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Supercooling, ice inoculation and freeze tolerance in the European common lizard, *Lacerta vivipara*

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Abstract The European common lizard (*Lacerta vivipara*) is widely distributed throughout Eurasia and is one of the few Palaearctic reptiles occurring above the Arctic Circle. We investigated the cold-hardiness of *L. vivipara* from France which routinely encounter subzero temperatures within their shallow hibernation burrows. In the laboratory, cold-acclimated lizards exposed to subfreezing temperatures as low as −3.5 °C could remain unfrozen (supercooled) for at least 3 weeks so long as their microenvironment was dry. In contrast, specimens cooled in contact with ambient ice crystals began to freeze within several hours. However, such susceptibility to inoculative freezing was not necessarily deleterious since *L. vivipara* readily tolerated the freezing of its tissues, with body surface temperatures as low as −3.0 °C during trials lasting up to 3 days. Freezing survival was promoted by relatively low post-nucleation cooling rates ($\leq 0.1\ °C \cdot h^{-1}$) and apparently was associated with an accumulation of the putative cryoprotectant, glucose. The cold-hardiness strategy of *L. vivipara* may depend on both supercooling and freeze tolerance capacities, since this combination would afford the greatest likelihood of surviving winter in its dynamic thermal and hydric microenvironment.

Key words Freeze tolerance · Supercooling · Cold-hardiness · Cryoprotection · Lizard · *Lacerta*

Abbreviations *bm* body mass · *SVL* · snout-vent length · T_b body surface temperature · T_c crystallization temperature

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Introduction

The European common lizard (*Lacerta vivipara*) is perhaps the most widely distributed of extant lizards. Its current range in Eurasia essentially spans the continent (12 000 km) and reaches ~3000 km northward, from northwest Spain to above the Arctic Circle. *L. vivipara* is a small (40–75 mm SVL; 3.5–4.0 g) viviparous, shuttling heliotherm that inhabits low dense vegetation, moist heathlands and open woodlands in cool-temperate and subarctic climatic regions (Spellerberg 1976). This species thrives in such diverse habitats owing to its remarkable physiological plasticity (Grenot and Heulin 1990).

As *L. vivipara* is one of only four Palaearctic reptiles inhabiting the subarctic region (Spellerberg 1976), it is curious that the cold hardiness of this species has received relatively little attention. Previous investigations have addressed compensatory shifts in metabolism at low temperature (Patterson and Davies 1978), the role of hibernation in promoting reproductive readiness (Gavaud 1983) and seasonal patterns of water flux and storage of energy-yielding substrates (Patterson et al. 1978; Grenot and Heulin 1988). However, the specific mechanisms by which *L. vivipara* tolerates subzero temperatures during winter are unknown.

In western Europe, *L. vivipara* hibernates from September or October to March in shallow terrestrial burrows within grass hummocks, abandoned rodent galleries or among tree roots (Bauwens 1981; Grenot and Heulin 1988). Despite the superficial position of their hibernacula, these lizards, which duly encounter temperatures at or below 0 °C (Gavaud 1983; Grenot and Heulin 1988), have exceptionally high survival rates (88–100%, all age classes) even in severe winters (Bauwens 1981).

The first detailed observations of overwintering *L. vivipara* were made by Grenot and Heulin (1988) who used ^{22}Na markers to locate individuals in hibernation

burrows. When examined during November and February most specimens were located in open areas, 2–4 cm beneath vegetative litter in grass hummocks. The lizards were alone or in small groups and were curled tightly with the snout drawn near the vent. These authors reported that temperatures $\leq -1.0\,^{\circ}C$ occurred within a burrow in five of the six months of hibernation. During January, the coldest month, minimum and maximum daily temperatures within the burrow averaged -6.0 and $-1.0\,^{\circ}C$, respectively; the lowest record was $-8.0\,^{\circ}C$.

Some investigators (Bauwens 1981; Grenot and Heulin 1988, 1990) have speculated that *L. vivipara* survives exposure to subfreezing temperatures by supercooling, i.e. remaining unfrozen at temperatures below the equilibrium crystallization temperature of body fluids. However, these authors have also reported that *L. vivipara* hibernates in wet substrates where frost occurs, conditions that might hamper supercooling by seeding the freezing of body tissues. Nevertheless, the possibility exists that *L. vivipara*, like a few other cold-hardy vertebrates exhibiting similar overwintering habits [review: Storey and Storey (1992)], is freeze tolerant. The purpose of the present study was to investigate the supercooling capacity, susceptibility to inoculative freezing, and potential to survive tissue freezing in *L. vivipara*.

Materials and methods

European common lizards (*Lacerta vivipara*) were captured soon after emergence from hibernation in mid-April (1992), or just prior to hibernation in October (1992 and 1993) from native populations in France. Lizards were collected from a lowland (150 m) population at the Station Biologique de Paimpont, Brittany and a highland (1450 m) population on Mont Lozère in the Cévennes. Habitats at the collection sites are described by Grenot et al. (1987). Lizards were kept for less than 2 weeks, initially indoors and subsequently in small outdoor enclosures before shipment by express carrier to Miami University.

Initial supercooling trials were conducted using five adult lizards collected in spring from the lowland population. Two male lizards were exposed to 15 °C, 10:14 (L:D) for 10 days and subsequently kept at 4 °C, 0:24 for 14 days prior to testing. Three female lizards, housed in a vivarium at 23 °C and fed beetle (*Tenebrio*) larvae until early August, were acclimated sequentially to 15 and 4 °C as before. All specimens were kept in plastic boxes containing damp moss (*Sphagnum*) during thermal acclimation.

Additional experiments involving supercooling, susceptibility to inoculative freezing and freeze tolerance were conducted using adult lizards collected in autumn from both lowland (1993) and highland (1992 and 1993) populations. As before, lizards were denied food, initially exposed to 15 °C for 10 days and thereafter kept at 4 °C. These specimens were kept in ventilated plastic jars loosely filled with damp paper, exposed to 4 °C for at least 12 weeks and used in various experiments during January–early March.

Cooling protocol

Lizards were weighed to 0.01 g, individually placed inside plastic tubes kept on ice and permitted to assume the coiled body posture characteristic of hibernating lizards (Grenot and Heulin 1988). Unless otherwise specified, specimens were placed on a pad of absorbent paper at the bottom of the tube. A thermocouple passed through the tube's cap and placed against the lizard's side was used with a multichannel data logger (Omega Engineering) to make continuous recordings of T_b during cooling. Each thermocouple was insulated from the tube wall with a small piece of plastic foam so that it more effectively measured T_b. Notably, our T_b data reflect surface rather than core body temperatures, although the small size of the specimens undoubtedly diminished the core-to-surface gradient during cooling. In the supercooling trials, lizards were insulated by loosely filling the empty space in the tube above each specimen with plastic foam. This protocol was modified for the experiments involving inoculative freezing and freeze tolerance by substituting damp moss or pulverized ice for the plastic foam. All lizards were cooled by placing the tubes in an insulated 2.5-l jar submerged in a refrigerated bath (Forma Scientific). In some trials, cooling rate was varied as part of the experimental protocol by adjusting the amount of insulation in the jar, whereas the target T_b was determined by manipulating bath temperature.

From the T_b recordings we measured the supercooling duration, an interval commencing when the cooling lizard reached the equilibrium freezing/melting point of the body tissues ($-0.6\,^{\circ}C$) and ending at either the onset of crystallization or the conclusion of the trial. In trials that resulted in the freezing of the specimen, ice nucleation was conspicuously marked by an exothermic response to the change in physical state of body water. The associated T_c was taken to represent the lowest T_b attained during supercooling. Cooling rates of lizards undergoing ice crystallization were calculated from the time elapsed during cooling of the specimen from -1.0 to $-1.5\,^{\circ}C$. Timing of the freezing episode was initiated at ice nucleation and concluded upon the removal of the lizard from bath. Generally, specimens remained at the minimum T_b (i.e., the lowest T_b reached during supercooling or freeze tolerance trials) for at least 6 h to ensure that the entire body attained thermoequilibrium.

Tests of supercooling capacity

The initial sample of five lizards (bm = 3.1 ± 0.3 g; mean ± SEM) were placed directly on the floor of the plastic tube and cooled to minimum T_bs ranging from -0.8 to $-4.2\,^{\circ}C$. In subsequent trials, three additional lizards (bm = 2.8 ± 0.1 g) were tested similarly, except that a pad of absorbent paper was added to prevent lizards from contacting moisture accumulating inside the tube.

Tests of inoculative freezing

The objective of this experiment was to determine whether direct contact of *L. vivipara* with ambient ice hampers supercooling by seeding the freezing of body fluids. Five lizards (bm = 2.5 ± 0.2 g) were cooled in plastic tubes after being covered with a 2-cm layer of moist sphagnum moss (water content = 9.5 ± 0.4 g water·g dry mass^{-1}; $n = 5$). Freezing of the moss was initiated via a brief application of aerosol coolant after the supercooled specimens had attained thermoequilibrium ($T_b = -1.9 \pm 0.1\,^{\circ}C$). Lizards were kept in situ for an additional 24 h, promptly removed from the tubes, forcibly separated from the adhering frozen moss and inspected for freezing status.

Tests of freeze tolerance

Initial tests of freeze tolerance were conducted in early March 1993 using four lizards (bm = 2.5 ± 0.3 g) placed individually in plastic tubes and insulated with an overlying piece of plastic foam. Two of the lizards were kept supercooled at thermoequilibrium ($T_b = -2.2$

and −2.3 °C, respectively) for 72 h before the foam was replaced with a 2-cm layer of pulverized ice whose application seeded the freezing of body tissues. Two additional lizards were tested in this manner, except that the ice was applied earlier during cooling ($T_b \sim 0$ °C).

Subsequent tests of freeze tolerance were conducted during January and February 1994 using fourteen additional lizards (bm = 3.4 ± 0.2 g). In these experiments, contact of the lizard with seed crystals was maximized by cooling lizards on a substrate of pulverized ice (~ 10 cm^3) in addition to covering the specimen with a 2-cm layer of ice.

After remaining frozen for 10.5–72.0 h, lizards were removed from the cold bath (whose temperature was maintained at −2.0 to −3.5 °C depending on the desired minimum T_b) and transferred to a cold room (4 °C) where they were carefully removed from their tubes and separated from the adhering ice mass. After being inspected for freezing status, lizards were placed individually on damp paper inside plastic cups and monitored for up to 12–46 days. We judged that lizards had recovered from freezing if they exhibited normal postures and responses to tactile stimulation and were capable of spontaneous locomotion.

Glucose assays

Tissue analyses were conducted in late February 1994 to determine whether freezing was associated with an accumulation of glucose, a principal cryoprotective response of some freeze-tolerant vertebrates. After attaining a minimum T_b of −1.9 ± 0.1 °C during freezing episodes lasting 32.3 ± 0.7 h, three lizards (bm = 3.5 ± 0.4 g) were thawed at 4 °C for 1.5 h to facilitate blood sampling, killed by decapitation and promptly dissected. Blood from the aorta was drawn into heparinized microhematocrit tubes and separated by centrifugation at 3000 *g*. The heart and samples of liver and spinatus (skeletal) muscle were isolated and deproteinized by homogenization in 6% perchloric acid. The plasma and neutralized organ extracts were analysed in triplicate for glucose concentration using an enzymatic, spectrophotometric procedure (Sigma).

Owing to the limited availability of specimens, it was necessary to use lizards in these experiments that had been previously used in cooling trials. Specifically, the "frozen" treatment group was comprised of two specimens that had survived earlier freezing episodes and one individual that had remained supercooled; they were allowed to recuperate for 11–16 days at 4 °C prior to being frozen in the cryoprotectant experiment. For comparative purposes, glucose concentrations were measured in two lizards that were sampled (unfrozen) directly from their cages in the cold room. These lizards, the first and last specimens used in the 1994 tests of freeze tolerance, were assayed 52 and 18 days after their recovery from the earlier freezing episodes.

Results

Supercooling capacity

In the initial supercooling trials, three of five specimens remained supercooled, with surface T_b's ranging from −0.8 to −3.0 °C, for up to 1 week (Table 1). Two lizards froze during the trials, perhaps because they contacted ice which apparently had formed from water vapor condensing on the walls of the tubes. Ice nucleation occurred in one of these specimens after having been supercooled, with minimum $T_b = -3.0$ °C for ~5 days. Freezing of the other lizard, initially supercooled with minimum $T_b = -3.0$ °C for 42.5 h, began 11.0 h after the specimen was further cooled to a lower T_b of −4.2 °C. Whereas neither of these frozen individuals recovered, all of the lizards that remained supercooled reanimated rapidly upon warming and subsequently appeared healthy.

Table 1 Trials of supercooling capacity of the European common lizard (*Lacerta vivipara*)

Sex	Body mass (g)	Cooling trial		Result
		Min. T_b (°C)	Duration (h)	
Male[a,d]	2.7	−0.8	72.0	Unfrozen
Male[a,d]	3.8	−2.1	45.0	Unfrozen
Female[b,d]	2.7	−3.0	167.0	Frozen[e]
Female[b,d]	3.8	−3.0	168.0	Unfrozen
Female[b,d]	2.3	−4.3	67.5	Frozen[f]
Male[c]	2.8	−3.5	504.0	Unfrozen[g]
Male[c]	2.6	−3.5	504.0	Unfrozen[g]
Male[c]	3.0	−3.5	504.0	Unfrozen[g]

[a] collected and tested in spring
[b] collected in spring, tested in autumn
[c] collected in autumn, tested in winter
[d] lowland population; others were collected from the highland population
[e] specimen nucleated ($T_c = -3.0$ °C) after remaining supercooled for 119.0 h, subsequently cooling (0.22 °C · h^{-1}) and ultimately reaching equilibrium at −3.0 °C during the next 48 h
[f] specimen nucleated ($T_c = -4.2$ °C) after remaining supercooled for 53.5 h, subsequently cooling (0.46 °C · h^{-1}) and ultimately reaching equilibrium at −4.3 °C during the next 14 h
[g] testing protocol was modified by cooling lizards on a substrate of dry paper to eliminate the risk of inoculative freezing

In the supercooling experiments in which lizards were cooled on a dry paper substrate, three males remained supercooled (minimum $T_b = -3.5$ °C) from 12 February to 5 March (21 days), at which time the experiment was terminated (Table 1). The body posture and position of the lizards within the tubes were similar at the beginning and end of the trials, suggesting that the supercooled lizards had remained quiescent.

Susceptibility to inoculative freezing

Five lizards cooled in contact with frozen moss (minimum $T_b = -1.8 \pm 0.1$ °C) had themselves frozen by the end of the 24-h trial. The frozen status of these individuals was determined from their rigid limbs, frost or ice adhering to their skin and the change in skin color (from grey to bluish-green) on the ventral surface and gular area; these characteristics were also reported for frozen specimens of the lacertid, *Podarcis muralis* (Claussen et al. 1990). None of these lizards recovered, perhaps because they thawed rapidly and were vigorously handled during the examination.

Additional data concerning the susceptibility of *L. vivipara* to inoculative freezing were collected in the

freeze tolerance trials, since ice nucleation in these lizards was intentionally induced by cooling them in contact with pulverized ice. Freezing of the four lizards used in the 1993 trials began 5.6 ± 2.1 h (range 2.3–11.6 h) after they initially contacted ice. The T_c of these lizards was -1.8 ± 0.2 °C. In the 1994 trials, 13 of 14 lizards froze, with ice nucleation occurring 2.6 ± 0.9 h (range 0.4–12.8 h) after the onset of supercooling in contact with ice. The mean T_c for these lizards was -1.2 ± 0.2 °C. One specimen, a 3.0-g female, resisted ice inoculation throughout the entire 46.0-h trial. Overall, however, two-thirds of the lizards that ultimately froze had commenced freezing within 3 h of contacting ice (Fig. 1).

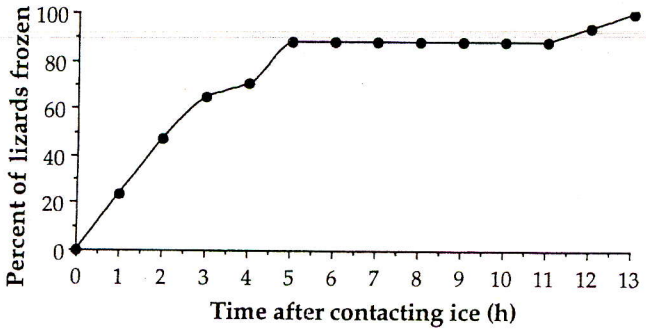

Fig. 1 Time-course for inoculative freezing of 17 European common lizards (*Lacerta vivipara*) cooled from −0.6 to ~ −2.4 °C in direct contact with pulverized ice

Freezing survival

Lizards used in the 1993 freeze tolerance trials attained a mean minimum T_b of -2.3 ± 0.1 °C during freezing episodes lasting 10.5–40.0 h (Table 2). Three of these four specimens survived. The specimen that succumbed, a 3.0-g male, was frozen for the shortest period (10.5 h) but also had cooled the fastest (0.23 °C · h^{-1}).

In the 1994 trials, 13 lizards attained a mean minimum T_b of -2.5 ± 0.1 °C during freezing episodes lasting 24.0–72.0 h (Table 2). Of the 8 lizards that ultimately recovered, the lowest T_b and longest freezing episode was −3.0 °C and 72 h, respectively (Table 2). Four of the 5 lizards that succumbed were male, although sex was not a significant determinant of freezing survival (Fisher's exact test, $P = 0.56$). Post-hoc analyses, using two-tailed Student's t-tests, indicated no significant differences between surviving and succumbing lizards concerning the duration of supercooling prior to crystallization ($t = 1.2$, $df = 11$; $P = 0.24$), T_c ($t = 0.13$, $df = 11$; $P = 0.90$), minimum T_b in the frozen state ($t = 1.6$, $df = 11$; $P = 0.15$) or duration of the freezing episode ($t = 1.8$, $df = 11$; $P = 0.10$). However, the lizards that died had a mean post-nucleation cooling rate of 0.30 ± 0.09 °C · h^{-1}, a value 4.1-fold higher ($t = 3.2$, $df = 11$, $P = 0.009$) than that of the surviving lizards (0.07 ± 0.02 °C · h^{-1}). Generally, lizards subjected to cooling rates > 0.1 °C · h^{-1} did not recover (Table 2).

Vital signs in some lizards were observed as early as 15 h after thawing commenced, although most lizards

Table 2 Trials of freeze tolerance of the European common lizard (*Lacerta vivipara*)

Sex	Body mass (g)	T_c (°C)	Cooling rate (°C · h^{-1})[b]	Freezing trial[a]		Result
				Min. T_b (°C)	Duration (h)	
1993						
Female	1.8	−2.0	0.07	−2.3	16.0	Lived
Female	2.2	−1.4	0.06	−2.0	36.0	Lived
Male	3.0	−1.5	0.06	−2.2	40.0	Lived
Male	3.0	−2.2	0.23	−2.2	10.5	Died
1994						
Male	4.7	−1.4	0.07	−2.9	24.0	Lived
Female	2.6	−2.3	0.04	−3.0	24.0	Lived
Female	3.4	−1.4	0.15	−2.8	24.0	Lived
Female	4.0	−0.8	0.03	−2.0	44.0	Lived
Male	3.0	−1.4	0.08	−2.0	59.0	Lived
Male	3.7	−0.8	0.08	−2.3	64.0	Lived
Male	2.8	−0.9	0.04	−1.8	69.0	Lived
Female	3.2	−0.9	0.10	−2.5	72.0	Lived
Male[c]	2.8	−0.9	0.59	−3.2	24.0	Died
Female[c]	3.4	−0.9	0.46	−2.9	24.0	Died
Male	4.3	−2.4	0.18	−2.5	24.0	Died
Male[c]	3.0	−0.8	0.15	−2.6	36.0	Died
Male	3.9	−0.9	0.14	−2.6	40.0	Died

[a] all specimens were collected in autumn and tested in winter
[b] post-nucleation cooling rate was calculated from the time elapsed during cooling of the specimen from −1.0 to −1.5 °C
[c] lowland population; others were collected from the highland population

Table 3 Glucose concentrations in plasma ($\mu mol \cdot ml^{-1}$) and organs ($\mu mol \cdot g^{-1}$) of unfrozen and frozen European common lizards (*Lacerta vivipara*)

	Unfrozen[a]			Frozen			
	No. 1	No. 2	Mean ± SD	No. 1	No. 2	No. 3	Mean ± SD
Plasma	19.8	9.7	14.8 ± 7.1	22.1	29.6	12.7	21.5 ± 8.5
Heart	29.3	9.7	19.5 ± 13.9	23.7	39.6	11.4	24.9 ± 14.1
Liver	31.3	12.6	22.0 ± 13.2	71.8	73.5	17.6	54.3 ± 31.8
Muscle	8.3	2.1	5.2 ± 4.4	4.3	11.3	11.2	8.9 ± 4.0

[a] specimens no. 1 and 2 were assayed 18 days and 52 days, respectively, after they were used in tests of freeze tolerance

recovered physiological and neurobehavioral faculties more slowly. In an extreme case, one 3.0-g male, which remained supercooled for a relatively long period (12.8 h) before nucleating at a relatively low T_b ($T_c = -1.4\,°C$), appeared lifeless when examined 24 h after thawing, and after 48 h showed only feeble responses to tactile stimulation. However, this specimen ultimately recovered fully and appeared healthy 5 weeks later when the investigation was concluded. Generally, early vital signs (e.g. rhythmic breathing and the opening of the eyes) appeared 18–24 h after thawing commenced, whereas restoration of more complex neurobehavioral functions (e.g. response to tactile stimulation, spontaneous locomotion) usually required at least 72 h. During recovery from freezing, some lizards showed transient debilities such as a paralysis of the forelimbs or a persistent curvature of the trunk.

Glucose concentrations in tissues

Tissues of three frozen lizards, sampled after brief thawing, contained significant quantities of glucose, which were highest in the liver (up to 73.5 $\mu mol \cdot g^{-1}$), the compound's probable source (Table 3). Unfrozen lizards had comparatively lower levels of tissue glucose, which, however, were inversely related to the time interval between the initial use of the specimen in freeze tolerance tests and the glucose assay (Table 3).

Discussion

One of the most significant liabilities confronting vertebrate ectotherms inhabiting cold regions is the potential for the freezing of tissues. Freezing risk is largely governed by climate, altitude and latitude, but also is influenced by local topographical relief and prevailing microenvironmental conditions such as ambient temperature, moisture and the efficacy of available insulation, e.g. snow cover, leaf litter, organic debris, etc. Of the few species that overwinter within the frost zone, it is generally believed that survival is promoted by either avoiding freezing via supercooling or a profound tolerance of tissue freezing [review: Costanzo and Lee (1995)].

Under idealized laboratory conditions many reptiles, including lacertid lizards (Weigmann 1929; Claussen et al. 1990), are capable of supercooling for at least short periods to T_bs as low as −8 °C (Lowe et al. 1971; Spellerberg 1972). Some small specimens can remain supercooled for lengthy periods. Notably, the mountain spiny lizard (*Sceloporus jarrovi*) remained unfrozen at −3 °C for more than 30 h (Halpern 1979) and some southeast Australian lizards were supercooled for as long as 8 days [data presented by Spellerberg (1972)]. Furthermore, Packard and Packard (1993) supercooled hatchling painted turtles (*Chrysemys picta*) for ~12 days. Our data for *L. vivipara*, which apparently represent the longest record of continuous supercooling for any vertebrate, suggest that this species can tolerate supercooling at a T_b of −3.5 °C for at least several weeks.

Among reptiles, inoculative freezing is promoted when ice crystals in the environment contact the mucous membranes of the cloaca, nostrils, or eyes (Lowe et al. 1971; Spellerberg 1972). In our tests of inoculative freezing, in which lizards were supercooled in direct contact with ambient ice, two specimens remained unfrozen for as long as 11.6 and 12.8 h, and another resisted ice inoculation throughout a 46.0-h cooling trial. However, inoculative freezing is subject to stochastic variation, as is clearly evidenced by the latter specimen which, when retested 2 weeks later (in conjunction with the cryoprotection experiments) was inoculated less than 1 h after contacting ice. Considering that most lizards while in contact with ice remained supercooled for only several hours and could not supercool below −1 to −2 °C, it is evident that such susceptibility to inoculative freezing may effectively preclude supercooling in *L. vivipara* under certain conditions. In particular, the damp and occasionally saturated substrate encountered by *L. vivipara* in its hibernaculum (Bauwens 1981; Grenot and Heulin 1988) promotes water flux (Grenot and Heulin 1990) and thus may expose lizards to ambient seed crystals. Nevertheless, ice inoculation is not necessarily deleterious,

since *L. vivipara* readily tolerates the freezing of its tissues.

Our finding that *L. vivipara* is freeze tolerant is both novel and significant. Indeed, the adaptive significance of freeze tolerance among vertebrates was only recently recognized, initially for terrestrially-hibernating anurans and subsequently for certain temperate reptiles, including the garter snake, (*Thamnophis sirtalis*), painted turtle (*Chrysemys picta*) and eastern box turtle (*Terrapene carolina*). With the exception of the Siberian salamander (*Hynobius keyserlingii*), for which the original report of exceptional freeze tolerance remains unconfirmed, all known freeze-tolerant vertebrates are endemic to North America [review: Storey and Storey (1992)].

The few available data suggest that among vertebrate ectotherms, freeze tolerance is particularly uncommon among squamates. From their studies of various North American and Australian species, both Lowe et al. (1971) and Spellerberg (1972) concluded that ice crystallization was invariably debilitating or lethal to lizards and snakes. Interestingly, Fitch (1964) reported the survival of eggs and hatchlings of the scincid *Eumeces obsoletus* during transient (30 min) exposures to temperatures as low as −5 °C, and specimens of the high altitude lizard *Sceloporus grammicus* from Iztaccihuattl Volcano, Mexico reportedly recovered from exposures to −2.5 °C lasting as long as 37 h (Lemos-Espinal and Ballinger 1992). Unfortunately, the significance of these studies is equivocal because tissue freezing was not verified. Indeed, the rapid reanimation on warming rather suggests that the specimens were only supercooled.

Some lacertid lizards apparently tolerate very brief freezing episodes at relatively high T_bs. For example, Claussen et al. (1990) reported that five of eight specimens of the wall lizard (*Podarcis muralis*) recovered after freezing for 10–120 min with T_bs ranging from −0.6 to −1.0 °C (mean ± SD = −0.7 ± 0.2 °C), but seven other specimens, which attained T_bs at or below −1.1 °C, succumbed. In an earlier investigation of *P. muralis* (and also *L. agilis*), Weigmann (1929) reported that very few specimens could survive even short (25–38 min) freezing episodes at high T_bs (−0.9 °C to −1.0 °C) and concluded that these species cannot tolerate extensive tissue freezing and therefore must overwinter in frost-free microenvironments.

The present study provides conclusive evidence for a well-developed capacity for freeze tolerance in *L. vivipara*. Although the thermal and temporal limits for freezing survival were not determined, our data indicate that *L. vivipara* can survive freezing episodes lasting at least 3 days and tolerate T_bs at least as low as −3 °C. Unfortunately, a limited number of specimens precluded our measuring ice contents of lizards subjected to tolerable freezing episodes. However, theoretical estimates derived from a model of the colligative properties of solutions, equilibrium freezing/melting point and minimum T_b (Claussen and Costanzo 1990), and the assumption that 18% of the body water was unfreezable or "bound" (Claussen et al. 1990), suggest that ice contents of the 11 surviving lizards (Table 2) ranged from 55 to 66% of total body water. The maximum ice content survived by most species of freeze-tolerant vertebrates is 65–70% (Storey and Storey 1992; Lee and Costanzo 1993).

Many freeze-tolerant vertebrates rapidly synthesize and accumulate the cryoprotectant glucose during freezing, although the ultimate tissue concentrations attained in reptiles, including *L. vivipara* in the present study, generally are much lower than in amphibians (Storey and Storey 1992; Costanzo et al. 1993; Storey et al. 1993). Basal tissue glucose levels for hibernating *L. vivipara* are unavailable for direct comparison with those of recently frozen specimens. However, relative to the normal glycemic state in various lizard species [e.g. 6.6 ± 0.6 $\mu mol \cdot ml^{-1}$; mean ± SEM; $n = 17$; Dessauer (1970)], the two- to fivefold higher levels of blood glucose in recently frozen *L. vivipara* (Table 3) may indicate that this species utilizes glucose as a cryoprotectant. Presumably, such glucose-based cryoprotectant systems would be promoted by both the deposition of glycogen during late summer and autumn and the preferential catabolism of abdominal and caudal lipid reserves during hibernation (Patterson et al. 1978).

Our limited data indicate substantial interindividual variability in the glucose levels of frozen specimens (Table 3) which, in freeze-tolerant anurans, typically stems from large variation in hepatic glycogen levels (Storey and Storey 1992; Lee and Costanzo 1993). Because tissue glucose levels in a lizard sampled 18 days after freezing were two- to fourfold higher than those measured in a specimen sampled 52 days after freezing (Table 3), elevated glucose levels apparently persist long after thawing. Additional study is needed to more fully elucidate the cryoprotective response of *L. vivipara*.

Rapid cooling during the freezing episode compromised the freezing survival of *L. vivipara*, particularly at rates $> 0.1\,°C \cdot h^{-1}$. In the wood frog (*Rana sylvatica*), rapid cooling injury results from an inhibition of cryoprotective responses (Costanzo et al. 1991, 1992). Interestingly, the critical threshold for cooling rate is about tenfold higher for wood frogs (Costanzo et al. 1991) than *L. vivipara*. However, relatively slow cooling likely prevails in nature because leaf litter and snow cover (when present) substantially moderate thermal fluctuations within the hibernation burrow (Grenot and Heulin 1988).

The degree of cold tolerance exhibited by lizards may strongly depend on geographic origin (Spellerberg 1976). Our studies using *L. vivipara* from lowland and highland populations were not designed to compare populational levels of cold hardiness, but nevertheless indicate that lizards from both regions are adapted for

survival at subfreezing temperatures. Subsequent studies should directly compare cold-hardiness attributes in populations from different altitudes, as well as those from southern European and Arctic locales. Also needed are investigations of freeze tolerance in other Palearctic reptiles inhabiting high latitudes, such as the lizard *Anguis fragilis* and the ophidians *Natrix natrix* and *Vipera berus*.

Our study suggests that the survival of the subfreezing T_bs encountered by *L. vivipara* during hibernation (Bauwens 1981; Grenot and Heulin 1988, 1990) may be promoted by both supercooling and freeze tolerance capacities. The principal benefits of freeze avoidance as a cold-hardiness strategy are that, unlike freeze tolerance, supercooling invokes relatively little physiological stress, allows rapid reanimation and physiological recovery on warming and presumably offers protection at markedly lower T_bs (Costanzo and Lee 1995). However, the efficacy of supercooling may be constrained in individuals which contact ice in their hibernacula, in which case survival would depend on a tolerance of tissue freezing. Fortuitously, the susceptibility to inoculative freezing may obviate the injury associated with spontaneous nucleation of deeply supercooled body fluids (Claussen et al. 1990). Although supercooling and freeze tolerance usually are dichotomous strategies for coping with subzero temperatures (Costanzo and Lee 1995), in *L. vivipara* such a dual-factor system may promote winter survival in its dynamic thermal and hydric microenvironment.

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