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Freezing survival, body ice content and blood composition of the freeze-tolerant European common lizard, *Lacerta vivipara*

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Abstract To investigate the freeze tolerance of the European common lizard, *Lacerta vivipara*, we froze 17 individuals to body temperatures as low as –4 °C under controlled laboratory conditions. The data show that this species tolerates the freezing of 50% of total body water and can survive freezing exposures of at least 24-h duration. Currently, this represents the best known development of freeze tolerance among squamate reptiles. Freezing stimulated a significant increase in blood glucose levels (16.15 ± 1.73 μmol.ml^{-1} for controls versus 25.06 ± 2.92 μmol.ml^{-1} after thawing) but this increase had no significant effect on serum osmolality which was unchanged between control and freeze-exposed lizards (506.0 ± 23.8 mosmol.l^{-1} versus 501.0 ± 25.3 mosmol.l^{-1}, respectively). Tests that assessed the possible presence of antifreeze proteins in lizard blood were negative. Recovery at 5 °C after freezing was assessed by measurements of the mean time for the return of breathing (5.9 ± 0.5 h) and of the righting reflex (44.8 ± 4.5 h). Because this species hibernates in wet substrates inoculative freezing may frequently occur in nature and the substantial freeze tolerance of this lizard should play a key role in its winter survival.

Keywords Freeze tolerance · Reptile · Ice content · Glucose · Osmolality

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Abbreviations *AFP* antifreeze protein · *Tc* crystallization temperature

Introduction

The subzero temperatures of winter provide one of the greatest challenges to the survival of ectothermic animals. Nevertheless, many species cope with extreme cold using behavioural, physiological and biochemical adaptations that promote their survival. Two strategies of subzero survival are commonly found among ectotherms: the "freeze avoidance strategy" allows animals to preserve a liquid state even at temperatures well below the equilibrium freezing point of body fluids, and the "freeze tolerance strategy" allows organisms to endure the conversion of a significant portion of their body fluids into ice (Somme 1982; Storey and Storey 1988). Among vertebrates, only a few amphibian and reptile species are freeze tolerant and most of these are found in North America. Elsewhere, the European common lizard, *Lacerta vivipara*, is one of the few terrestrially hibernating reptiles that overwinters in sites that receive significant subzero exposure. The animals typically overwinter in shallow hibernacula 2–4 cm beneath the vegetative litter in grass hummocks (Grenot and Heulin 1988). The cold hardiness strategy of *L. vivipara* was first studied by Grenot and Heulin (1988) who used radioactive markers to locate overwintering individuals in the field and found some of them in sites where ambient temperature was as low as –8 °C, yet lizards were unfrozen. Subsequent work has located lizards in the field in either supercooled or frozen states within the same winter and within the same site (Grenot et al. 2000). Hence, it appears that the species can make use of either strategy for survival, supercooling substantially in some instances and freezing in others. Since humidity within the grass hummocks is ~100% all the time, the ability to survive freezing may be essential because supercooled organisms are susceptible to inoculative freezing if they come in contact with environmental ice. Laboratory

tests showed that *L. vivipara* has the ability to remain supercooled for at least 21 days at –3.5 °C and can also tolerate 3 days of freezing at an mean minimum body temperature of –2.5 °C (Costanzo et al. 1995). These dual strategies of cold hardiness may explain both the high survival rate observed for these lizards even during harsh winters (Bauwens 1981) and the impressive northern range limits of the species that extend past the Arctic circle.

However, several important features of the freeze tolerance of *L. vivipara* remain to be assessed. The present study assesses some of these unknown elements:

1. The amount of ice formed is a crucial determinant of the survival of freeze-tolerant animals and the limits tolerated are different for each species; therefore, we analysed the time/temperature influence on body ice content.
2. Both freeze tolerance and freeze avoidance are generally characterized by the accumulation of low molecular weight carbohydrates that provide colligative cryoprotection and by the presence of specific blood proteins that have either antifreeze or ice nucleating actions (Somme 1982; Storey and Storey 1988). To characterize *L. vivipara* in this regard, we analysed selected blood/serum parameters including glucose levels, osmolality, thermal hysteresis activity and the capacity to inhibit ice recrystallization.
3. Finally, we studied the recovery time after thawing by observing the return of breathing and the righting reflexes (the animal's ability to return to an upright posture within 60 s after being placed on its dorsum).

Materials and methods

Animals

Seventeen lizards, *L. vivipara*, mean body mass 2.63 ± 1.14 g, were captured in late September from a highland population (1450 m) in the Cévennes mountains in France. They were held in boxes with sand and wet moss and cold acclimated at 4 °C for 6–7 weeks in the dark without feeding; experiments were performed in November and December.

Freezing protocol

For freezing exposure, individuals maintained at 4 °C were rapidly placed on a pad of paper towelling with a thermocouple in contact with the abdomen of the animal. A band of masking tape was used to secure the animal and thermocouple in place. Using a pipette, the towelling around the animal was wet with water so that the lower surface of body and limbs were in contact with a damp substrate. Each animal was then placed in an incubator set at –2 °C, –3 °C or –4 °C and the course of cooling and freezing was monitored. The initiation of freezing was detected by the jump in body temperature due to the release of heat of crystallization (exotherm). If freezing did not begin before body temperature equilibrated with the chosen incubator temperature, then air temperature was adjusted downwards in 0.5 °C intervals until the exotherm was observed. Incubator temperature was then immediately returned to the chosen value and maintained for the duration of the freeze. Freezing exposure was timed from the appearance of this exotherm, and lizards were sampled after different intervals of freezing ranging from 0.5 h to 72 h. Some specimens exposed to very short freezing times, which they survived, were used a second time. They were allowed to recuperate for 5–7 days at 4 °C prior to the second freezing exposure.

Ice content

To determine the ice content of frozen lizards, we used the whole-body calorimetry technique described by Layne and Lee (1987, 1991). The calorimeter consisted of an insulated flask that was imbedded in a block of stryofoam insulation and fitted with a styrofoam plug that fitted down into the flask leaving a space of only about 100 ml at the bottom of the flask. Thawing was done in a volume of 50 ml water for most animals or 30 ml for animals weighing < 1 g. A thermocouple was positioned below the water surface and connected to a digital thermometer; stirring was provided by a magnetic stirrer. The change in water temperature caused by thawing the lizard was recorded. Calculations of body ice content used experimentally determined values for our system which were: F factor for the calorimeter = 1.06, the percentage of body mass that is water for *L. vivipara* = 70.09 ± 1.39% (from measurements of wet and dry masses of lizards that died), specific heat of the dry mass measured by calorimetry = 0.25 ± 0.04 (SD) and the melting point of body fluids as estimated from osmolality determinations = –0.9 °C (Table 2). Body water content was determined for seven lizards by drying carcasses at 80 °C to constant mass. Ice content was expressed both in terms of absolute mass and fraction of total body water.

Osmolality, glucose content and antifreeze proteins

To determine whether freezing was associated with an accumulation of low molecular weight cryoprotectants, blood samples were taken from the infra-orbital sinus of the animals. Analysis of blood glucose content was performed on whole blood immediately after sampling using a Freestyle glucose monitor. For osmolality, blood samples were separated by centrifugation at 5 °C (10,000 rpm, 5 min), and then serum was collected and analysed using a Wescor INC 51 vapour pressure osmometer. The possible presence of antifreeze proteins (AFPs) in *L. vivipara* serum was tested using two standard methods: (1) analysis of thermal hysteresis between freezing and melting points of serum using the method of Chakrabartty and Hew (1991) and (2) inhibition of recrystallization using the method of Smallwood et al. (1999).

Statistical analysis

Mean values are presented as means ± SD where appropriate. No statistical tests (with data of glucose/osmolality values and time for the righting reflexes) used repeat measurements from the same animals. Statistical analyses were performed with an SAS computer statistical package. A 5% ($P < 0.05$) level of significance was used in all tests.

Results

Freezing survival and ice content

During the freezing exposure experiment, lizards cooled on a wet substrate showed a mean crystallization temperature (Tc) of –2.9 ± 0.6 °C ($n = 23$, range –1.8 °C to –4.3 °C). The time course of ice accumulation was analysed in animals subjected to freezing at –3 °C (Fig. 1). At such temperature, freezing progressed quickly during

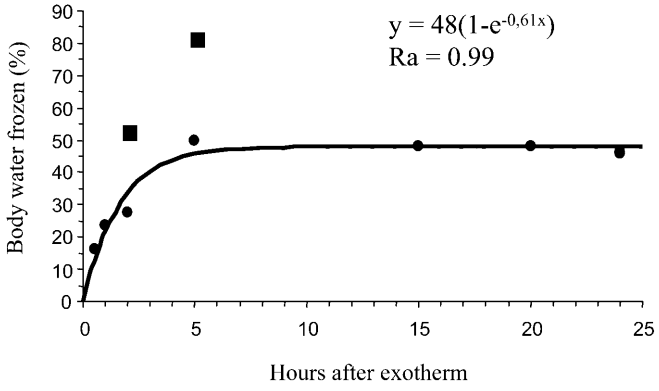

Fig. 1 The time course of ice accumulation of lizards subjected to freezing at –3 °C. A non-linear regression fitted the data. The hyperbolic plot [$y = 48(1-\exp^{(-0.61x)}$] represents the best fit to the percentage of body water frozen in lizards over time. We also give the "*Ra*" as the ratio between the totals of sums of squares explained by the regression and uncorrected (equivalent of the R^2 of a linear regression). *Circles* show ice content of lizards that survived freezing; symbols for two lizards frozen for 24 h overlap. *Squares* indicate animals that died during freezing; data for these animals were not used in fitting the curve

the early stages of the time course with a mean rate of $20.3 \pm 10.4\%$ of total body water frozen per hour ($n = 4$, animals frozen for 0.5, 1, 2 and 5 h). Equilibrium ice content was attained about 5 h after the onset of freezing. The data for individual animals used in the freeze tolerance trials are presented in Table 1. All lizards survived when ice content was lower than 41% and some of them also survived with as much as 50.1% ice within their body. However, higher ice contents were constantly lethal for the animals (Table 1).

After freezing, four lizards frozen 20 h and more (ice content between 40.8% and 48.5%) and then thawed in the calorimeter were used for an analysis of the recovery of breathing function and righting reflex over time at 4 °C. The mean time for breathing recovery was 5.9 ± 0.5 h; for righting reflex, the mean recovery time was 44.8 ± 4.5 h.

Blood osmolality, glucose concentration and antifreeze activity

Values for serum osmolality and whole blood glucose concentration for control (never frozen) and freeze-exposed (sampled 3–48 h after thawing) lizards are presented in Tafble 2. Statistical testing found no significant difference in osmolality between control and freeze-exposed animals ($U = 15.5$, $P = 0.54$). By contrast, freezing was associated with an accumulation of glucose, the mean blood glucose concentration rising by 55% in freeze-exposed animals ($U = 20.0$, $P = 0.016$). The mean is probably a good measure of peak blood glucose since no rank order to the values has been detected over the 48-h time span. No thermal hysteresis between the freezing and melting points of lizard serum was detected in any sample from either control or freeze-exposed lizards ($n = 5$ of each). Furthermore, lizard serum lacked

Table 2 Serum osmolality and whole blood glucose concentration of winter *L. vivipara*: control versus freezing exposed

	Control animals	Frozen animals (with ice content >45%)
Osmolality (mosmol.l^{-1})	506.0 ± 23.8 $n = 5$	501.0 ± 25.3 $n = 5$
Glucose concentration (μmol.ml^{-1})	16.15 ± 1.73 $n = 4$	25.06 ± 2.92 $sn = 5$

Table 1 Trials of freezing tolerance of the European common lizard (*Lacerta vivipara*). *J* juvenile, *S.A.* sub-adult, *A* adult, *Tc* crystallization temperature (or supercooling point), T_a is the ambient air temperature maintained in the incubator for the duration of the freeze. In instances where animals cooled to T_a without freezing, T_a was then lowered in intervals (–0.5 °C.15 min^{-1}) until nucleation occurred and then temperature was immediately readjusted upward to the chosen T_a value and held there for the duration of the freeze

Animal stage	Body mass (g)	*Tc* (°C)	Freezing trial		Ice mass (g)	Ice content (% body water)	Result
			Ta (°C)	duration (h)			
S.A.	1.7	–3.5	–2	5	0.27	22.6	Alive
A	2.9	–2.3	–2	36	0.83	40.8	Alive
A	3.0	–4.2	–2	22	1.16	54.4	Dead
A	4.2	–1.8	–2	47	1.05	41.8	Dead
A	3.0	–2.3	–2	72	1.05	49.9	Dead
J	1.1	–3.5	–2.4	0.5	0.06	8.1	Alive
A	2.9	–2.5	–3	0.5	0.20	16.6	Alive
S.A.	1.8	–2.4	–3	1	0.49	24.0	Alive
A	2.9	–2.5	–3	2	0.57	27.9	Alive
A	3.1	–3.1	–3	5	1.07	50.1	Alive
A	3.1	–2.5	–3	15	1.06	48.5	Alive
A	2.6	–2.6	–3	20	0.87	48.5	Alive
A	4.2	–2.7	–3	24	1.37	46.4	Alive
A	4.0	–2.4	–3	24	1.29	46.5	Alive
S.A.	1.4	–2.8	–3	5	0.80	81.7	Dead
J	0.7	–2.5	–3	2	0.26	52.7	Dead
J	1.0	–3.6	–3.6	0.5	0.25	37.2	Alive
S.A.	1.8	–3.6	–3.6	1	0.31	17.6	Alive
S.A.	1.7	–3.0	–4	0.5	0.29	24.4	Alive
A	4.2	–4.3	–4	1	0.87	29.5	Alive
A	4.3	–2.8	–4	2	0.95	32.3	Alive
A	4.2	–2.6	–4	5.3	2.06	70.5	Dead
A	4.5	–2.5	–4	15	1.89	59.5	Dead

the ability to modify the recrystallization of ice, another defining action of AFPs. The failure of lizard serum in both of these standard tests for detecting AFP activity indicates that AFPs are not present in the blood of this species in the winter nor are they induced by freezing exposure.

Discussion

L. vivipara occupy shallow terrestrial hibernacula (Grenot and Heulin 1988), and therefore it is reasonable to assume that they are well adapted to the subzero temperature exposures that they experience over the winter. Our data corroborate the previous work on the freeze tolerance capacities of these lizards (Costanzo et al. 1995) and demonstrate that this species has the ability to tolerate the freezing of a substantial portion of body fluids. About 48% ice was routinely accumulated (maximum 50.1%) by lizards in our tests with full survival of 24 h frozen (or 36 h survival with an ice content of 40.8%). This experimentally measured value for 50.1% ice is close to the theoretically estimated value of 55–66% ice that Costanzo et al. (1995) proposed for lizards frozen for 1–3 days at –2 °C to –3 °C. The present report of endurance of 48% ice by *L. vivipara* is the highest level of freeze tolerance reported to date for squamate reptiles (lizards and snakes). However, literature on the subject is quite scarce and very few other species of lizards have been tested. This is partly because there are very few lizard species that have ranges that extend into northern or alpine regions where subzero temperature exposures are common during winter, and thus few species likely to be freeze tolerant. To date, only a few lacertid lizards have been shown to tolerate even very brief freezing episodes at relatively high subzero temperatures. Wall lizards (*Podarcis muralis*) endured 10–120 min with minimal body temperatures of around –1 °C (Claussen et al. 1990) and *Lacerta agilis* recovered after 25–38 min freezing with the same minimal body temperature (Weigmann 1929). These very poor survival capacities suggest that neither of these species would survive if ice content rose to its equilibrium value and thus neither species has the long term capacity for freezing survival that is needed to be truly considered a freeze-tolerant animal.

By contrast, the present study provides evidence that the European common lizard has developed ecologically relevant freeze tolerance. The lizards tolerated a high percentage of body ice and showed relatively low supercooling capacity (mean $T_c = -2.9$ °C) when frozen in contact with a moist substrate. This suggests that the lizards are highly susceptible to inoculative freezing via skin contact with ice. This may be the normal situation during hibernation because the lizards overwinter in sites that experience 100% humidity in the micro-environment (Grenot and Heulin 1988, 1990). Hence, it is highly likely they would come in contact with ice crystals when these form on the plant material or soil around them. Inoculative freezing at relatively high subzero temperatures is advantageous for it promotes a slow rate of ice formation that often enhances survival. Such a relationship between the characteristics of the overwintering site and inoculative freezing is already known for several other reptiles, amphibians and insects (Costanzo et al. 1997, 1998). However, as the current data show, during the early hours of freezing, the rate of ice formation in *L. vivpara* is actually quite high, $20.3 \pm 10.4\%$ of total body water freezing per hour at an ambient temperature of –3 °C. With such a rate, the equilibrium ice content of ~48% was rapidly attained. This rapid ice growth may be explained by the lack of large cryoprotectant pools such as occur in freeze-tolerant frogs and which help to minimize the amount of ice accumulated (Storey and Storey 1988).

Many freeze-tolerant animals accumulate high concentrations of sugars or polyhydric alcohols that act as cryoprotectants (Storey and Storey 1996; Layne 1999). Freeze-tolerant frogs typically use glucose for this function whereas most insects use glycerol or other polyols (Storey and Storey 1988). Among reptiles that have been studied to date, cryoprotectants are generally lacking. Garter snakes (*Thamnophis sirtalis*), painted turtle hatchlings (*Chrysemys picta*) and adult box turtles (*Terrapene carolina*) show elevated glucose in blood and some organs during freezing, but levels rarely exceed 25 mM and so their value as colligative protectants is questionable (Costanzo and Lee 1988; Storey et al. 1988, 1993). A similar response was seen in *L. vivipara*; freezing stimulated a 55% increase in blood glucose levels but the mean concentration in freezing-exposed lizards was only 25 mM (Table 2). Measurements of serum osmolality further suggested that no other osmolyte was being produced during freezing since there was no significant difference in osmolality between control and frozen groups (Table 2).

Many cold-hardy species employ special proteins to manage ice. AFPs are well known in cold-water fish and freeze-avoiding insects where their primary role is to adhere to the growth planes of microscopic ice crystals and prevent their growth to a size that could do injury (Ewart et al. 1999). AFPs have also been reported in some freeze-tolerant insects and plants where their role seems to be to modulate ice growth during freezing and minimize the recrystallization of ice during prolonged freezing (Ewart et al. 1999). Hence, the proteins may characterize both freeze avoiding and freeze-tolerant species and could, therefore, be a valuable asset to species like *L. vivipara* that seem to employ both strategies of cold hardiness to survive the winter. However, a search for AFP activity using standard methods to assess the thermal hysteresis and recrystallization inhibition properties of lizard serum was negative for both control and freeze-exposed animals. We conclude, therefore, that the use of AFPs is not part of the cold-hardiness strategy of *L. vivipara*.

Although Costanzo et al. (1995) reported that some specimens of highland *L. vivipara* survived 3 days of

freezing (at –2 °C to –2.5 °C), no individual in the present study endured more than 36 h frozen. This difference might be related to the blood glucose concentration which seemed to increase throughout the winter season from 1.8 $g.l^{-1}$ in October to 6.0 $g.l^{-1}$ in March (Grenot et al. 2000). Seasonal differences in this or other factors could also be responsible for the different results for freezing survival between the two studies, the present work having been done in November-December whereas the studies of Costanzo et al. (1995) were done in January-March. Another factor that could reduce survival is fast thawing in the calorimeter which subjects the animal to both temperature and osmotic shocks. Hence, estimates of survival based on animals thawed in calorimeters are likely to be lower than those from animals thawed slowly. However, the comparison between the two studies is hazardous since Costanzo et al. (1995) did not measure the ice contents of their frozen lizards. Thus, we do not know whether the lizards in the two studies are really being compared at similar ice content. In the present study we found both dead and alive animals at ice contents of about 48%, but if the ice was slightly lower, e.g. 35–40%, values for long survival might have been similar to those reported by Costanzo et al. (1995).

Our data suggest that all the life stages of this species that overwinter are freeze tolerant (one juvenile endured 37.2% ice and several sub-adults recovered from 24% ice). However, the low number of individuals did not allow us to determine if mass has an important effect on freeze tolerance capacity. It would be interesting to know if juveniles can endure the same maximum ice content as adults and if the predictably higher rates of ice accumulation and post-exotherm cooling in juveniles (due to lower body masses) would result in higher freezing injury to juveniles than to adults hibernating under comparable field conditions. Our actual observations from excavation of hibernation sites in midwinter is that juveniles are often found at greater depths than adults within the common overwintering site (C. Grenot and Y. Voituron unpublished data). Such behaviour could exposure juveniles to less severe temperature conditions and thus could increase their chances of winter survival.

Data on the post-freeze recovery of vital signs and muscle coordination are scarce (Layne and First 1991). One easy way to assess post-freeze recovery of physiological functions is via the recovery of the righting reflex. This reflex requires complex neuronal integration and therefore represents a good index of global recovery. For *Rana sylvatica* frozen for 28 days, the righting reflex was re-established within 24 h after thawing (Layne et al. 1998). When *L. vivipara* with high ice contents ($>40\%$) were observed just after thawing no signs of recovery were seen. However, breathing movements were re-established within about 6 h but the righting reflex took much longer, occurring about 45 h after thawing. In a wider integration of studies on cold hardiness strategies, such a substantial delay for recovery could have an important ecological impact especially in spring when sub-zero temperatures may occur again after the mating period has begun. The survival rate is often the only index of interest in cold hardiness studies, however, a number of life history parameters such as future reproduction may be strongly modified by the winter periods (Bale 1987; Kozlowski 1991). Thus, it would now be interesting to incorporate this physiological aspect into a larger life history context.

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