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Effects of Low Temperature on Embryonic Development of *Sceloporus* Lizards

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The most widely accepted explanation for the evolution of viviparity at high elevations and latitudes (cold climates) is that, by retaining eggs either for short periods (in the transition between oviparity and viviparity) or for the entire gestation period, females can keep embryos warmer than they would be in nests and, thus, enhance their development. However, an increase in the length of egg retention is not the only mechanism that would allow squamate embryos to cope with the low ambient temperatures in nests at high elevations or latitudes. We tested the hypothesis that short-term exposure to cold temperatures has less effect on embryonic development of species or populations from cold than warm climates, indicating physiological adaptation of embryos to cold temperatures. Our experimental subjects were four species (five populations) of *Sceloporus* lizards from a wide range of elevations: *Sceloporus scalaris* (Arizona, 1460 m) and *Sceloporus aeneus* (Mexico, 2800 m) from the *scalaris* species group; and *Sceloporus undulatus* (Virginia, 600 m) and *Sceloporus virgatus* (Arizona, low and high elevation populations at 1800 and 2400 m) from the *undulatus* species group. We incubated eggs under simulated natural temperature regimes, but experimental eggs were exposed to cold (8, 11, 14, or 17 C) for five days to determine the mortality and the delay in hatching relative to control eggs that were incubated under the same simulated natural temperature regimes. The mortality of eggs that were exposed to cold temperatures during incubation did not differ from that of control eggs, and mortality did not vary with elevation. These experimental eggs hatched later than control eggs, but the delay in hatching was again not related to elevation. The hypothesis of physiological adaptation to cold by embryos was thus rejected.

THE relatively low temperatures at high elevations and latitudes are thought to favor an increase in the length of egg retention by female squamates and, ultimately, the evolution of viviparity (Tinkle and Gibbons, 1977; Packard et al., 1977; Shine, 1985). The putative selective basis for increasingly longer periods of egg retention is that embryos will experience warmer temperatures (and hence develop more rapidly) while retained in utero than they would in nests because of the thermoregulatory capability of females. In addition, the female could prevent exposure of the embryos to lethal low temperatures. However, an increase in the length of egg retention is not the only mechanism that would allow squamate embryos to cope with the low ambient temperatures in nests at high elevations or latitudes. One alternative response is physiological adaptation of embryos to cold.

Shine (1984) speculated that cold climates could select for tolerance to low temperatures by embryos and enhanced embryonic development at these low temperatures. These ideas have not been tested systematically, but interspecific variation in the lower temperature limits for successful embryonic development at constant temperatures (Sexton and Marion, 1974; Muth, 1980) suggests that the thermal response curves of embryos may be modified by selection. Few studies, however, have focused on the thermal biology of squamate embryos, and their results are equivocal in this regard. Shine (1983) found little variation in the lengths of incubation, or in the low temperature limits for development, among species of skinks at one site in Australia. On the other hand, DeMarco (1992) and Qualls (1996) report more rapid embryonic development for high elevation (HE) than low elevation (LE) species of *Sceloporus* lizards and populations of the skink *Lampropholis guichenoti*, respectively, when eggs were incubated at same temperature.

The objective of this research was to further test the physiological adaptation hypothesis by evaluating the low temperature limits for embryonic development of *Sceloporus* lizards. We tested the hypothesis that embryos from high elevation species or populations, that is, cold climates as judged by mean nest temperature, have lower mortality following exposure to low temperature and continue development at lower temperatures than do embryos from low elevation species or populations.

TABLE 1. SPECIES, ELEVATION OF STUDY SITES, MEAN HATCHLING MASSES, MEAN POSTGRAVID FEMALE MASSES, MEAN TEMPERATURES OF NATURAL NESTS, AND TEMPERATURE REGIMES EXPERIENCED BY EACH SPECIES DURING INCUBATION. The number of females/clutches (in parentheses) follows the species name. Mean hatchling mass is based on clutch means. Means are given ± SE.

	Elev. (m)	Hatchling mass (mg)	Female mass (g)	Mean nest temp. (C)	Incubation temperature regimes
scalaris sp. group					
S. scalaris (14)	1460	268 (5.8)	4.3 (0.21)	~25	20–30
S. aeneus (10)	2700	243 (7.1)	3.4 (0.15)	16	8–29
undulatus sp. group					
S. undulatus (4)	600	488 (31.6)	10.0 (1.20)	~25	20–30
S. virgatus LE (25)	1800	430 (10.1)	6.0 (0.18)	25	15–25, 20–30
S. virgatus HE (17)	2400	448 (6.6)	5.9 (0.26)	~20	15–25, 20–30

MATERIALS AND METHODS

Source of eggs.—Gravid female *Sceloporus* from five species/populations (Table 1) were collected just prior to the time of natural oviposition. Females, with the exception of *S. undulatus*, were injected with oxytocin to induce oviposition. For all species except *S. virgatus*, oviposition occurred in our laboratory in Blacksburg, Virginia, and eggs were immediately placed under experimental conditions. *Sceloporus scalaris* were collected from the Appleton-Whittell Research Ranch Sanctuary, Arizona, on 28 and 29 June 1995, and 14 clutches of eggs (mean clutch size ± SE = 12 ± 0.7) were obtained from these females on 15 July. *Sceloporus aeneus* were collected near Milpa Alta, Estado de Mexico, Mexico, on 28 July 1995, and 10 clutches of eggs (mean clutch size ± SE = 5 ± 0.3) were obtained from these females on 14 August (voucher specimens were deposited in the Museo de Zoología, Facultad de Ciencias, Universidad Nacional Autonoma de México). *Sceloporus undulatus* were collected on 8 and 14 June 1995 near Blacksburg, Virginia, and eggs were obtained from four females (mean clutch size ± SE = 10 ± 0.9) that oviposited in the laboratory (26 June through 2 July). *Sceloporus virgatus* were collected between 23 June and 4 July 1995, at two sites in the Chiricahua Mountains, Arizona; the LE (1800 m) site was located near the Southwestern Research Station (SWRS), and the HE (2400 m) site was near Barfoot Park. Eggs from 25 LE clutches (mean clutch size ± SE = 8 ± 0.4) and 17 HE clutches (mean clutch size ± SE = 10 ± 0.6) were obtained from these females at SWRS on 9 July. The eggs were packed in moist vermiculite and housed in an air conditioned room, then transported to Blacksburg, Virginia, where they were placed under experimental conditions on 15 July.

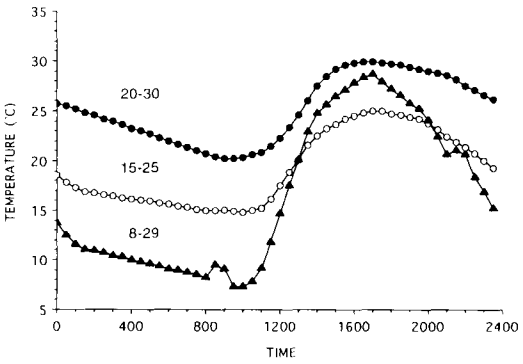

Fig. 1. Representative thermal profiles for the three incubation temperature regimes. Values shown are half-hour means over 48 h (27–28 July) for two probes per chamber. Probes were in the same jars used to incubate eggs. One jar with a probe was placed toward the center of each chamber and the other at the periphery. Overall mean temperatures taken at four different periods during the incubation period did not vary by more than 0.4 C. The 8–29 (solid triangles), 15–25 (open circles), and 20–30 (filled circles) regimes had overall mean temperatures of 16.8, 19.5, and 25.3 C, respectively.

Thermal regimes for incubation of eggs.—Eggs were incubated in controlled temperature chambers (Percival model no. I-30BL with B1 option) representing three experimental temperature regimes (Table 1; Fig. 1). These regimes were based on the nest temperatures of *S. aeneus*, HE *S. virgatus*, and LE *S. virgatus*. The thermal regimes were designated as 8–29, 15–25, and 20–30, respectively, indicating the diel range of temperature (C) in each case.

Temperatures of *S. aeneus* nests were measured over four days in July 1993, at Ajusco (2800 m), Estado de Mexico, Mexico, and over two weeks in July 1996, at Milpa Alta (RMA, unpubl. data), and our 8–29 temperature regime

corresponded to these measurements. Nest temperatures of LE *S. virgatus* are from Andrews and Rose (1994) and corresponded to our 20–30 temperature regime. Nest temperatures of HE *S. virgatus* were estimated from soil temperature by assuming that nests would be buried at depths and exposures similar to those of *S. virgatus* at the LE site. Soil temperature was measured during the incubation period (July and August) during two years (1993 and 1994). Thermocouple probes were placed at two locations that corresponded to nest sites at the low elevation site, and temperatures at a depth of 6 cm were recorded hourly by data loggers. Our 15–25 temperature regime corresponded to these estimated nest temperatures for HE *S. virgatus*.

Temperatures of the nests of *S. scalaris* and *S. undulatus* have not been measured. In Arizona, *S. scalaris* and LE *S. virgatus* live at similar elevations (Andrews and Rose, 1994; Mathies and Andrews, 1995), and gravid females have similar activity temperatures and nest site locations (RMA, unpubl. data) suggesting that nest temperatures would be similar as well. *Sceloporus undulatus* is geographically widespread at low to moderate elevations with optimal incubation temperatures of 25 to 30 C (Sexton and Marion, 1974). We therefore assumed that the 20–30 C regime would provide appropriate "normal" thermal conditions for both of these species.

Eggs remained under one of the three experimental temperature regimes throughout development, except for the following manipulation. To determine the low temperature limits for development, we followed the protocol of Christian et al. (1986) and removed preassigned eggs from the incubation chambers and placed them into one of four constant temperature chambers for 5 d. These chambers were set at 8, 11, 14, or 17 C (± 0.8 C). At the end of the five days of cold exposure, eggs were returned to their original incubation chambers until hatching. We then determined the length of time that hatching of these eggs was delayed, in comparison with eggs from the same clutch that were incubated throughout development at the normal thermal regime for the species/population. Our rationale was that if some development occurred at a particular cold temperature, then hatching would be delayed proportionally to the degree at which the rate of development was reduced or arrested. For example, when Christian et al. (1986) exposed eggs of *S. undulatus* (collected at 2290 m in the Guadalupe Mountains, TX) to 15 C for five days, hatching was delayed by four to five days relative to a control group incubated at a constant 32 C, suggesting that embryonic development had been arrested during the period of cold exposure; if development had occurred at 15 C, the delay in hatching would have been less than five days. We selected relatively low constant temperatures because we predicted that embryos of *S. aeneus*, from the highest elevation, and the coolest site, would continue development at lower temperatures than would *S. undulatus*. Because Christian et al. (1986) found hatching was not delayed when eggs were exposed to low temperatures a week or less before hatching, we exposed eggs to low temperatures at least two weeks prior to hatching (16–50 d).

Allocation of eggs to temperature regimes and cold temperature treatments.—At oviposition, eggs were marked individually and weighed. At the time that eggs were placed under experimental conditions, one or two eggs from each clutch were selected for determination of developmental stage. Embryos were staged according to criteria of Dufaure and Hubert (1961), except that half stages were assigned if embryos had features intermediate between two developmental stages. Embryos from these eggs were dried and weighed. The remaining eggs within each clutch were randomly assigned to temperature regimes and treatments. Eggs of all species/populations were incubated under the temperature regime representing their normal nest temperature and at the cold temperature treatments (Table 1). Specific assignments for the experiments follow for each species (some eggs were assigned to other temperature regimes for experiments not reported here).

Two or more *S. virgatus* eggs (in multiples of two) from each clutch (HE and LE populations) were assigned to the 15–25 and the 20–30 temperature regimes, half in each regime. Any remaining eggs from 20–30 regime for the LE population were assigned, one per temperature, to the cold temperature treatments; and any remaining eggs from the 15–25 temperature regime for the HE population were assigned, one per temperature, to the cold temperature treatments. Not all clutches had four eggs available for the cold temperature treatments, so available eggs were assigned sequentially to these treatments such that sample sizes in each treatment remained approximately equal.

Two *S. scalaris* eggs from each clutch were assigned to the 20–30 temperature regime. Any remaining eggs were assigned, one per temperature, to the cold temperature treatments.

One or two *S. aeneus* eggs per clutch were incubated under the 8–29 temperature regime.

The remaining eggs were assigned, one per temperature, to the cold temperature treatments with the same protocol used for *S. virgatus*.

Two *S. undulatus* eggs per clutch were assigned to the 20–30 temperature regime. The remaining eggs from each clutch, one or two per temperature, were assigned to the cold temperature treatments.

Eggs were placed individually in 65-mL glass jars containing moistened vermiculite. The jars were sealed with plastic kitchen wrap and secured with rubber bands. The initial ratio of distilled water to dry vermiculite was 0.7:1.0 by mass: approximately −200 kPa (Tracy et al., 1978; Packard et al., 1987). The range of water potential observed in the field for *S. virgatus* is −300 to > −100 kPa (BRR, unpubl.). No additional water was added to the jars during incubation. Small samples of eggs were weighed within a week of hatching to determine their water uptake. The ratios of final egg mass to initial egg mass for *S. virgatus* incubated under the 20–30 and 15–20 temperature regimes averaged 2.8 (n = 8) and 3.1 (n = 4), respectively. For *S. scalaris* in the 20–30 temperature regime, the ratio of final egg mass to initial egg mass averaged 3.2 (n = 14). Eggs thus tripled their initial masses during incubation; this magnitude of water uptake is associated with high hatching success for parchment-shelled eggs in general (Packard, 1991) and for *Sceloporus* in particular (Tracy, 1980; Vleck, 1991). Thus, hydric conditions in the jars, although they would not have been identical under all temperature regimes and incubation periods (Packard, 1991), produced eggs that had comparable ratios of water uptake during incubation.

Jars containing eggs were placed randomly within assigned environmental chambers. Within each chamber, jars were rotated within shelves, and shelves were rotated among positions within the chambers twice weekly to minimize any position effects. Chambers were checked twice daily for hatchlings. Hatchlings were weighed to the nearest 0.1 mg within in a few hours of hatching.

Because embryos from different clutches varied in developmental stage when incubation was initiated, the total length of the incubation period varied among clutches. Therefore, to determine a standard incubation period for each species or population, we regressed the mean length of the incubation period for control eggs from each clutch against initial embryo stage. We used the resultant regression equations to predict the length of the incubation period starting from developmental stage 30 for each species. Stage 30 is the modal period of oviposition for sceloporines and other lizards (Blackburn, 1995).

Statistics.—All parametric analyses (ANOVA, ANCOVA) were conducted with Statistical Analysis Systems software (SAS Institute Inc., Cary, NC, 1985, unpubl.), and nonparametric analyses follow Siegel (1956). The results of ANCOVAs are reported only if the interaction between the covariate and the class variable(s) was not significant ($P > 0.05$). Means are presented with their standard errors.

Results and Discussion

Egg mortality and development at low temperature.—Mortality of eggs during incubation was generally low (Table 2). For *S. aeneus*, *S. scalaris*, *S. undulatus*, and LE *S. virgatus*, mortality ranged from 0–17%, and mortality rates of eggs incubated continuously under one of the incubation regimes did not differ from those of eggs removed from these regimes placed temporarily in one of the cold temperature treatments (*P*s > 0.05, independent 2 × 2 chi-squared tests for each species). In contrast, for the HE population of *S. virgatus*, mortality was relatively high and was also greater for eggs exposed to the cold temperature treatments than for eggs in the 15–25 incubation regime (41 vs 15%, respectively). However, because mortality was only 5% for eggs of LE and HE populations incubated under the 20–30 temperature regime, the particularly high mortality of eggs of HE *S. virgatus* in the cold temperature treatments suggested a cumulative effect of cold temperature on embryonic development.

The physiological adaptation hypothesis predicts that high elevation species, which have the lowest mean and minimum nest temperatures (Fig. 1), should be most tolerant of cold. Our data do not support this prediction; mortality of eggs incubated at cold temperatures for five days did not differ from that of control eggs for any of the species we tested except for the HE population of *S. virgatus*. The predicted greater egg mortality of low elevation than high elevation species in response to short-term exposure to cold temperatures was thus not observed.

Exposure of eggs to cold temperatures for five days during incubation delayed hatching (Table 3). Preliminary ANCOVAs and ANOVAs indicated that the delay in hatching was independent of the time before hatching that eggs were exposed to the treatments as well as to the temperature during cold exposure. The only exception was for *S. scalaris*. Delay in hatching var-

TABLE 2. MORTALITY OF EGGS INCUBATED CONTINUOUSLY UNDER THE EXPERIMENTAL TEMPERATURE REGIMES (8–29, 15–25, OR 20–30) AND FOR EGGS THAT WERE TEMPORARILY (5 d) EXPOSED TO CONSTANT COLD TEMPERATURES (8, 11, 14, and 17 C Treatments Pooled and Indicated as Cold Temp. Trt.) and then Returned to Previous Incubation Conditions. Observations of eggs of *Sceloporus virgatus* incubated under reciprocal temperature regimes are enclosed in brackets (eggs from these regimes were not exposed to the cold temperature treatments). Died/hatched = the total number of eggs in each category.

Species	Incubation regime cold temp. trts.	% Mortality (died/hatched)	Intraspecific contrast
S. aeneus	8–29	17 (3/14)	$X^2 = 0.1$, $P = 0.8$
	Cold temp. trt.	14 (5/30)	
S. scalaris	20–30	5 (3/58)	$X^2 < 0.1$, $P > 0.9$
	Cold temp. trt.	3 (1/36)	
S. undulatus	20–30	11 (1/8)	$X^2 = 0.3$, $P = 0.6$
	Cold temp. trt.	0 (0/27)	
S. virgatus (LE)	20–30	5 (4/77)	$X^2 = 0.4$, $P > 0.5$
	Cold temp. trt.	11 (3/25)	
	[15–25]	[20 (16/65)]	
S. virgatus (HE)	15–25	15 (10/55)	$X^2 = 10.0$, $P < 0.001$
	Cold temp. trt.	41 (11/16)	
	[20–30]	[5 (3/62)]	

ied among cold temperature treatments ($F_{4,26} = 3.4$, $P = 0.032$, one-way ANOVA); and although the delay in hatching did not differ between eggs exposed to 14 and 17 C and eggs exposed to 8 and 11 C, eggs exposed to 14 C hatched faster than those exposed to 11 C (least-squared means comparisons, $P = 0.010$). Therefore, we compared species/populations irrespective of treatment, except for *S. scalaris*, in which the eggs exposed to 8 and 11 C and to 14 and 17 C were combined, using the delay in hatching as the dependent variable (each observation was a clutch mean) and species as the class variable (Table 3). In this analysis, the delay in hatching varied among species/populations ($F_{5,48} = 3.6$, $P = 0.008$, one-way ANOVA). The delay for *S. aeneus* was significantly greater than that for *S. scalaris* (14 and 17 C treatments) and for LE *S. virgatus* (Ps < 0.003, Bonferroni correction of P for 15 possible pairwise comparisons), but no other comparisons differed. These results indicated that, although exposure to temperatures of 17 C or lower slowed or arrested the development of all species/populations, embryos of *S. scalaris* and HE *S. virgatus* were least affected as hatching was delayed by one to two days less than the period of cold exposure.

The delay in hatching associated with exposure to the cold temperature treatments was not related to the length of the incubation period (Table 3). *Sceloporus aeneus* and *S. scalaris* had the longest delay in hatchling relative to the length of the incubation period, and *S. undulatus* and *S. virgatus* had the shortest. This was also true for the delay in hatching relative to the length of the incubation period. For exam-

TABLE 3. DELAY IN HATCHING OF EGGS EXPOSED TO ONE OF FOUR CONSTANT COLD TEMPERATURES FOR FIVE DAYS RELATIVE TO EGGS FROM THE SAME CLUTCH THAT WERE INCUBATED CONTINUOUSLY IN THE NORMAL INCUBATION REGIME. Unless indicated, 8, 11, 14, and 17 C treatments were pooled. The incubation regime for each species is indicated. The mean delay is the grand mean of individual clutch means for each species. The total incubation period was determined for control eggs, and the delay in hatching is also expressed as a percentage of this period. For *Sceloporus scalaris*, the total number of clutches was 11; of these, six were represented in both treatment groups.

Species (no. of clutches)	Incubation regime	Mean delay d (± SE)	Incubation period d (% delay)
S. aeneus (10)	8–29	5.6 (0.52)	52 (10.8)
S. scalaris (10)	20–30: 8 and 11 C	4.4 (0.23)	36 (12.2)
(7)	20–30: 14 and 17 C	3.4 (0.17)	36 (9.4)
S. undulatus (4)	20–30	5.2 (0.22)	60 (8.7)
S. virgatus (LE) (15)	20–30	3.8 (0.31)	54 (7.0)
S. virgatus (HE) (11)	15–25	4.8 (0.65)	106 (4.5)

ple, eggs of *S. aeneus*, the species from the highest elevation and the coldest climate, had the greatest absolute delay in hatching (5.6 d) in response to cold exposure and the second highest relative delay (11% of its total incubation period). According to the cold adaptation hypothesis, this species should have been affected the least.

In conclusion, our studies did not reveal any systematic variation in the mortality or the cold tolerance of eggs of the *Sceloporus* lizards that we studied after five days of exposure to cold. Thus, the conservative thermal biology of eggs may parallel the conservative thermal biology of the posthatching stages of *Sceloporus* (Bogert, 1949; Brattstrom, 1965).

Cold temperatures and development.—Incubation at constant temperatures is the norm for laboratory studies on reptile eggs. However, for many small squamates whose nests are located superficially, nest temperature may vary considerably on a diel cycle (Andrews and Rose, 1994; Castilla and Swallow, 1996; Shine and Harlow, 1996; this paper). In this situation, incubation at constant temperature does not provide ecologically relevant data about the temperature limits for development (see also Shine, 1983; Overall, 1994). For example, eggs of *S. undulatus* that were incubated at constant 6, 15, and 20 C failed to hatch (Sexton and Marion, 1974). In contrast, the eggs of *S. undulatus* that were exposed to temperatures of 8, 11, 14, or 17 C for five days in our experiments had the same high survival as eggs incubated in the 20–30 temperature regime. At the other extreme, about half the eggs of *Sceloporus merriami* incubated at 31 C hatched when exposed to 37 C for periods of 1–3 h/d, whereas no eggs hatched when incubated at a constant 37 C (Overall, 1994). Thus, high and low temperatures that are lethal to embryos with constant exposure, are experienced for short periods on a daily basis without increasing mortality. Although daily exposure of embryos to low temperatures may temporarily slow or arrest development, daily exposure of embryos to high temperatures may actually enhance development (Andrews and Rose, 1994; Shine and Harlow, 1996). Observations at constant temperatures thus underestimate the range of temperatures that normally support successful embryonic development.

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