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Comparison of the Cold Hardiness Capacities of the Oviparous and Viviparous Forms of *Lacerta vivipara*

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ABSTRACT The lizard *Lacerta vivipara* has allopatric oviparous and viviparous populations. The cold hardiness strategy of *L. vivipara* has previously been studied in viviparous populations, but never in oviparous ones. The present study reveals that both the oviparous and viviparous individuals of this species are able to survive in a supercooled state at −3°C for at least one week when kept on dry substrates. The mean crystallisation temperatures of the body, around −4°C on dry substrata and −2°C on wet substrata, do not differ between oviparous and viviparous individuals. All the individuals are able to tolerate up to 48–50% of their body fluid converted into ice, but only viviparous individuals were able to stabilize their body ice content at 48%, and hence were able to survive even when frozen at −3°C for times of up 24 hours. Ice contents higher than 51% have been constantly found lethal for oviparous individuals. This suggests that, in *L. vivipara*, the evolution towards a higher degree of freezing tolerance could parallel the evolution of the viviparous reproductive mode, a feature believed to be strongly selected under cold climatic conditions. This is the first report, among reptiles, of an intraspecific variation regarding the freeze tolerance capacities. *J. Exp. Zool. 301A:367–373, 2004.*

INTRODUCTION

Reptiles are ectotherms, and therefore, can only partially regulate their body temperature through behavioral thermoregulation during the active season. Cold winters in temperate and Arctic regions represent a serious challenge for these animals, especially regarding the subzero temperatures that can induce freezing of their body fluids. Although numerous species cope with this problem by selecting adequate overwintering sites (e.g., below the frost line), there are also species whose superficial overwintering sites require a cold hardiness to ensure survival. Cold hardiness strategies are commonly divided into two main categories: freeze avoidance via an extensive supercooling capacity, and freeze tolerance. Most reptilian species exhibiting cold hardiness do not tolerate freezing and, therefore, rely exclusively on supercooling to face subzero body temperatures (Halpern and Lowe, '68; Lowe et al., '71; Costanzo and Lee, '94). There are a few species of reptiles, however, known to have various degrees of freeze tolerance. Some of them, e.g., the wall lizards, *Podarcis muralis* and *P. sicula*, and the boreal adder, *Vipera berus*, only tolerate conversion of a limited proportion of their body fluid into ice for a very brief period (less than 30% for less than 4h) (Claussen et al., '90; Andersson and Johansson, 2001; Burke et al., 2002). Others, such as the European common lizard *Lacerta vivipara*, the common garter snake *Thamnophis sirtalis*, the painted turtle *Chrysemys picta*, and the box turtles *Terrapene carolina* and *T. ornata*, are able to survive freezing for 24 hours and more with 30–50% of body fluids converted into ice (Storey et al., '88; Costanzo and Lee, '88; Costanzo and Claussen, '90; Costanzo et al., '95; Voituron et al., 2002a). Among these species, *L. vivipara* is particularly interesting because it exhibits the rare capacity to tolerate subzero temperatures by both supercooling and freeze tolerance. Yet, laboratory experiments have shown that this

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lizard can remain in the supercooled state at −3°C for at least three weeks, the longest record for vertebrates (Costanzo et al., '95), and also survive the conversion of 50% of its total body water into ice for 24 hours at −3°C (Voituron et al., 2002a).

L. vivipara is an interesting model species, not only because of its unusual cold hardiness strategy but also because it has both oviparous and viviparous populations. Such a reproductive bimodality is a rare phenomenon that has been reliably documented for only 11 species of Squamates (Shine '83; Heulin et al., '91). Viviparous populations of *L. vivipara* are widely distributed from the British Isles and central France into Scandinavia and eastern Russia. Oviparous populations of this species were first identified on the southwestern margin of the species' range, from the Cantabrian and Pyrenean Mountains into the Aquitaine region in southern France (Brana and Bea, '87; Heulin and Guillaume, '89). Another group of oviparous populations, also located on the southern margin of the species' range, was more recently discovered in northern Italy and Slovenia (Heulin et al., 2000; Ghielmi et al., 2001; Surget-Groba et al., 2002). Ecological and morphological resemblance, successful laboratory hybridization, and genetic data from enzymes and mitochondrial DNA all indicate that the oviparous and viviparous forms of *L. vivipara* are very closely related (Bea et al., '90; Heulin et al., '93, '99; Arrayago et al., '96). Divergence times calculated from mitochondrial DNA data indicate that the radiation of *L. vivipara,* and in particular the emergence of viviparity in this species, occurred during the Pleistocene; that is, during a period including numerous glacial phases (Heulin et al., '99; Surget-Groba et al., 2001; Surget-Groba, 2002). This is in accord with the widely accepted ''cold climate theory'' positing that cold climatic conditions are one of the most important selective forces acting in favor of the evolution of viviparity in Squamates (Shine, '83). Hence, one may think that cold climatic conditions, such as the glacial phases of the Pleistocene, have probably strongly selected both the evolution of viviparity and the evolution of cold hardiness, including supercooling and freeze tolerance in *L. vivipara*.

So far, the cold hardiness strategy of *L. vivipara* has only been investigated by studying individuals coming from several French lowland and mountain viviparous populations (Costanzo et al., '95; Grenot et al., 2000; Voituron et al., 2000, 2002a, b). It is therefore of obvious interest whether the oviparous populations of this species present similar cold hardiness characteristics. This may enable us to know whether the evolution of cold hardiness paralleled the evolution of viviparity in this species.

We present here the first data allowing a comparison of the supercooling capacities, the survival time in frozen state, and the ice content tolerated between oviparous and viviparous populations of *L. vivipara*. We discuss the extent to which the characteristics observed could be related to the ecological characteristics of the species and to the phylogeographic history of its oviparous and viviparous forms.

MATERIALS AND METHODS

Animals

We used 15 viviparous individuals of *L. vivipara*, weighing 3.52 ± 0.62 g (mean $\pm$ SD). All were captured in a mountain population in the Cévennes in France (44.30°N, 3.45°E, altitude 1000 m). We used 23 oviparous individuals of different origins: six of them, weighing 4.24 ± 0.83 g, were captured at altitudes between 900 and 1100m in the Tarvisio valley (46.50°N, 13.25°E) in northeastern Italy; twelve others (3.80 ± 0.33 g) were captured at an altitude of 900m in the Ossau Valley (43.05°N, 0.40°W) in southwestern France, and the last five, weighing 3.43 ± 0.25 g, were captured in the Ossau Valley, but at lower (350m) altitude.

These lizards were captured in August, and kept in terraria in the laboratory; they were allowed to thermoregulate with lamps providing heat for 8 hours/day and to feed ad libitum for one month. The animals were starved one week before hibernation. Afterwards they were placed in small boxes, containing damp sand and wet mosses, that were kept in the dark in a hibernation chamber. The temperature in the hibernation chamber was progressively cooled from 10 to 4°C during the first ten days, and maintained at a constant 4°C for 3 months before experimentations. Supercooling and freezing experiments were conducted in January.

Cooling protocol and crystallisation temperatures

Each lizard was weighed to 0.01g, placed on a pad of absorbent paper, either wet or dry, at the bottom of a 50 ml Falcon plastic tube and progressively cooled at a constant rate of $0.2°C.min^{-1}$ in a Low Temperature Incubator

815 from PRECISION, from 4°C (initial temperature) down to the crystallisation temperature (Tc). A thermocouple placed against the lizard's side was used with a multichannel data logger to continuously record body temperatures during cooling. The onset of crystallisation was detected for each lizard by a sudden increase of its body temperature, due to the release of the heat of crystallisation, the exotherm. This indicated the crystallisation temperature (Tc), the lowest body temperature attained by each lizard during supercooling.

Supercooling test

The lizards (n=5 viviparous and n=5 oviparous) that were cooled in contact with a dry absorbent paper were not allowed to freeze and were immediately removed from their tubes at the onset of crystallisation. They were then kept in their hibernation boxes at 4°C for one week before being used again in a test of long term survival in the supercooled state. Lizards were re-introduced into individual tubes, where they were again kept for seven days in contact with a dry absorbent paper at a temperature of -3 ± 0.1°C, slightly above their crystallisation temperatures.

Freezing tolerance test

The lizards (n=18 oviparous, n=10 viviparous) that were cooled in contact with a wet absorbent paper remained in their tubes after the onset of crystallisation and were kept frozen for various lengths of time, ranging from 0.5 to 30 hours. We considered that the freezing exposure of each lizard began immediately after its exotherm and ended when this individual was removed from its tube. During this period all the individual tubes were placed in an incubation chamber set at −3°C (± 0.1°C). At the end of the freezing exposure we checked whether the individual had survived and we calculated its body ice content (% of body water frozen).

Body ice content

To determine the ice content of frozen lizards, we used the whole-body calorimetry technique described by Layne and Lee ('87, '91). Calculations of body ice content used experimentally determined values for our system which were: F factor for the calorimeter =1.05, the melting point of body fluids as estimated from osmolality determinations=−0.8°C for both strains, the percentage of body mass that is water for *L. vivipara* is $71.6 \pm 0.9\%$ for viviparous individuals and $72.4 \pm 1.2\%$ for oviparous individuals, calculated for 5 lizards of each strain by drying carcasses at 105°C to constant mass, specific heat of the dry mass measured by calorimetry (Sd)=0.25 ± 0.04 for both strains. For Sd values, the sets of oviparous and viviparous values were not significantly different.

RESULTS

Crystallisation temperatures

Statistical comparisons of the oviparous subgroups did not reveal any significant difference of the crystallisation temperatures (Tc) between lowland (350m) and mountain (above 900m) populations, nor between French and Italian populations (Kruskal-Wallis Statistic (KW)=1.93; p=0.41 for wet substrate treatment and KW=2.21; p=0.46 for dry substrate treatment). Hence it was possible to calculate the mean value of Tc for all the oviparous individuals pooled together and to compare it to the mean Tc value obtained for the viviparous individuals. A strong treatment effect (between dry vs. wet substrate) was observed in the two groups, but we did not find any significant differences between oviparous and viviparous populations (Table 1). The mean Tc values calculated for all oviparous and viviparous individuals was -2.0 ± 0.8 °C (n=28, range from −1.1 to −3.1 °C) in the wet substrate treatment and -4.0 ± 0.3°C (n=10, range from −3.5 to −4.5°C) in the dry substrate treatment.

Supercooling test

Among the ten lizards used in this experiment, only one oviparous individual began to freeze, as indicated by an exotherm, after 2 days of supercooling, and was immediately removed from the cooling chamber. The other nine remained supercooled at −3°C for the seven days and

TABLE 1. Tc values of viviparous and oviparous strains of L. vivipara *on dry and wet substrate*

	Viviparous strain of *L. vivipara*	Oviparous strain of *L. vivipara*	Statistic
Dry substrate	-4.1 ± 0.4	-3.9 ± 0.3	U′=16.5
	n=5	n=5	p=0.42
Wet substrate	-2.0 ± 0.5	-2.1 ± 0.4	U′=103
	n=10	n=18	p=0.55
Statistic	U′=50.0	U′=90.0	
	p<0.001	p<0.001	

subsequently reanimated rapidly upon warming. No mortality occurred during the following 3 weeks.

Freezing survival and ice content

The time course of ice accumulation (% of body water frozen), and the individuals' survival are presented in Figure 1.

During the first 5 hours of freezing, all the sampled individuals (5 viviparous and 9 oviparous) were alive. All the oviparous individuals (n=9) that were sampled after 5 hours of freezing were dead, whereas 3 out of 5 viviparous individuals were still alive when sampled after 9 to 24 hours of freezing (difference of survival between the 2 groups significant at p=0.03, Fisher exact probability test).

The ice accumulation rate of live individuals was very high during the first 5 hours (respectively $11.1 \pm 2.4\%$ and $14.9 \pm 6.2\%$ of total body water frozen per hour, for oviparous and viviparous individuals) and did not differ between the two reproductive forms ($U'=27.0$; $p=0.37$). The ice content of the body reached a plateau, at around 48%, for the individuals that survived after 5 hours of freezing. In contrast, for the eleven individuals that died during freezing, the ice content of their bodies increased continuously between 5 and 15 hours, reaching equilibrium at $77.5 \pm 1.7\%$.

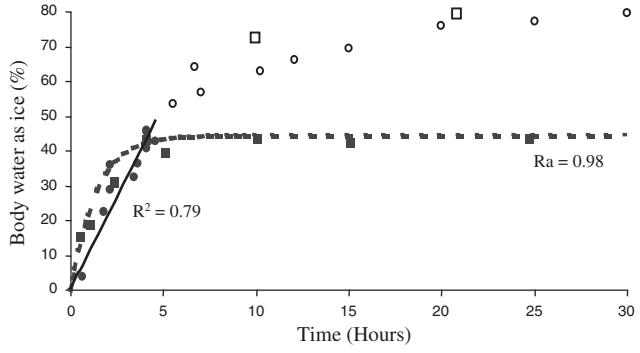

Fig. 1. The time course of ice accumulation in oviparous (circles) and viviparous (squares) lizards subjected to freezing at −3°C. Open symbols correspond to the animals that died during freezing; data for these animals were not used in fitting the curves. A non-linear regression fitted the data from the viviparous strain. The hyperbolic plot: $y=47.8\ (1-\exp^{(-0.72x)})$ represent the best fit to the percentage of body water frozen in viviparous lizards over time. We 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). The time course of ice accumulation from surviving oviparous individuals (those sampled between 0.5 and 5 hours of freezing exposure) followed a linear regression ($y=10.8x$; $R^2=0.79$; $n=10$).

DISCUSSION

The present study provides conclusive evidence for a well-developed capacity for tolerance below 0°C in *L. vivipara*. Both the oviparous and the viviparous individuals of this species were able to survive for at least 7 days in a supercooled state at −3°C, have similar crystallisation temperatures (−4 and −2°C under dry and wet conditions respectively), and were also able to tolerate up to 50% of their body fluid converted into ice for at least 5 hours. The present study, however, indicates that oviparous individuals are not able to survive to freezing for more than 5 hours. On the contrary, it has been shown that some viviparous individuals (3 out of 5 in the present study and 5 out of 6 in the study of Voituron et al., 2002a) are able to survive and to stabilize their body ice content below 50 % for up to 24h when frozen at −3°C. Hence, the only difference observed between oviparous and viviparous *L. vivipara* was their capacity for freeze tolerance. The oviparous *L. vivipara* is able to tolerate having 50% of its body water frozen, but is unable to stabilize ice accumulation, and therefore, to survive. Hence, according to Sinclair's nomenclature (Sinclair, '99), this strain might be considered as partially freeze-tolerant. In contrast, the viviparous individuals of this species are able to stabilize ice accumulation at 50% and thus can survive for much longer periods; they might be classified as moderate freeze-tolerant. The ice rate accumulation and the equilibrium ice content attained are dependent upon the temperature of freezing exposure (Costanzo et al., '91); thus, it would be interesting to test higher sub-zero temperatures between −0.5°C and −1°C which might allow some individuals of this oviparous strain to survive longer than 5 hours. Nevertheless, our data show that, at least at −3°C, there is an intraspecific variation in the degree of freezing tolerance between the oviparous and viviparous forms of *L. vivipara*. The data also suggest that this evolution could parallel the evolution of viviparity which is also very likely to be strongly selected under cold climatic conditions. This is also congruent with the phylogeographical investigations, based on mtDNA sequences analysis, indicating that the differentiation of the oviparous and viviparous *L. vivipara* occurred during the Pleistocene, a period with

severe glacial phases. The viviparous populations subsequently colonized the northern part of the distribution range, whereas the oviparous populations remained restricted on the southern margin of this range (Heulin et al., '99; Surget-Groba et al., 2001; Surget-Groba, 2002).

The cold tolerance capacities are much more pronounced in the oviparous and viviparous *L. vivipara* than in the other European Lacertids so far studied. The sand lizard *L. agilis* and the wall lizards *Podarcis sicula* and *Podarcis muralis* are not able to survive even for short periods (30 to 120 min) when kept below −1.1°C and never tolerated more than 28% of their body fluid converted into ice (Weigmann, '29; Claussen et al., '90; Burke et al., 2002).

The fact that the viviparous form of *L. vivipara* has successfully colonized northern regions up to latitude 69°N, which is the northernmost record for reptiles (Borkin et al., '84), and that both the oviparous and viviparous forms of this lizard have colonised relatively high altitude areas (2200–2500m) (Heulin and Guillaume, '89) may be related to the cold tolerance observed in this species. It should be noted, however, that the oviparous populations of *L. vivipara* are restricted to latitudes below 47°N (Heulin and Guillaume, '89, Surget-Groba et al., 2002), and that this is actually below the northernmost limits of 51°N and 60°N, respectively, observed for *Podarcis muralis* and *L. agilis* (Matz and Weber, '99). In addition, the altitudinal distributions of *Podarcis muralis* and *L. agilis* also extend close to 2200m. Therefore, one may suspect that additional ecological factors, other than the altitudinal and latitudinal distribution, have also contributed to the evolution of a stronger cold tolerance in *L. vivipara* than in other Lacertid species. With regard to this, it should be stressed that the most striking ecological particularity of *L. vivipara* is that this species preferentially lives in wet biotopes, in particular peatbogs, fens, and wet heathlands, and that this may expose the individuals of this species to increased risks of freezing due to the ice-inoculation phenomenon. This inoculative freezing, which is promoted when ice crystals in the environment come into contact with the body (Costanzo and Lee, '96; Lee and Costanzo, '98), is clearly illustrated by our data; in *L. vivipara* the crystallisation of body fluids began at −4°C under dry conditions without ambient ice crystals but at −2°C under wet conditions with ambient ice crystals. It was previously observed that both the oviparous and viviparous individuals have overwintering sites located at shallow depths (1–8 cm), within damp substrates such as mosses, grass hummocks, and tuft (Grenot and Heulin, '88; Osenegg, '95). Consequently, it may be suspected that both forms have to cope with the risks of subzero temperature and inoculative freezing. The dual-factor system of cold hardiness identified in oviparous (supercooling+partial freeze tolerance) and viviparous (supercooling+moderate freeze tolerance) individuals may promote winter survival in their dynamic thermal and hydric environment. Bauwens ('81) previously observed that viviparous individuals of *L. vivipara* had a very high survival rate between 88–100% for all age classes during a severe winter, but there are no data in the literature on the survival rates of oviparous individuals during winter.

Intraspecific variations in cold tolerance have been rarely studied in insects (Baust and Lee, '81, '83; Horwath and Duman, '84; Duman et al., '91) and in some ectothermic vertebrates such as the hatchling turtles *Chrysemys picta* (Packard and Janzen, '96). All these intraspecific variations, however, concerned only the crystallisation temperatures and were caused by the influence of some external factors such as winter intensity, soil moisture, or presence of undigested food in the guts (Baust and Lee, '83; Costanzo et al., 2001). Our data on *L. vivipara* are original because they did not reveal a variation of the crystallisation temperatures, nor a differential effect of dry and wet microenvironments on the crystallisation temperature between the oviparous and viviparous individuals. Our study provides the first evidence of the existence of an intraspecific variation of freeze tolerance in a reptile. Such a model species is therefore of considerable interest for future studies aimed at identifying the physiological processes underlying the evolution of freeze tolerance in reptiles. Previous investigations suggest that, as is known for Anurans, the physiological responses of reptiles to freezing also involves, to a lesser extent, osmotic variations and a glucose mobilization process (Storey et al., '88; Costanzo et al., '93; Voituron et al., 2002a). Further experiments including assessment during freezing of the ice distribution within body, organ dehydration, amino acid variations, and gene expression are thus needed. Such comparative studies will provide the opportunity to test the extent to which such physiological and genetic responses differ between the oviparous and viviparous individuals, and whether they are involved in the

transition from a partially freeze tolerant to a moderate freeze tolerant strategy.

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