leq .001$	7.9 < 13.8 = 18.5	$F_{1, 15} = 4.8$, $P = .045$	18.5 < control
Embryo dry mass	$F_{2, 17} = 14.3$,[a] $P < .001$	7.9 < 13.8 = 18.5	$F_{1, 15} = 10.4$, $P = .006$	18.5 < control
Egg wet mass	$F_{1, 10} = 5.9$, $P = .036$	13.8 < 18.5	$F_{1, 15} = 156.2$, $P \leq .001$	18.5 > control
Absolute area of CAM	$F_{2, 12} = 56.1$, $P < .001$	7.9 < 13.8 = 18.5	$F_{1, 13} = 7.6$, $P = .016$	18.5 > control
Relative area of CAM	$F_{2, 14} = 16.4$,[a] $P < .001$	7.9 < 13.8 = 18.5	$F_{1, 15} = 8.7$,[a] $P = .010$	18.5 < control
S. virgatus:				
Stage	$F_{2, 19} = 10.1$, $P \leq .001$	7.9 < 13.8 = 18.5	$F_{1, 13} = 4.1$, $P = .065$	18.5 = control
Embryo dry mass	$F_{2, 19} = 16.8$, $P \leq .001$	7.9 < 13.8 < 18.5	$F_{1, 13} = 7.1$, $P = .019$	18.5 < control
Egg wet mass	$F_{2, 19} = 7.9$, $P \leq .003$	7.9 < 13.8 = 18.5	$F_{1, 14} = 28.8$, $P = .001$	18.5 > control
Absolute area of CAM	$F_{2, 20} = 5.1$,[a] $P \leq .016$	7.9 = 13.8, 7.9 < 18.5, 13.8 = 18.5	$F_{1, 13} = 18.7$, $P < .001$	18.5 < control
Relative area of CAM	$F_{2, 20} = 7.7$,[a] $P \leq .003$	7.9 = 13.8, 7.9 < 18.5, 13.8 = 18.5	$F_{1, 14} = 6.9$,[a] $P = .019$	18.5 < control

Note. Tests were ANOVAs and ANCOVAs comparing treatment effects for eggs incubated under simulated in utero conditions (second and third columns) and simulated in utero conditions at 18.5 kPa Po_2 versus simulated nest conditions at 18.5 kPa Po_2 (fourth and fifth columns). Post hoc pair-wise comparisons were made using a Tukey's honestly significant difference test (ANOVAs) or a least significant difference test on least squared means (ANCOVAs).

[a] Analyzed using single-factor ANCOVAs.

after 15 d of incubation at 7.9 kPa Po_2 contained living embryos at stage 35 (S. L. Parker, unpublished data). In previous studies, embryos of *S. virgatus* retained in utero for as long as 1 mo beyond the normal time of oviposition developed as far as stage 37. Development of retained embryos, however, was retarded and survival to hatching was reduced compared with control eggs laid at the normal time of oviposition (Andrews and Rose 1994; Andrews 1997). For example, after 30 d of retention in utero, the dry masses of *S. virgatus* embryos were less than half those of eggs laid at the normal time of oviposition. Similarly, in this study, *S. virgatus* embryos incubated at 7.9 kPa Po_2 were less advanced developmentally, by about three stages, and had dry masses less than half those of embryos incubated under simulated nest conditions (Fig. 1). Embryos of *S. virgatus* were still developing, albeit relatively slowly, at a predicted in utero Po_2 of 10–11 kPa. Whether this Po_2 would be sufficient to support the metabolic demands of the embryo throughout development until hatching is not known, however.

Sceloporus scalaris embryos exhibited the highest survival and greatest growth at low Po_2 of the three species (Tables 2, 4; Fig. 2). Because our observations were restricted to two clutches of eggs, we cannot rule out the possibility that our results may not represent a typical response of *S. scalaris* embryos to incubation at low Po_2. Our results (albeit based on a small sample) are supported, however, by observations from previous studies demonstrating that development of *S. scalaris* embryos is not retarded during prolonged egg retention (Mathies and Andrews 1996; Andrews 1997). Unlike embryos of *U. ornatus* and *S. virgatus*, *S. scalaris* embryos incubated in the 5.3 kPa Po_2 treatment survived and increased in mass over the 10-d experimental period. Furthermore, hatching occurred in all treatments, including the two eggs in the 5.3 kPa Po_2 treatment and the one egg in the 7.9 kPa Po_2 treatment. Finally, in contrast to *U. ornatus* and *S. virgatus* embryos, the dry masses of experimental embryos in the 18.5 kPa Po_2 treatment were very similar to those of control embryos incubated at 18.5 kPa Po_2 (18.5 kPa experimental [60.8 ± 1.15 mg] vs. 18.5 kPa control [58.3 ± 2.91 mg]; Fig. 2). Experimental embryos thus grew nearly the same amount as embryos incubated under simulated nest conditions. Retained embryos from both *S. scalaris* clutches were laid at stage 39; thus, embryos had completed the majority of their development in utero by the time oviposition occurred.

While we were not able to estimate in utero Po_2 directly for *S. scalaris*, the ability of embryos to survive and develop at 5.3 and 7.9 kPa Po_2 suggests that *S. scalaris* possess physiological features that enhance oxygen uptake during egg retention. Because oxygen consumption by embryos increases throughout development, the negative effects of low oxygen on embryo survival and growth should be greatest late in development, when oxygen demand of embryos is highest (Dmi'el 1970; Birchard et al. 1984; Vleck and Hoyt 1991). For example, when eggs of *S. undulatus* were incubated under simulated nest conditions at low (7 kPa) Po_2 during the latter half of development (stage 38 and later), survival and growth of embryos were reduced relative to those of embryos incubated at low Po_2 during the first half of development (Andrews 2002). In contrast, *S. scalaris* embryos were nearly fully developed (stage 39) when

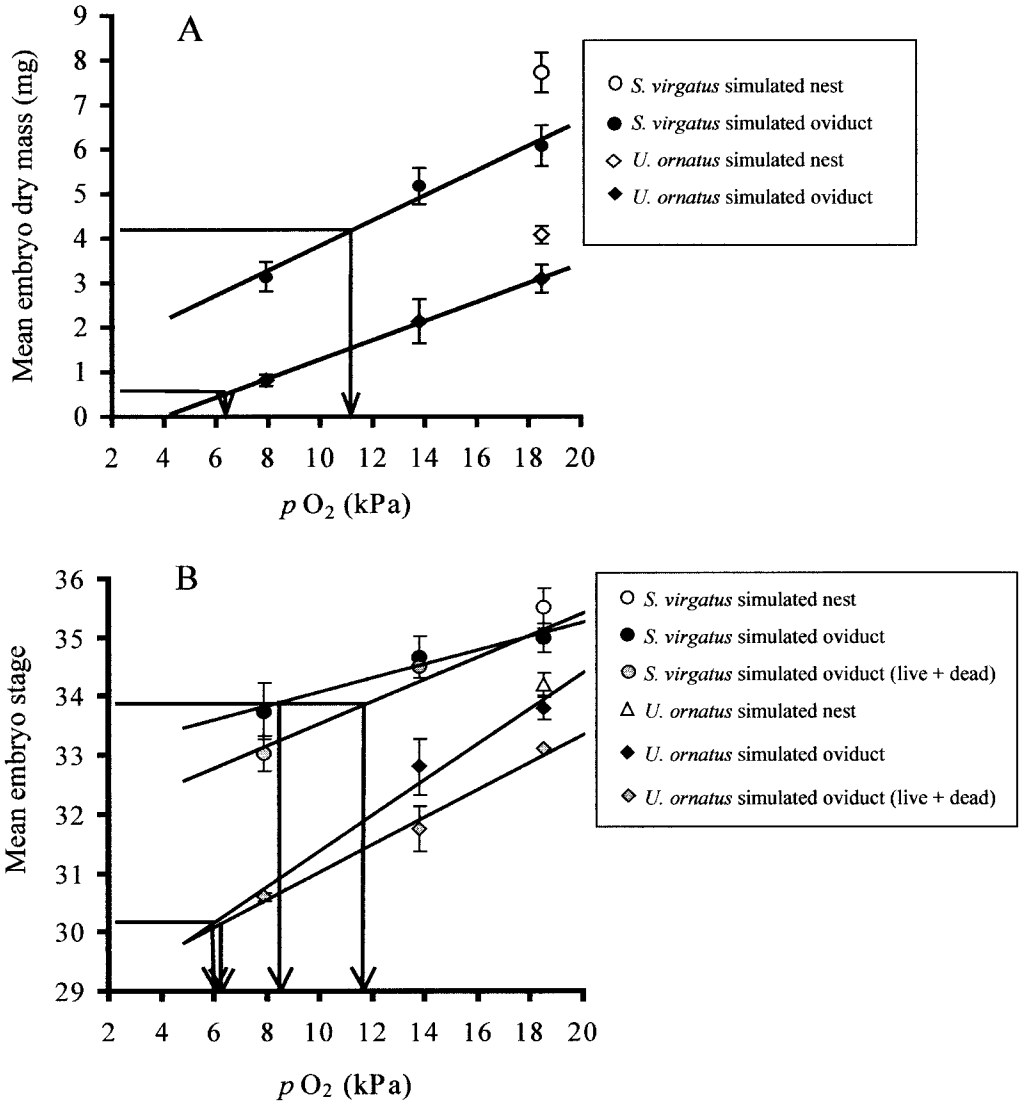

Figure 1. Embryonic growth (*A*) and stage (*B*; mean ± SEM) at 10 d in response to incubation at 7.9, 13.8, and 18.5 kPa Po_2 (simulated oviduct; *filled symbols*) and 18.5 kPa Po_2 control (simulated nest; *open symbols*) treatments. Light gray filled symbols represent values based on both live and dead embryos. Circles represent *Sceloporus virgatus*, and diamonds represent *Urosaurus ornatus*. Intersection of horizontal line with the *Y*-axis indicates the mean embryo dry mass or stage attained after 10 d of retention in utero. Arrows indicate estimated Po_2 associated with the degree of in utero development of lizard embryos during egg retention. Statistical comparisons of treatment effects are presented in Table 5.

placed under experimental conditions; yet embryos survived and increased in mass at low Po_2.

The results of our experiments confirmed the prediction that the degree of embryonic development attained during egg retention is directly related to in utero Po_2. Because our method of estimating in utero Po_2 is indirect, however, our results provide a relative estimate of the Po_2 experienced by lizard embryos retained in utero. Estimated in utero Po_2 was lowest for *U. ornatus* (5–6.5 kPa), higher for *S. virgatus* (10–11 kPa), and highest for *S. scalaris* (>11 kPa). Moreover, our results are consistent with observations that embryonic development of *Sceloporus undulatus* becomes arrested at stage 30 and at a partial pressure of 8.6 kPa (9% O_2) when retained past the normal time of oviposition (Mathies 1998; Parker et al. 2004). Thus, the relatively low estimate of in utero Po_2 for retained *U. ornatus* embryos is consistent with values observed in *S. undulatus*. Unlike *U. ornatus*, *S. virgatus*, and *S. undulatus*, extended egg retention has relatively little effect on growth and development of *S. scalaris* embryos. For example, when *S. scalaris* embryos were experimentally retained in utero past the

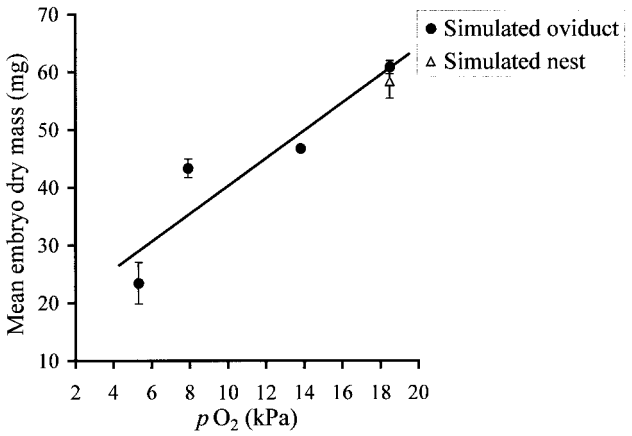

Figure 2. Dry mass of *Sceloporus scalaris* embryos at 10 d in response to incubation at 5.3, 7.9, 13.8, 18.5 kPa Po_2 (simulated oviduct; *circles*) and 18.5 kPa control (simulated nest; *triangles*) treatments. Stage is not illustrated because all embryos were at stage 39.

normal time of oviposition, embryos grew at nearly the same rate as control embryos incubated at ambient Po_2 (Mathies and Andrews 1996; Andrews 1997). These observations suggest that in utero Po_2 is substantially higher in *S. scalaris* than in the other species.

O_2 Availability and Embryonic Development

Previous studies on embryos from a wide variety of taxa, including insects (Frazier et al. 2001; Woods and Hill 2004), frogs (Seymour et al. 2000), turtles (Kam 1992), alligators (Deeming and Ferguson 1991), and chicks (Black and Snyder 1980), have demonstrated that embryonic development is reduced or arrested at low Po_2. For example, development of *Drosophila* embryos is completely arrested when embryos are incubated under severe hypoxic conditions, but embryos reinitiate development when oxygen content is increased (Teodoro and Farrell 2003). The results of our study implicates in utero oxygen availability as the primary factor responsible for interspecific differences in the capacity to support intrauterine development in sceloporine lizards. The stage attained by embryos retained in utero is apparently determined by the ability of gravid females to provide sufficient oxygen to meet the metabolic demands of developing embryos. Thus, embryonic development is presumably arrested when the oxygen demand of the growing embryos equals or exceeds the oxygen availability in the oviduct.

What physiological and morphological features affect in utero Po_2 for embryos retained past the normal time of oviposition? Oxygen must first diffuse from oviductal capillaries, through the fluid film surrounding the egg, and across the eggshell before being taken up by the embryonic blood supply in the chorioallantois. Thus, a combination of both maternal and embryonic features could potentially affect oxygen availability in utero. Of these features, a reduction in eggshell thickness, associated with extended egg retention, has been observed in several species (Guillette and Jones 1985; Mathies and Andrews 1995; Qualls and Shine 1998; Heulin et al. 2001). Reduced eggshell thickness could enhance oxygen availability to the embryo by decreasing the diffusion distance between maternal and embryonic circulation. In phrynosomatid lizards, however, eggshell structure and thickness are not associated with the capacity to support embryonic development during extended egg retention (Mathies and Andrews 2000). The most likely features mediating gas exchange in the oviduct are thus the vascularized respiratory surfaces of the oviduct and CAM and the oxygen-binding properties of embryonic blood.

The oviduct and the CAM are both believed to play an integral role in gas exchange (Guillette and Jones 1985; Yaron 1985; Masson and Guillette 1987; Blackburn 1993; Stewart and Thompson 1993). In two closely related species of *Sceloporus* lizards, for example, the vascular density of the oviduct is higher in the viviparous species (*Sceloporus bicanthalis*) than in the oviparous species (*Sceloporus aeneus*; Guillette and Jones 1985). The CAM covers the inner surface of the eggshell and functions as the primary respiratory membrane for the late-stage embryo. In this study, because the embryo stage at oviposition varied dramatically between species (mean stage at oviposition: *U. ornatus*, 29.6; *S. virgatus*, 32.1; *S. scalaris*, 39), we could not directly compare the absolute and relative sizes of the CAM among the three species. A previous study, however, demonstrated that at comparable embryonic stages, both absolute and relative CAM areas were larger for *S. scalaris* than for *S. virgatus* (Andrews 1997). The larger CAM of *S. scalaris* eggs would provide a greater surface area for gas exchange and thus enhance oxygen availability to the embryo. In addition to surface area, vascular density of the CAM could also play an important role in embryonic gas exchange. To our knowledge, no comparative studies of CAM vascular density have been conducted on any species of squamate reptile.

Oxygen-binding affinity of embryonic blood could also facilitate increased oxygen uptake of embryos retained in the oviduct. Several studies on viviparous squamates have demonstrated that the oxygen-binding affinity of fetal blood is greater than that of maternal blood (Grigg and Harlow 1981; Birchard et al. 1984; Holland et al. 1990; Ragsdale and Ingermann 1991). Increased blood oxygen affinity has been documented in chick embryos incubated under hypoxic conditions (Baumann et al. 1983; Ingermann 1992). The oxygen-binding properties of embryonic blood in oviparous reptiles is less well documented (Ingermann 1992), and whether oxygen-binding affinity of embryonic blood in oviparous reptile species differs from that of adults is unknown.

The results of this study support the hypothesis that selection for progressively longer periods of egg retention will not lead to viviparity unless features that enhance oxygen availability to

embryos evolve concurrently with egg retention. The contributions of maternal and embryonic physiological features and of shell morphology associated with gas exchange, however, have yet to be studied in a broadly comparative sense. Future studies should examine the mechanisms of embryonic gas exchange both among squamate families with diverse reproductive natural histories and among closely related taxa within these families.

Acknowledgments

We thank the staff of the Southwestern Research Station of the American Museum of Natural History for their logistic support, Anne McNabb for helpful comments on the manuscript, and Geoff Adams for assistance in the laboratory. This research was funded by a grant from the Virginia Tech Graduate Research and Development Program awarded to S.L.P.

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