

Low Oxygen: A Constraint on the Evolution of Viviparity in Reptiles

Robin M. Andrews*

Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

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ABSTRACT

The evolution of reptilian viviparity (live bearing) from oviparity (egg laying) is thought to require transitional stages of increasingly longer periods of embryonic development in utero, that is, longer periods of egg retention by the gravid female. Studies on sceloporine lizards demonstrate that embryonic responses to egg retention that is extended beyond the time of normal oviposition range from developmental arrest to normal development. The present study was designed to test the hypothesis that O₂ availability is the proximate factor that determines the rate and degree of development that reptilian embryos undergo in utero. Eggs of *Sceloporus undulatus* were incubated under conditions of low (LOX), normal (NOX), and high (HOX) oxygen both early and late in development. The LOX treatment consistently had a negative effect on development in terms of embryonic differentiation and growth, length of incubation, egg mortality, and hatchling size. Moreover, the LOX treatment had a stronger negative effect later in development than earlier in development. The results support the hypothesis that limited oxygen availability in utero acts as a developmental constraint. They further indicate that selection for extended egg retention, per se, will not lead to viviparity unless each incremental increase in the duration of egg retention is coupled with selection for traits (e.g., vascularity of oviduct and chorioallantois, hemoglobin oxygen affinity, etc.) that enhance O₂ availability to embryos. Such selection would be the most efficacious in cold climates where the effects of hypoxia would be the least likely to limit embryonic development.

Introduction

The evolution of viviparity (live bearing) from oviparity (egg laying) is thought to require transitional stages of increasingly

longer periods of embryonic development in utero, that is, longer periods of egg retention by the gravid female (Shine 1985). Lizards typically lay their eggs at developmental stage 30 (hatching or birth is at stage 40); few oviparous species lay eggs at stages >32, while about 20% of squamates are viviparous and thus give birth at stage 40 (Shine 1985; Andrews and Mathies 2000). The paucity of oviparous species that lay eggs with embryos at stage 33 or more suggests that egg retention much beyond stage 30 may have a detrimental effect on development or that the evolutionary transition between oviparity and viviparity occurs rapidly or both (Blackburn 1995). Studies on the lizard sister genera *Urosaurus* and *Sceloporus* demonstrate that the response to extended egg retention by embryos varies among species (Andrews and Mathies 2000). At one extreme, embryonic development in utero is arrested and does not resume until eggs are laid (*Urosaurus*). At the other extreme, embryonic development continues normally in utero, even up to hatching (*Sceloporus*). Moreover, the pattern of development documented for embryos in utero continues after oviposition; species that continue to develop beyond stage 30 in utero also develop more rapidly after oviposition than species whose development in utero is arrested (Andrews et al. 1999).

What factor or factors limit development in utero? The elaborate placentas of viviparous lizards and the reduction in shell thickness of oviparous lizards that retain eggs during most of the gestation period support the idea that the availability of oxygen is an important constraint on the evolution of viviparity (Weekes 1935; Packard et al. 1977; Guillette 1982; Qualls 1996). While this long-held idea is consistent with the morphological changes that are associated with the transition from oviparity to viviparity, the relationship between embryonic development in squamate reptiles and oxygen availability has never been determined directly. Indeed, two other factors could limit embryonic development in utero. One of these is the build up of CO₂ (hypercapnia) as a result of embryonic metabolism. CO₂, however, has a substantially higher rate of diffusion through water (egg and oviductal fluids) for an equivalent partial pressure gradient than does O₂ (Withers 1992). Embryos in the uterine environment are thus more likely to suffer from hypoxia before hypercapnia would limit development. The other factor is that water availability is highly restricted while eggs are in the oviduct (Andrews and Rose 1994; Mathies and Andrews 1996; Qualls and Andrews 1999). Because squamate eggs normally at least double their mass by water absorption during incubation in the nest, substantial water uptake may be a requirement for completing development (Packard and Packard 1988).

The objective of this research was to test the hypothesis that

* E-mail: randrews@vt.edu.

the rate and degree of development that reptilian embryos undergo in utero and after oviposition is related to the amount of O_2 available to embryos (Andrews and Mathies 2000). I did so by manipulating the partial pressures of oxygen (PO_2 's) for oviposited eggs of *Sceloporus undulatus hyacinthinus* Green, a phrynosomatid lizard that is widely distributed throughout the eastern United States. Oviposition by most oviparous species of *Sceloporus*, including *S. undulatus*, occurs after about 25% of development is completed (DeMarco 1993). Thus, the effects of variable PO_2 can be determined for the latter 75% of development. I incubated eggs under conditions of low (LOX), normal (NOX), and high (HOX) oxygen and did so for embryos that were either early or late in development. I evaluated responses including the rate of embryonic development, hatching success, and hatchling phenotypes, including size, running speed, growth, and survival. Based on previous studies on the effect of oxygen availability on the development of turtle, alligator, and chick embryos, I made two predictions: (1) the rate of development and hatchling quality would increase in the order $LOX < NOX < HOX$; (2) given that O_2 demands of embryos are substantially greater later in development than earlier in development (Vleck and Hoyt 1991), then the negative effects of low oxygen should be more pronounced for embryos exposed late than early in development.

Material and Methods

Collection and Maintenance of Females

Gravid female *Sceloporus undulatus* ($n = 15$) were collected at several localities in Montgomery County, Virginia, between May 12–18, 2000. They were taken to an animal room at Virginia Polytechnic Institute and State University, Blacksburg, Virginia, where they were housed individually in plastic tubs ($47 \times 26 \times 21$ cm) containing damp sand as a substrate and boards for shelters and basking sites. Windows provided an ambient light cycle. Tubers were also illuminated by fluorescent Vita-lites (0800–1800 hours), and a 100-W spotlight placed at one end of each tub (0900–1800 hours) allowed females to thermoregulate. Females were fed (crickets and wax worms) and watered daily. Cages were checked several times daily for eggs. Females were released at the place of capture shortly after oviposition.

Collection and Initial Incubation of Eggs

Four of the 15 females laid eggs between May 19 and 23. Because *S. undulatus* females in the laboratory may retain eggs beyond the time of normal oviposition (Andrews and Mathies 2000; Mathies and Andrews 2000), oviposition was induced in the remainder on May 26 by injection of oxytocin. Embryos from one egg per clutch (see below) were staged at oviposition according to Dufaure and Hubert (1961). The mean stages of embryos at oviposition for the females that oviposited normally

and those for females in which oviposition was induced with oxytocin on May 26 were 28.3 and 29.0 (range 28–29 in both cases), respectively. Mean clutch size was 11.7 and ranged from eight to 17.

Each egg was weighed within a few hours of oviposition and numbered consecutively within a clutch. Each clutch was placed in a small plastic container with a loosely fitting lid. The incubation substrate was vermiculite moistened with distilled water. The initial ratio of water to dry vermiculite was 0.7 : 1.0, which provided a water potential of -200 kPa (determined by thermocouple psychrometry). The containers were placed in an incubator in which the daytime temperature was 33°C (8 h) and nighttime temperature was 23°C (8 h), and the temperature ramped linearly for 4 h between these temperatures in the morning and the evening (mean = 28°C). This temperature regime is similar to the temperature fluctuation in natural nests of *S. undulatus* (Andrews et al. 2000). Eggs were maintained under these “normal” conditions until the dates that experimental conditions were imposed (see “Experimental Design”).

Experimental Design

Eggs were allocated to three treatments during incubation: LOX ($PO_2 = 7$ kPa, 53 mmHg), NOX ($PO_2 = 18$ kPa, 138 mmHg), or HOX ($PO_2 = 28$ kPa, 209 mmHg). The LOX treatment was designed to simulate in utero PO_2 's. Because the PO_2 that reptilian embryos experience in the oviduct is unknown, the PO_2 in the LOX treatment was arbitrarily set to the upper part of the range recorded for embryonic tissues of fish and birds (Ar and Mover 1994). PO_2 of the NOX treatment was that of Blacksburg, Virginia. PO_2 of the HOX treatment was chosen to increase the percentage of oxygen in the air by about 50%. Once oviposited, eggs in the shallow nests (4–6 cm) that characterized *Sceloporus* and other small squamates are unlikely to experience conditions in which oxygen would limit development (Packard and Packard 1988). For example, even toward the end of development in the relatively deep nest (16 cm) of the turtle *Chelodina expansa*, oxygen tension averaged 18.6 kPa (Booth 1998). Oxygen tensions in the LOX treatment were thus substantially lower than those squamate eggs experience in a nest.

Treatments were imposed at two times during development. One set of eggs (nine clutches) was exposed to the experimental treatments during the first half of the anticipated 52-d developmental period (based on Andrews et al. 2000). On May 26 (day 0), embryos in this group were at stages 28 and 29. Because of technical problems, however (the boxes initially used were not airtight), experimental conditions were not imposed until day 7, when airtight boxes were obtained. Experimental conditions thus extended from day 7 to day 28 with oviposition at day 0 (group 1). Eggs in this group were removed from experimental conditions on day 28 (June 23) and allowed to complete development under normal conditions. Another set

of eggs (six clutches that had been incubated under normal conditions since oviposition) was exposed to experimental conditions from June 23 to hatching during the last half of development (group 2). On June 23, when observations on group 2 were initiated, embryos from the six clutches were all at stage 38. The allocation of eggs from each clutch to the three treatments within the two groups is shown in Tables 1 and 2.

Egg Incubation and Manipulation of P_{O_2}

The plastic containers with eggs allocated to groups 1 and 2 were placed, according to their treatment, into one of three airtight metal boxes on June 2 and June 23, respectively. The boxes were fitted with inlet and outlet ports and were flushed with appropriate gas mixtures using a gas-mixing flowmeter (Cameron Instrument, Model GF-3/MP) connected to tanks of compressed N_2 and O_2 . Boxes were flushed with the appropriate gas mixture at least every 3 d initially and daily once hatching began. Lids from the small plastic egg containers were removed while eggs were in the boxes to ensure that eggs would be exposed to experimental P_{O_2} 's. To reduce moisture loss from the vermiculite and eggs, the gas stream was bubbled through distilled water to saturate the air flowing through the boxes. The amount of oxygen (%) in the boxes was checked during incubation with an Applied Electrochemistry S-3A/II oxygen analyzer. Preliminary observations 1–3 d after the boxes were flushed showed that the amount of O_2 in the boxes had varied only slightly (maximally $\pm 0.6\%$) over this period. Boxes were therefore flushed with gas at intervals of 1–3 d during the experimental period. Mean oxygen levels (in dry air) during incubation for the LOX, NOX, and HOX treatments were 7.8%,

Table 1: Sampling dates and allocation of eggs from each clutch into experimental treatments (low oxygen [LOX], normal oxygen [NOX], or high oxygen [HOX]) for group 1

Egg (No.)	Day 0 (May 26)	Day 28 (June 23)	Hatching
1	Sampled
2, 3	LOX	Sampled	...
4	LOX	...	Hatched
5, 6	NOX	Sampled	...
7	NOX	...	Hatched
8, 9	HOX	Sampled	...
10	HOX	...	Hatched
11+	All ^a	...	Hatched

Note. Early development only: 7–28 d of incubation. Day 0 is oviposition. Experimental conditions for group 1 were imposed on day 7 of incubation (see “Material and Methods” for details).

^a Allocated systematically to all three treatments.

Table 2: Sampling dates and allocation of eggs from each clutch into experimental treatments (low oxygen [LOX], normal oxygen [NOX], or high oxygen [HOX]) for group 2

Egg (No.)	Day 0 (May 19–26)	Stage 38 (June 23)	Hatching
1, 2	...	Sampled	...
3, 4	...	LOX	Hatched
5, 6	...	NOX	Hatched
7, 8	...	HOX	Hatched
9+	...	All ^a	Hatched

Note. Late development only: stage 38 to stage 40 (hatching)

^a Allocated systematically to all three treatments.

20.3%, and 30.6%, respectively. Given that the mean air pressure at Blacksburg (elevation 625 m) during the incubation period was 94.5 kPa (711 mmHg), that the average incubation temperature was 28°C, that the gas stream was humidified, and that water vapor at saturation has a P_{H_2O} of 3.8 kPa, the actual P_{O_2} 's in the boxes would have been 7.1, 18.4, and 27.8 kPa, respectively.

Sampling of Embryos and Observations on Hatchlings

One egg from each clutch in group 1 was sampled on May 26, and one egg from each clutch and treatment combination was sampled at the end of the treatment period (June 23). Eggs sampled were weighed. Embryos were then removed from the eggs and staged according to Dufaure and Hubert (1961) with the modification that half stages were assigned for embryos with intermediate suites of traits. Embryos were dried at 40°C to a constant mass and weighed.

Two eggs in group 2 were sampled on the day that they were placed under experimental conditions (June 23) using protocols described for group 1. One of the eggs was used for studies not reported here.

Containers were checked daily for hatchlings. On the day of hatching, each individual was weighed (mass), its snout-vent length (SVL) and tail length (TL) were measured, and it was given a unique toe clip for identification. Hatchlings were placed in small groups in the same tubs and under the same ambient conditions as those used for the adult females. Hatchlings were watered and fed to satiation twice daily on *Tribolium* larvae, wax worm larvae, and pinhead crickets that were dusted with a vitamin-mineral mixture.

I assessed hatchling quality in terms of sprint speed, survival, and growth in mass in the laboratory over 40–65 d after hatching and survival for 1 mo after release in the field. Sprint speed was measured 1 d after hatching in a 1-m-long electronically timed racetrack (see Qualls and Andrews 1999). Five infrared

photocells (connected to an electronic stopwatch) were spaced at 0.25-m intervals along the length of the track. The racetrack was in a walk-in environmental chamber set at a constant 31°C. Observations were made at midday when hatchlings were most active. They were given a half hour to acclimate to the temperature of the environmental chamber before their speed was measured. Hatchlings were placed at the beginning of the track and prodded gently with a paintbrush if they did not run. Running speed was measured three times for each individual with at least a 5-min resting period between trials. The time it took hatchlings to cover each 0.25-m interval and the total 1-m distance was recorded, and speed was expressed in meters per second in both cases.

On September 16, hatchlings were weighed and then released at one of the sites where females were captured. The release site was a forest clearing about 1,500 m² in area. The clearing, initially used as a log deck, included a number of large piles of woody debris, stumps, and scattered small shrubs. It was bounded on two sides by a gravel road and on the other sides by forest (described by Andrews et al. 2000). Hatchlings remain in the clearing at least for the first month of life (Warner 2001). The release site was searched thoroughly five times between October 13 and 28.

Data Manipulation and Statistical Analyses

The effect of treatment and clutch on embryonic and hatchling features was evaluated using two-factor ANOVAs (GLM procedure; SAS Institute 1996). Clutch was used as a class variable because of the strong influence of clutch on the embryonic development and hatchling phenotypes of reptiles (Van Berkum and Tsuji 1987; Bohn and Brooks 1994; Olsson et al. 1996; Andrews et al. 2000). Analyses were based on clutch means for each group and treatment combination. Water uptake by eggs in group 1 was the difference between the mean mass of eggs in each group and treatment combination on day 28 and the mass of the egg sampled on day 0 from that clutch. Embryonic differentiation for group 1 was assessed as the embryo stage reached by day 28 and as the difference between the stage reached by day 28 and the stage of the embryo sampled at oviposition. Embryonic growth was assessed as the dry mass of the embryo on day 28 and as the difference between the dry mass on day 28 and the dry mass of the embryo sampled at oviposition. Because all embryos allocated to group 2 were at stage 38 when they were exposed to experimental treatments, and hatching is at stage 40 (by definition), all embryos in this treatment completed two stage units during the observation period. I therefore used the time interval (d) between stage 38 and hatching to assess the rate of differentiation. Embryonic growth for group 2 was assessed as the difference between the estimated mean dry mass of hatchlings and the dry mass of the embryo sampled on day 28 from that clutch (dry mass of hatchlings could not be determined because hatchlings were to

be released). The dry mass of hatchlings was estimated as live mass times 0.15 (Mathies and Andrews 1999). Egg mass at hatching (and thus mass gain due to water uptake during incubation) could not be determined because eggs lose substantial amounts of water during the process of hatching. TL was assessed directly and as TL relative to SVL (TL/SVL). Length of the incubation period was the number of days between the day of oviposition and hatching. The rate of growth by hatchlings in the laboratory was calculated as the mass at release minus the mass at hatching divided by the number of days between hatching and release. Because some hatchlings were necessarily older than others and rate of growth decreases with age, I used the Julian date of hatching as a covariate in two-factor ANCOVAs so growth would be assessed independently of time of hatching. For this analysis, least squared means are reported. Sprint speeds used in analyses were the fastest speed (m/s) over any 0.25 m and 1 m of track. In preliminary analyses of sprint speed using ANCOVAs with SVL as a covariate, neither the covariate nor any of the interaction terms were significant. Therefore differences among groups/treatments were evaluated using two-factor ANOVAs. Because the results for analyses of speed over 0.25 m and 1.0 m were similar, only results for speeds over 0.25 m are reported here. Because of significant interactions, comparisons between group 1 and 2 hatchlings were made with one-factor ANOVAs for each treatment independently. Means and least squared means are presented \pm SEs, and *P* values <.05 are considered statistically significant.

Results

Oviposition in the laboratory (May 19–26) coincided with oviposition in the field. Two females were observed ovipositing at the field site on May 12, and three of nine adult females collected on May 19 had recently oviposited. Most of the adult females seen on June 9 were nongravid. These observations at the field sites indicate that eggs were obtained at the normal time of oviposition for *Sceloporus undulatus* at this site.

Embryonic development was strongly affected by the amount of O₂ available during development (Table 3); with one exception, treatment effects were significant for all variables measured (*P* < 0.001). The exception was the amount of water taken up by day 28 by eggs in group 1 that did not vary among treatments.

Embryos in group 1 were exposed to experimental conditions for 21 d (day 7–28). During this time, embryos from the LOX, HOX, and NOX treatments reached average stages of 35.6, 37.0, and 37.8, with increases of 6.6, 8.1, and 8.8 stages (Table 3). The amount of differentiation was thus the least for embryos in the LOX treatment but also differed between the NOX and HOX treatments; differentiation was greatest in the NOX treatment. Embryos from the NOX and HOX treatments attained greater dry masses (19 mg) than those attained by embryos (9 mg) from the LOX treatment. Growth in mass by embryos in

Table 3: Egg and embryo responses to the low-oxygen (LOX), normal-oxygen (NOX), and high-oxygen (HOX) treatments

Response	LOX ^a	NOX ^b	HOX ^c	Statistical Test		
				Clutch	Treatment	Results
Group 1:						
Water uptake by eggs (mg)	775 (40.0)	797 (38.1)	786 (41.5)	$F_{8,16} = 4.1,$ $P = .008$	$F_{2,16} = .2,$ $P = .855$	LOX = NOX = HOX
Stage on day 28	35.6 (.06)	37.8 (.15)	37.0 (.06)	$F_{8,16} = 1.9,$ $P = .139$	$F_{2,16} = 178.4,$ $P < .001$	LOX < HOX < NOX
Stage increment, days 0–28	6.6 (.15)	8.8 (.15)	8.1 (.15)	$F_{8,16} = 7.6,$ $P < .001$	$F_{2,16} = 178.4,$ $P < .001$	LOX < HOX < NOX
Embryo dry mass (mg) on day 28	9.3 (.29)	19.4 (.53)	19.5 (.53)	$F_{8,16} = 3.7,$ $P = .013$	$F_{2,16} = 304.2,$ $P < .001$	LOX < NOX = HOX
Embryo dry mass, increment (mg), days 0–28	8.8 (.27)	19.0 (.48)	19.0 (.49)	$F_{8,16} = 2.9,$ $P = .034$	$F_{2,16} = 304.2,$ $P < .001$	LOX < NOX = HOX
Group 2:						
Incubation length from day 28 (d)	25.4 (.77)	22.8 (.93)	23.5 (.72)	$F_{5,8} = 13.4,$ $P = .001$	$F_{2,8} = 19.9,$ $P < .001$	LOX > NOX = HOX
Embryo dry mass, increment (mg), day 28–hatching	42.3 (1.44)	59.0 (1.35)	58.4 (1.97)	$F_{5,8} = .9,$ $P = .513$	$F_{2,8} = 31.3,$ $P < .001$	LOX < NOX = HOX

Note. Data are reported as mean (SE). Statistical tests were two-way ANOVAs. Overall models were significant in all cases ($P < 0.02$). n = number of clutches.

^a Group 1: $n = 9$; group 2: $n = 5$.

^b Group 1: $n = 9$; group 2: $n = 6$.

^c Group 1: $n = 9$; group 2: $n = 5$.

the LOX treatment was thus retarded relative to that of embryos in the HOX and NOX treatments, which did not differ.

Embryos in group 2 were exposed to experimental conditions from stage 38 to stage 40 (hatching). This period averaged 23 d for embryos in the NOX and HOX treatments and 25 d for embryos in the LOX treatment (Table 3). Embryos from both groups 1 and 2 were thus exposed to experimental conditions about the same length of time (21–25 d). During the experimental period, group 2 embryos in the HOX and NOX treatments increased in dry mass by almost 60 mg while embryos in the LOX treatment increased by only 42 mg. Thus, during late development as well as during early development, differentiation and growth of embryos in the LOX treatment was retarded relative to that of embryos in the NOX and HOX treatments.

Mortality during incubation was low overall; of the 81 eggs that were not sampled during incubation, only nine did not hatch (11% mortality). Mortality, however, was distributed unevenly among groups and treatments. For group 1, only one (NOX treatment) of 38 eggs did not hatch. For group 2, one egg from the NOX treatment did not hatch, whereas seven eggs did not hatch in the LOX treatment (2×2 χ^2 -test, survivors

vs. nonsurvivors in the NOX/HOX vs. the LOX treatment: $\chi^2 = 10.22, P < 0.01$). Dissections of the unhatched eggs revealed that the seven embryos that died in the LOX treatment had developed to stage 40, but they apparently died after failing to escape the egg.

Hatchling attributes were also affected by variation in O_2 availability during development (Tables 4, 5). For both groups 1 and 2, hatchlings from the LOX treatment weighed less and were shorter in SVL, TL (not plotted), and relative TL than hatchlings from the NOX and HOX treatments (Fig. 1A–1C). Incubation length differed among treatments within groups (Fig. 1D). For group 1, HOX hatchlings had a slightly, but significantly, shorter incubation period than the NOX hatchlings, and both had substantially shorter incubation periods than the LOX hatchlings. For group 2, NOX and HOX hatchlings had similar incubation periods, and both were substantially shorter than were those of the LOX hatchlings.

In contrast, hatchling quality, as defined by sprint speed over 0.25 m and growth in mass in the laboratory, did not vary among experimental treatments for either group 1 or 2 (Tables 4, 5). Recovery of hatchlings after release in the field ranged from 11 survivors from the HOX treatment (38% of 29 re-

Table 4: Hatchling responses to the low-oxygen (LOX), normal-oxygen (NOX), and high-oxygen (HOX) treatments

Response	LOX	NOX	HOX
Snout-vent length (mm):			
Group 1	24.9 (.19, 9)	25.8 (.14, 8)	25.4 (.18, 9)
Group 2	23.3 (.16, 5)	25.4 (.18, 6)	25.6 (.22, 5)
Live mass (g):			
Group 1	.503 (.0218, 9)	.554 (.0138, 8)	.551 (.0145, 9)
Group 2	.462 (.0200, 5)	.547 (.0071, 6)	.561 (.0132, 5)
Tail length (mm):			
Group 1	27.4 (.69, 8)	30.2 (.36, 8)	29.7 (.56, 9)
Group 2	23.8 (.68, 5)	29.4 (.26, 6)	29.9 (.47, 5)
Relative tail length: ^a			
Group 1	1.107 (.0198, 8)	1.171 (.0146, 8)	1.168 (.0164, 9)
Group 2	1.014 (.0248, 5)	1.160 (.0104, 6)	1.160 (.0144, 5)
Growth (mg/d):			
Group 1	.018 (.0050, 8)	.022 (.0022, 7)	.021 (.0030, 9)
Group 2	.022 (.0037, 4)	.022 (.0020, 6)	.019 (.0017, 5)
Incubation length (d):			
Group 1	59.0 (.41, 9)	53.2 (.31, 8)	52.6 (.42, 9)
Group 2	57.8 (.68, 5)	54.1 (.46, 6)	54.3 (.46, 5)
Speed (m/s):			
Group 1	.69 (.075, 9)	.79 (.089, 8)	.87 (.091, 9)
Group 2	.57 (.088, 5)	.59 (.095, 6)	.80 (.072, 5)

Note. Results presented as mean (SE, *n* clutches). Group 1 represents individuals from eggs exposed to the experimental treatment during early development, and group 2 represents individuals from eggs exposed during late development (see Tables 1, 2). Results of statistical tests are presented in Table 5.

^a Tail length/snout-vent length.

leased), seven survivors from the NOX treatment (28% of 25 released), and four survivors from the LOX treatment (23% of 17 released; $P > 0.05$, $2 \times 3 \chi^2$ -test, groups 1 and 2 pooled).

Treatment had a greater impact on the size of hatchlings that were exposed to experimental conditions late in development (group 2) than individuals that were exposed early (group 1) in development (Fig. 1). Hatchlings in the LOX treatment from group 2 were shorter in SVL and had relatively shorter tails than hatchlings in the LOX treatment from group 1 (Fig. 1A, 1C). Similarly, hatchlings in the LOX treatment from group 2 weighed less than hatchlings in the LOX treatment from group 1 (Fig. 1B), but in this case, the difference was not statistically significant ($P = 0.094$). In contrast to the effect on hatchling size, time of exposure to experimental conditions had no effect on hatchling performance; no contrast of sprint speed over 0.25 m or growth in mass in the laboratory was significant ($P < 0.05$).

The only positive effect of HOX treatment was on incubation length for group 1; hatchlings in the HOX treatment hatched sooner (had faster development) than hatchlings from all other treatments. This effect was not observed for group 2, for which the incubation periods of the NOX and HOX treatments did not differ.

Mortality of hatchlings in the laboratory was low overall; of the 69 (three additional hatchlings escaped in the lab and were not recovered) individuals that hatched successfully, only 10 (14% mortality) did not survive for the 51–65 d that they were in the laboratory before release. The distribution of mortality appeared to be random with respect to treatment and group; two, four, and four hatchlings died from the NOX, HOX, and LOX treatments, respectively, and four were from group 1 and six were from group 2.

Pervasive clutch effects in this (Tables 3, 5) and other studies indicate the importance of including clutch as part of experimental designs for ecophysiological studies. Such designs allow the assessment of treatment effects independent of any clutch effects. Clutch effects are the result, in part, of the genetic contributions of both the maternal and paternal parents (Olsson et al. 1996). Strictly maternal effects also contribute to clutch effects. For example, egg mass at oviposition is a function, in part, of the size of the clutch; large clutches have less water at the time of oviposition than small clutches, presumably because increased pressure in the oviduct reduces water uptake (Qualls and Andrews 1999). In this study, a substantial amount of variation in embryonic development and hatchling phenotypes was explained by clutch. Most developmental attributes

Table 5: Statistical tests of treatment and clutch effects on hatchling responses

Response	Treatment	Clutch	Overall Model
Group 1:			
Snout-vent length	$F_{2,15} = 9.0, P = .003$	$F_{8,15} = 3.2, P = .024$	$F_{10,15} = 4.7, P = .004$
Mass	$F_{2,15} = 5.9, P = .013$	$F_{8,15} = 4.3, P = .007$	$F_{10,15} = 4.6, P = .004$
Tail length	$F_{2,14} = 14.0, P < .001$	$F_{8,14} = 4.3, P = .008$	$F_{10,14} = 6.6, P < .001$
Relative tail length ^a	$F_{2,14} = 9.1, P = .003$	$F_{8,14} = 4.7, P = .006$	$F_{10,14} = 5.8, P = .002$
Incubation length	$F_{2,15} = 255.8, P < .001$	$F_{8,15} = 7.0, P < .001$	$F_{10,15} = 58.0, P < .001$
Growth	$F_{2,12} = .3, P = .723$	$F_{8,12} = 3.0, P = .043$	$F_{11,12} = 2.5, P = .066$
Speed	$F_{2,15} = 1.1, P = .373$	$F_{8,15} = 0.8, P = .589$	$F_{10,15} = .9, P = .573$
Group 2:			
Snout-vent length	$F_{2,8} = 36.7, P < .001$	$F_{5,8} = .6, P = .709$	$F_{7,8} = 11.6, P < .001$
Mass	$F_{2,8} = 31.3, P < .001$	$F_{5,8} = 1.0, P = .457$	$F_{7,8} = 9.2, P = .003$
Tail length	$F_{2,8} = 66.8, P < .001$	$F_{5,8} = 1.2, P = .383$	$F_{7,8} = 21.4, P < .001$
Relative tail length ^a	$F_{2,8} = 36.3, P < .001$	$F_{5,8} = 1.5, P = .298$	$F_{7,8} = 12.3, P = .001$
Incubation length	$F_{2,8} = 19.9, P < .001$	$F_{5,8} = 8.0, P = .006$	$F_{7,8} = 14.0, P < .001$
Growth	$F_{2,6} = 1.1, P = .396$	$F_{5,6} = 2.8, P = .125$	$F_{8,6} = 2.8, P = .115$
Speed	$F_{2,8} = 1.7, P = .250$	$F_{5,8} = 0.9, P = .521$	$F_{7,8} = 1.2, P = .389$

Note. Analyses were two-factor ANOVAs except for estimated growth in mass, which was a two-factor ANCOVA. When treatment effects were significant ($P < 0.05$), the LOX treatment differed from the NOX and HOX treatments ($P < 0.05$, REGWQ a posteriori tests) and the NOX and HOX treatments did not differ from one another ($P > 0.05$). The only exception is group 1 incubation length, where all treatments differed from one another ($P < 0.05$).

^a Tail length/snout-vent length.

varied as a function of clutch (Table 3), and hatchling size and growth rate for group 1 exhibited significant variation associated with clutch. This was not true, however, for group 2, where strong treatment effects apparently overrode clutch effects (Table 5).

Discussion

Tests of Hypotheses

In general, the results of the experiments support the hypothesis that low levels of oxygen act as a developmental constraint. The LOX treatment consistently had a negative effect on development in terms of embryonic differentiation and growth, length of incubation, egg mortality, and hatchling size. An incubation environment with a P_{O_2} of 7 kPa clearly provided hypoxic conditions for development. Chick, turtle, and alligator embryos incubated under hypoxic conditions (McCutcheon et al. 1982; Kam 1993; Warburton et al. 1995) exhibit similar kinds of developmental inhibition. In addition, I found that inhibition of development and embryo mortality under hypoxic conditions was the greatest late in development, at the time when the oxygen needs of embryos are the highest. Both the slow rate of differentiation and growth of embryos in the LOX treatment in general and the especially negative effects of low oxygen late in development support the hypothesis that oxygen availability acts as a constraint on development.

If oxygen availability acts as a constraint on development, then even moderate increases in oxygen should enhance de-

velopment. In contrast to this prediction, the HOX treatment generally did not enhance development. The only trait that was enhanced was the length of incubation for group 1; it was slightly, but significantly, shorter for the HOX than the NOX treatment. Would development have been enhanced under levels of oxygen higher than the 30% O_2 in the HOX treatment? Development of chicken embryos exposed to 40%–70% O_2 is enhanced (McCutcheon et al. 1982; Stock et al. 1983). It is thus possible that oxygen levels in this study were not high enough to elicit a generally positive response from the lizard embryos. However, if the blood of embryos is saturated with oxygen under normal conditions (NOX treatment), then enhancing oxygen in the atmosphere would not increase oxygen availability to the embryos. Regardless of the reasons why the HOX treatment was not effective, the strong negative response to low oxygen in this study parallels observations on lizard embryos that have been retained in utero.

When oviparous lizards in the sister genera *Sceloporus* and *Urosaurus* retain eggs beyond the stage of normal oviposition, embryonic development is typically retarded and hatchlings are smaller than those from oviposited eggs incubated under normal conditions (Andrews and Rose 1994; Andrews 1997; Mathies and Andrews 1999; Andrews and Mathies 2000). For example, in this study, after 28 d of incubation under hypoxic conditions, *Sceloporus undulatus* embryos were about half the dry mass of control embryos. Similarly, after 30 d of retention in utero, embryos of *Sceloporus virgatus*, a comparably sized and closely related species, were somewhat less than half the

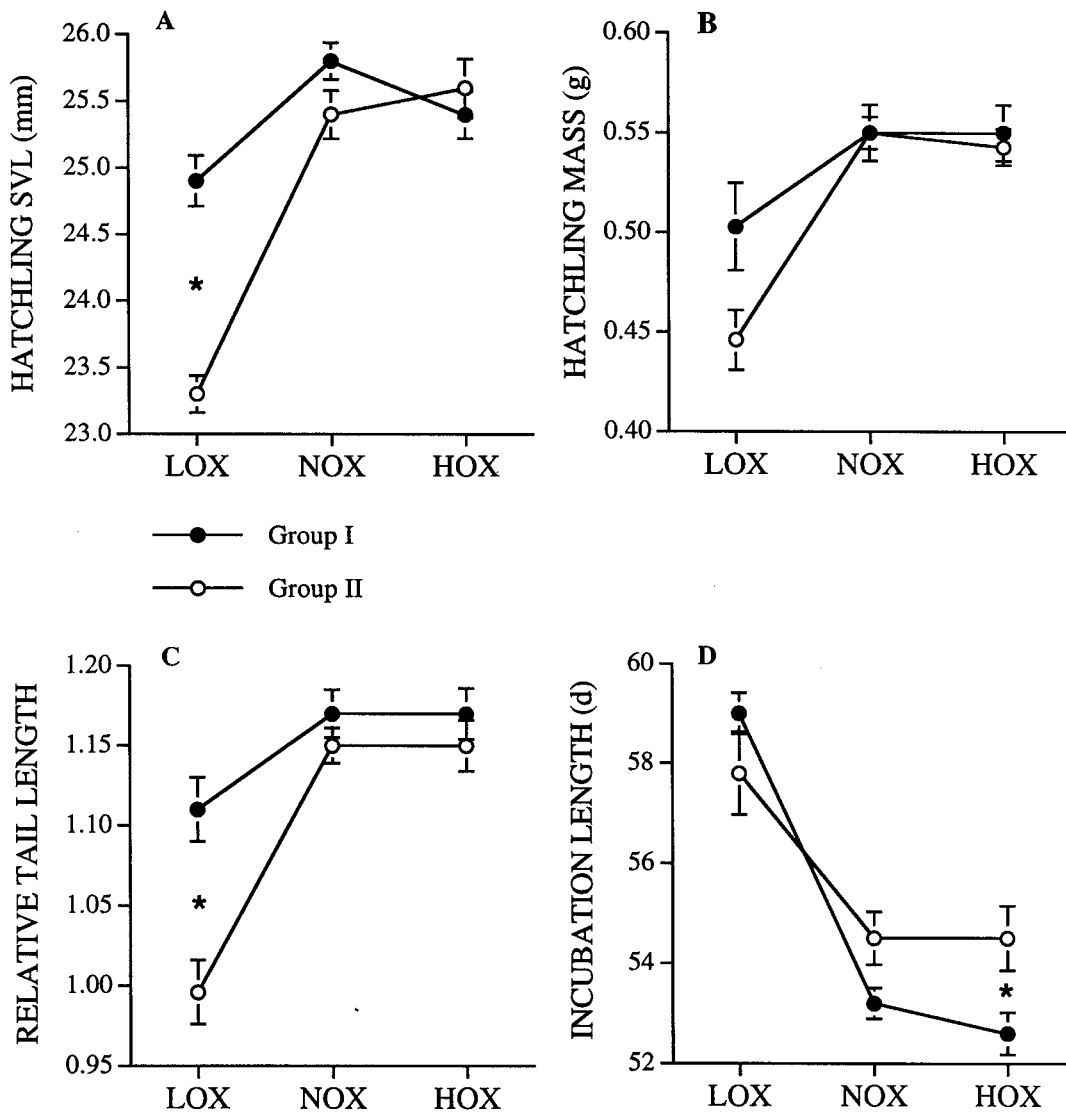


Figure 1. Hatchling responses (mean, SE) to the low-oxygen (LOX), normal-oxygen (NOX), and high-oxygen (HOX) treatments for groups 1 and 2. Group 1 (filled circles) represents individuals from eggs exposed to the experimental treatments during early development, and group 2 (open circles) represents individuals exposed during late development. Results of statistical comparisons of treatment effects within groups are presented in Table 5. Asterisks indicate significant contrasts between group 1 and group 2 for each treatment (one-factor ANOVA: $P < 0.001$, $P = 0.004$, and $P = 0.024$ for hatchling snout-vent length [SVL], relative tail length, and incubation length, respectively).

dry mass of control embryos (Andrews and Rose 1994). These parallels occurred despite the limited amount of water uptake by eggs during incubation in utero and the ample amounts of water available to embryos under experimental conditions in this study. Moreover, despite the negative effect of the LOX treatment on development in this study, eggs in all treatments took up similar amounts of water. Water limitation is thus not the proximate factor limiting development in retained eggs (see also Mathies and Andrews 1996). The parallel responses of embryos to extended retention in utero and to low Po_2 thus

support the hypothesis that negative effects of egg retention on embryonic development are the result of hypoxia.

The effects of hypoxia during incubation were limited to immediate effects on the development of the embryo itself. Hypoxia had no residual effects on hatchlings as measured by their survival, sprint speed, and growth in the laboratory over 40–65 d after hatching or their survival during the first month after release in the field. This conclusion, however, requires some qualification. First, in contrast to this study, Mathies and Andrews (1999) found that hatchlings from retained eggs of

Urosaurus ornatus had slower sprint speeds than hatchlings from control eggs. Second, only four phenotypic traits (growth, sprint speed, and survival in the laboratory; survival in the field) were measured in this study; other measures of hatchling quality could have been affected by incubation under hypoxic conditions. Third, survival in the field was monitored for only 1 mo, and while the trend was not significant, individuals from the LOX treatment did have the lowest survival. Nonetheless, hypoxia during incubation clearly can have negative effects on embryonic development, and these negative effects can impact selection for extended egg retention, and ultimately viviparity.

Implications for the Evolution of Viviparity in Squamates

The most widely accepted model for the evolution of viviparity posits that viviparity has its origins in cold climates (Shine 1985). The putative benefit of increases in the length of egg retention, and ultimately viviparity, in cold climates is that females, because of their thermoregulatory capabilities, are able to maintain higher mean temperatures for their eggs than the eggs would experience if placed in a nest. Because development is temperature dependent, eggs that are retained during part or all of incubation will hatch earlier than eggs placed in a nest. This argument, of course, presumes that embryonic development in utero is not limited by any factor other than temperature. In fact, when eggs are retained in utero beyond the normal stage of oviposition, embryonic development is typically retarded or halted during the period of retention (Mathies and Andrews 1996, 1999; Andrews and Mathies 2000).

Because of hypoxic conditions in utero, selection for extended egg retention, per se, will not lead to viviparity unless the increase in the duration of egg retention is coupled with selection for traits that enhance O₂ availability to embryos (Mathies and Andrews 1999; Andrews and Mathies 2000). The efficacy of this constraint on the evolution of viviparity is likely temperature dependent, however. All other things being equal, hypoxia is greater at higher than at lower temperatures because of the enhancing effect of temperature on metabolic rate. In warm climates where gravid females can thermoregulate to preferred body temperatures, retained embryos would be relatively warm. For example, in the southwestern United States, for example, females of many lizard species retain eggs until the monsoon rains begin in early to mid-July (Andrews and Rose 1994). In this situation, facultative egg retention has a high fitness benefit because eggs would not survive in the dry soil before the rains. Because of the effects of hypoxia on development, however, such facultative egg retention is not necessarily an intermediate stage between oviparity and viviparity. In contrast, in cool climates at high latitudes and elevations, gravid females have lower mean body temperatures than gravid females at low elevations and altitudes because of low ambient temperature and short activity periods (Adolph 1990; Andrews 1998, 2000); retained embryos would be relatively cool. In this

situation, hypoxia would have a relatively small effect on development, and selection for longer and longer periods of egg retention would result in viviparity.

In sceloporine lizards, eggshell attributes are unrelated to the ability of embryos to develop in utero during extended egg retention (Mathies and Andrews 2000). In this group at least, the ability of embryos to develop in utero especially during the latter part of the incubation period must then be related to morphological and functional features of the vascular systems of the female, of the embryo, or both. Obvious systems associated with oxygen delivery to embryonic tissues are the vascular supply of the oviduct and the chorioallantois (Masson and Guillette 1987; Dusseau and Hutchins 1989) and the respiratory properties of the blood including the difference in hemoglobin oxygen affinity between embryos and their mothers (Grigg and Harlow 1981). Comparative studies of these and other relevant systems should be helpful in revealing the mechanisms that support the metabolism of the embryo during and after the transition between oviparity and viviparity.

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