

The respiratory metabolism of a lizard (*Lacerta vivipara*) in supercooled and frozen states

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The respiratory metabolism of a lizard (*Lacerta vivipara*) in supercooled and frozen states. *Am J Physiol Regulatory Integrative Comp Physiol* 283: R181–R186, 2002; 10.1152/ajpregu.00378.2001.—We investigated the respiratory metabolism of the overwintering lizard *Lacerta vivipara* while in either supercooled or frozen states. With a variable pressure and volume microrespirometer and a chromatograph, we show that the oxygen consumption of the supercooled animals showed a nonlinear relationship with temperature and an aerobic metabolism demand between 0.5 and -1.5°C . A significant increase in the respiratory quotient (RQ) values indicated an increasing contribution by the anaerobic pathways with decreasing temperature. In the frozen state, two phases are easily detectable and are probably linked to the ice formation within the body. During the first 5–6 h, the animals showed an oxygen consumption of $3.52 \pm 0.28 \mu\text{l}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ and a RQ value of 0.52 ± 0.09 . In contrast, after ice equilibrium, oxygen consumption decreased sharply ($0.55 \pm 0.09 \mu\text{l}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$) and the RQ values increased (2.49 ± 0.65). The present study confirms the fact that supercooled invertebrates and vertebrates respond differently to subzero temperatures, in terms of aerobic metabolism, and it shows that aerobic metabolism persists under freezing conditions.

oxygen consumption; anaerobiosis; respiratory quotient; Lacertidae

MOST TEMPERATE ECTOTHERMS avoid subfreezing temperatures by migration or by using insulated hibernacula. However, some species, particularly terrestrial hibernators (i.e., certain amphibians, reptiles, and insects), have developed specific physiological mechanisms that allow them to evade cold injuries even during Arctic winter. The cold-hardiness strategies of such animals can be divided into two main groups: freeze tolerance, in which the animal endures the conversion of a fraction of its body water into ice; and freeze avoidance, in which the animal prevents crystallization (i.e., remaining in a metastable supercooled state) and preserves the liquid state of body fluids even at very low temperatures.

Organs such as the lungs, heart, and liver are strongly affected by these two different strategies:

freezing induces a cessation of heart activity and circulation, imposing an anoxic state on tissue cells (19). In contrast, in the supercooled state, the heart continues to beat even if the rate of contraction changes with temperature (3), and the lungs remain functional even if anaerobic metabolism takes on greater importance as temperature decreases (12).

To compare the metabolic balance (anaerobic vs. aerobic metabolism) under these two physiological states, we focused our study on O_2 consumption and CO_2 release during cooling, supercooling, and freezing states. In fact, it is well known that the gas exchange rate in ectotherms is strongly dependent on environmental conditions and the physiological state of the animal (7, 24), but few studies have assessed the gas flux in these particular physiological states. Kalabuchov (13) only reported several values of oxygen consumption of supercooled *Tenebrio* larvae between -7.5 and -10°C . Scholander and collaborators (25) studied the respiratory metabolism of five species of vascular plants and one chironomid larva under freezing conditions and found a direct logarithmic relationship with temperature below the freezing point. Similar relationships have been found in supercooled and frozen insects (1, 23) and intertidal animals (14). However, even if the shape of O_2 consumption and CO_2 release responses to temperature are alike, the slope is generally far stronger for frozen individuals. For vertebrates, as far as we know, only one study with a supercooled lizard, *Uta stansburiana*, has been conducted (11). In contrast to invertebrates, *Uta stansburiana* exhibited a linear relationship between temperature and oxygen consumption in the supercooled state (11).

Given the lack of data in this field, especially for cold-adapted vertebrates, we chose to study the European common lizard, *Lacerta vivipara* Jacquin, which exhibits the rare capacity of surviving during winter by means of both freeze tolerance and freeze avoidance (5). This lacertidae endures the conversion of nearly 50% of its total body water into ice (30) and can also remain supercooled for at least 21 days at -3.5°C (5). Such a rare physiological capacity could explain the exceptional survival rates of this lizard (88–100%, all

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age classes) even during the extreme cold of winter (2) that it experiences in the northerly limit (beyond the Arctic circle) of its geographical range.

MATERIALS AND METHODS

Population studied. This species inhabits mainly damp habitats such as meadows, peatbogs, and heathlands and hibernates in shallow terrestrial hibernacula (8). *Lacerta vivipara* (2.94 ± 1.25 g) were collected from the Jura mountains, France (latitude: $46^{\circ}48'7''$ N; longitude: $6^{\circ}11'1''$ E; altitude 850 m) during August and September and kept in 15-m² outdoor enclosures within which the natural peatbog environment was reconstituted. In this manner, the animals were acclimated under natural conditions until the end of October.

Animals. At the beginning of the winter period, each individual was identified and marked by clipping a finger toe. Lizards were then maintained at 5°C in the laboratory until the start of experiments. All individuals were kept in plastic boxes containing sand and damp moss (*Sphagnum*).

Only nonparasitized lizards were used for these experiments. The presence of a hemogregarine detected in this population (Surget-Groba, personal communication) affects the physiology of these lizards, inducing a decrease of O₂ consumption (20). A smear of blood collected just after clipping the fingertips allowed us to determine the absence/presence of parasites. Smears were air dried, fixed in absolute methanol, and stained with Giemsa stain. For parasite detection, we used a magnification of $\times 800$.

Respirometric protocol. We estimated gas flux production using a variable pressure and volume microrespirometer (28). A Manovolumate (manufactured by AISSOR, SARL) was used for these experiments. This apparatus is a physical captor of microvariations of the gas parameters (pressure, volume, and temperature) into respirometric units (see Fig. 1). The principle of the measurement is based on the differential form of the ideal gas law: $dN \cdot R \cdot T = P \cdot dV + V \cdot dP$. A manovolumetric liquid flows out of the reservoir and fills the capillary by capillarity and gravity. The liquid columns reach an equilibrated length (L_0), which depends on the residual air volume in the respirometer cells and the physical properties of the liquid used. Variations in length (dL ; see Fig. 1) of the fluid column moving within the glass capillary tube of a given section (s) are dependent on pressure (dP) and volume (dV) variations occurring within the respirometric cell.

The lengthening of the liquid column measured at regular intervals allows the calculation of the simultaneous variation in pressure and volume inside the respirometric cells according to the relation

$$dV = s \cdot dL, \quad dP = \rho \cdot dL$$

with ρ = density of manovolumetric liquid and s = capillary section. The sensibility of the apparatus, defined as the quantity of consumed gases for 1 mm of index displacement, is ~ 0.1 μ l. If these pressure and volume variations are transformed into the differential form of the ideal gas law, $dN \cdot R \cdot T = P \cdot dV + V \cdot dP$, the gas quantity variations can be calculated. We expressed such variations in microliters (STP) of oxygen per gram of wet weight per hour.

In addition to the control unit (same respirometric cell without the animal) that allows estimation of the movement of the gas parameter variations independently of the respirometric activity of the animal, six microrespirometric units were used. A microrespirometric unit constituted a measure chamber (20-ml syringes) connected first to a CO₂ fixator (KOH 10%) reservoir and second to a manovolumetric probe (Fig. 1B). Because the temperature was kept constant (all respirometric chambers were immersed in a thermostat-controlled bath of ethylene glycol capable of regulating the chamber temperature to an accuracy of $\pm 0.05^{\circ}\text{C}$), all gas quantity variations corresponded to either oxygen consumption (with KOH) or a total flux (O₂ + CO₂; without KOH). Measurements of gas exchange of nonfrozen lizards between 4 and -4.5°C were conducted on 15 individuals. Before respirometric assessment, all the lizards were weighed to the nearest 0.001 g. Between six and eight values of oxygen consumption (at each temperature for each animal) were selected from the lowest but consistent readings to represent standard metabolism.

However, as Kanwisher (14) noticed, "manometric and volumetric techniques cannot be used (to quantify respiratory metabolism in a frozen living organism) because of the volume change when water turns to ice." We confirmed these observations because in the event of the animal freezing, the index rose, rendering the O₂ consumption measurements impossible. Such a response of the apparatus allowed us to be sure of the nonfrozen (and thus supercooled) state of the animal under observation. Thus we used a chromatographic technique to measure oxygen consumption and CO₂ rejection in frozen animals.

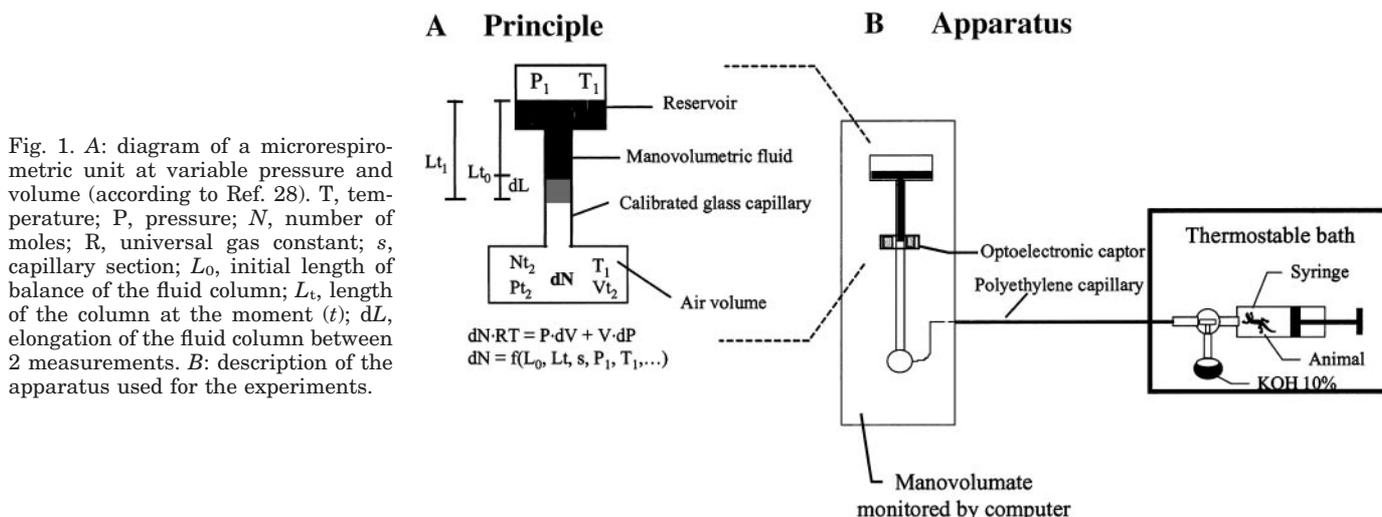


Fig. 1. A: diagram of a microrespirometric unit at variable pressure and volume (according to Ref. 28). T, temperature; P, pressure; N, number of moles; R, universal gas constant; s, capillary section; L_0 , initial length of balance of the fluid column; L_t , length of the column at the moment (t); dL , elongation of the fluid column between 2 measurements. B: description of the apparatus used for the experiments.

Chromatographic technique. Measurements of the gas exchange of frozen lizards were conducted on nine individuals frozen at -3°C over varying intervals between 30 min and 24 h with a gas chromatograph Micro GC (Chrompack CP 2002P) monitored with the software Maestro II version 2.4. These animals were maintained on a pad of paper towel with a Type-K thermocouple in contact with the abdomen of the animal. The thermocouple was connected to a Bioblock MT 100 KC datalogger so that temperature of the animal could be measured every 30 s. The linearity of the thermistor was previously verified between a water/ice mix (0°C) and water vapor (100°C). A band of masking tape was used to secure the animal and thermocouple in place. Water was pipetted onto the towel around the animal so that the lower surface of the body and limbs were in contact with a damp substrate. The secured animal was then placed in a 10-ml flask itself placed in a thermostat-controlled bath of ethylene glycol (capable of regulating the temperature to an accuracy of $\pm 0.05^{\circ}\text{C}$) at 2°C . The temperature was then slowly decreased until the animal froze. In the instances where animals cooled to -3°C without freezing, the temperature was then lowered in intervals (-0.5°C per 15 min) until nucleation occurred and temperature was then immediately readjusted upward to -3°C and held there for the duration of the observation. The onset of ice formation was detected by the exotherm (i.e., an abrupt increase in temperature caused by the release of latent heat of fusion by water undergoing a transition in phase from liquid to solid). Once the animal was frozen, the flask was sealed and a 500- μl control sample of the air chamber was processed. Because such sampling induces a depression in the chamber, the pressure was reequilibrated and atmosphere was renewed from an air reserve kept at the same temperature. Once the pressure reequilibrated, the sample chamber was hermetically closed and the air was sampled every 4 h. After each sampling, the renewal of the air chamber, the control sampling, and the pressure reequilibration were processed. At the end of the freezing trials, the animals were thawed at 3°C , during which gas exchange measurements were continued. The gas flux assessments ended after 48 h of thawing. The quantities of consumed O_2 or rejected CO_2 were calculated from the evolution of the relative quantities of gases measured at regular intervals with a 500- μl sample analyzed every 4 h with the chromatograph. Our experimental protocol was designed to show the time effect but not the temperature effect on frozen *Lacerta vivipara*. This is explainable by the biology of the lizard, which first never encounters very harsh temperatures and second freezes at a high subzero temperature because of the 100% humidity of its overwintering site (8, 10). Furthermore, the survival rate below -4°C is very low for frozen *Lacerta vivipara* (30). Thus the 2 – 3°C range was thought to be too small to estimate the temperature effect.

Statistics. During a pilot experiment conducted the winter before, the oxygen consumption of four frozen/thawed lizards was followed. In all the analyses (presented as means \pm SD), data were pooled for the statistical analyses and were performed with the SAS computer statistical package. A 5% ($P < 0.05$) level of significance was used in all tests.

RESULTS

Gas exchange in the supercooled state. Our study showed that oxygen consumption sharply decreases between 4 and -4.5°C ranging from 24.87 ± 2.17 to

$0.35 \pm 0.02 \mu\text{l}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$. The regression of the frequency distribution of oxygen consumption and CO_2 rejection as a function of temperature is shown in Fig. 2, A and B. The oxygen consumption values vs. temperature for the nonfrozen animals (supercooled and above melting point) were treated by linear regression ($y = 2.3845x + 12.57$; $R^2 = 0.88$; $F = 720$; $P < 0.0001$). However, a finer analysis using a third-degree polynomial ($y = 0.093x^3 + 0.123x^2 + 1.286x + 11.443$) significantly increased the percentage of variance explained ($R^2 = 0.93$; $F = 50.95$; $P < 0.0001$). This curve may be divided into three sections; a plateau, between 0.5 and -1.5°C with essentially no response to temperature change (slope value 0.86) flanked by two decreases with similar slopes (3.07 and 4.56). The pattern of CO_2 release differed from that of oxygen uptake and was indicated by the fact that a polynomial regression was not significantly better than the linear regression ($R^2 = 0.57$ for both analyses). The respiratory quotient (RQ) values show a significant tendency to increase with decreasing temperature ($F = 11.62$; $R^2 = 0.32$; $P = 0.001$) from 0.58 ± 0.11 at 4°C to 0.95 ± 0.004 at -4.5°C (Fig. 3). However, the pattern of RQ evolution did differ slightly among individuals. These interindi-

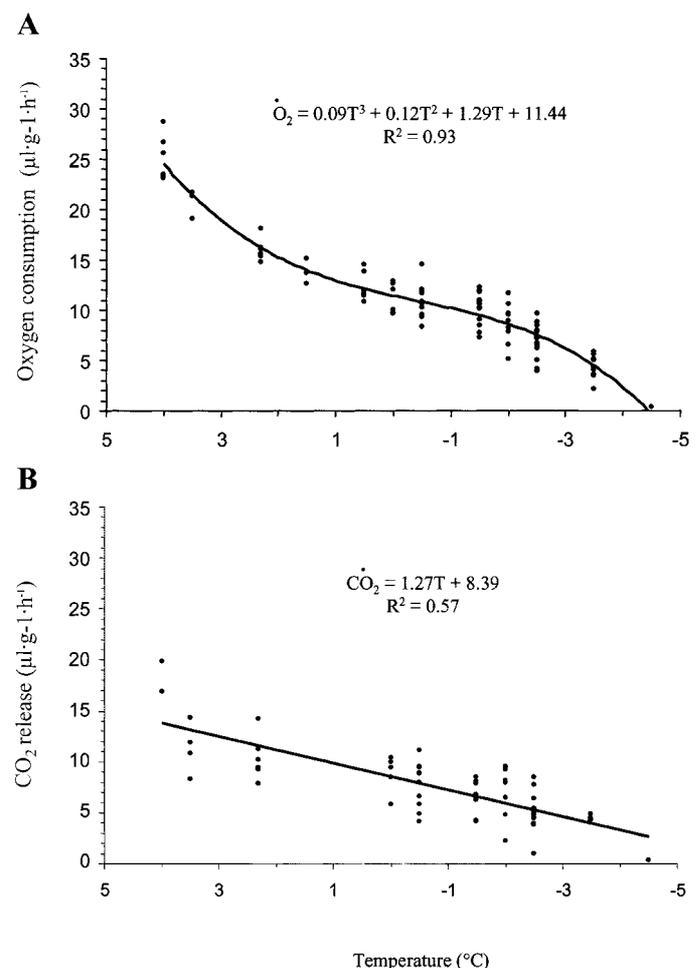


Fig. 2. O_2 consumption (A) and CO_2 rejection (B) of European common lizards (*Lacerta vivipara*) between 4 and -4.5°C .

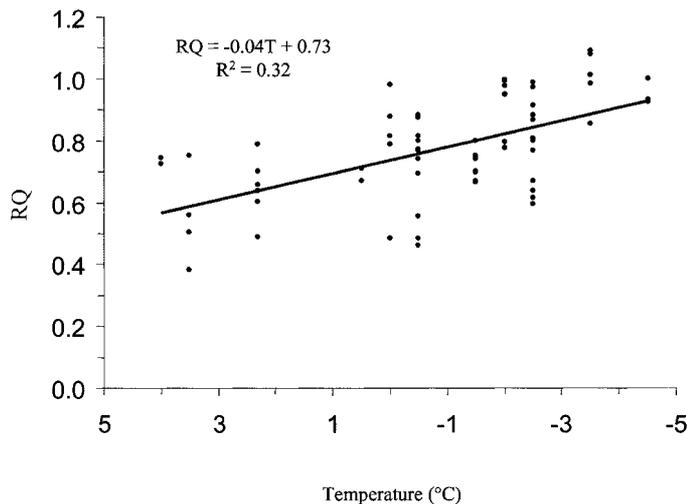


Fig. 3. Respiratory quotient (RQ) of *Lacerta vivipara* as a function of temperature between 4 and -4.5°C .

vidual differences explain the low R^2 value (0.32). It is noteworthy that the first value above 1.0 (presence of anaerobic metabolism) appears at -2.5°C and below. No lizard died in a supercooled state during the experiments.

Gas exchange in the frozen state. The lizards exhibited a mean crystallization temperature of $-2.0 \pm 0.02^{\circ}\text{C}$. Once the exotherm has been detected, two phases are easily detectable in the respirometric response of frozen *L. vivipara*. The onset of freezing induces a 42% decrease in oxygen consumption compared with supercooled animals at the same temperature. During the first 5–6 h, the animals consumed $3.52 \pm 0.28 \mu\text{l}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ of oxygen with a RQ value of 0.52 ± 0.09 . The second phase was characterized by a sharp decrease in the oxygen consumption ($0.55 \pm 0.09 \mu\text{l}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$) accompanied by a sharp increase of the RQ (2.49 ± 0.65) mainly explained by the cessation of the O_2 consumption (Fig. 4). These values remained constant until the end of the freezing trial, just after the rise in temperature leading to the thawing of the lizard; the oxygen consumption increased and the RQ slowly returned to below 1, indicating the return of aerobic metabolism. However, no oxygen debt was observed even during the 24-h freezing trials. A typical curve of oxygen consumption and RQ variation during the supercooling, freezing, and thawing periods is shown in Fig. 4.

Three lizards (representing 33% of the sample) died within 2 days following freezing exposure. Their oxygen consumption never returned to normal values at 3°C and remained between 3 and $7 \mu\text{l}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ and slowly decreased until the death of the animal. Thus similar to freeze-tolerant frogs *Pseudacris crucifer* and *Rana sylvatica* (17, 18), lethal freeze injury markedly reduces oxygen consumption. The corresponding values for *L. vivipara* were not taken into account for the statistical analysis.

DISCUSSION

L. vivipara is a very adaptable species that can inhabit a wide range of environments (meadows, peatbogs, heathlands, and from sea level to 3,000-m altitude) due to its remarkable physiological plasticity (9). Its geographic distribution extends from the mountains of northwest Spain to Sakhalin on the Pacific coast, a distance of 12,000 km, and from northern Spain to beyond the Arctic Circle, a span of nearly 3,000 km in latitude. During winter, this small lacertidae increases its anaerobic metabolism by $\sim 20\%$ leading to the accumulation of lactate and D-3-hydroxybutyrate (29). It was hypothesized that the relative contribution of anaerobic pathways would be greater at subzero temperatures in both a supercooled state and in a frozen state. Because of the observed tendency for the RQ values to increase with decreasing tempera-

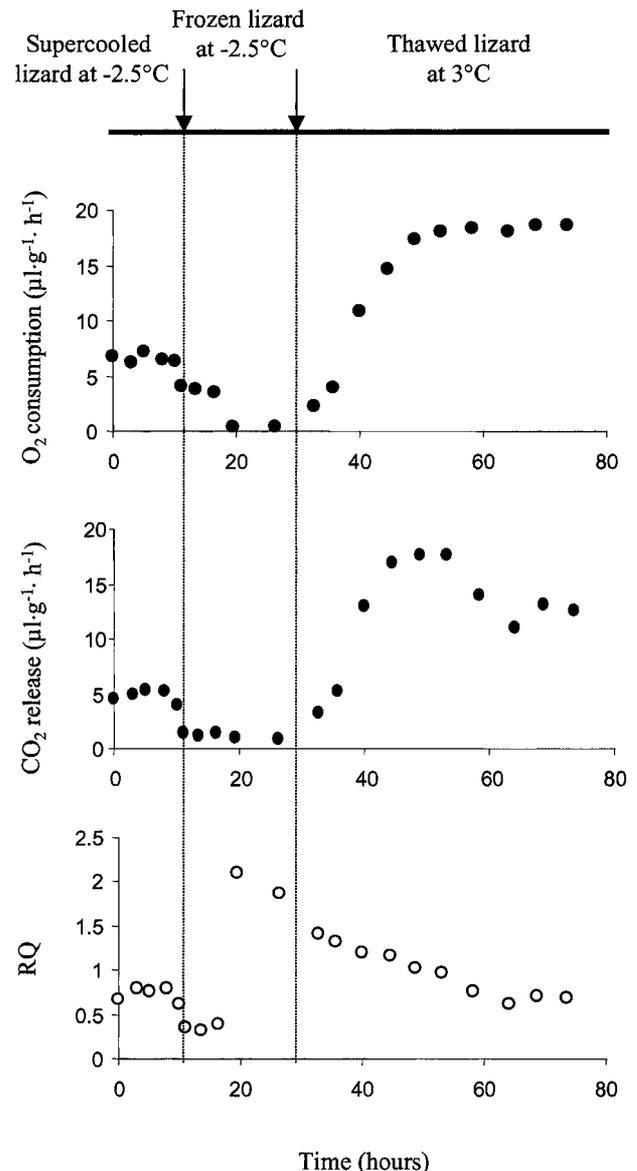


Fig. 4. Time course of O_2 consumption and RQ values of a 22-h frozen *Lacerta vivipara*.

tures in nonfrozen lizards, we conclude that this species increases its anaerobic metabolism with the decreasing temperature. This is probably a response of the tissues facing a decrease in available oxygen. Recent studies showed that heart rate and the oxygen delivery system are hampered by such low temperatures inducing a functional hypoxia (6, 12). Our data also show that aerobic metabolism is still dependent on temperature even in a supercooled state. The consumption rate at -2.5°C is only about one-fourth of that measured at 4°C , indicating a pronounced metabolic depression that certainly constitutes an energy conservation adaptation during the winter period. However, oxygen consumption declines linearly rather than logarithmically as it does when temperature is above 5°C (21). Furthermore, a slope damping appears between 0.5 and -1.5°C . This observation can be explained by an activation of different aerobic metabolic pathways leading to the synthesis of different metabolites that probably play a role in the two cold-hardiness strategies of *L. vivipara* (freeze tolerance and freeze avoidance) (5). It is noteworthy that an adequate cryoprotective system is activated before attainment of the crystallization temperature of the animal. Similar phenomena are known in cold-hardy insects where the production of polyols is often initiated below 5°C and is maximal between ~ 0 and -5°C both for freeze-tolerant and freeze-avoiding animals (15, 22, 26). However, the mechanisms involved are likely to be different from those of insects as *L. vivipara*, similar to other freeze-tolerant reptiles, does not accumulate large amounts of low molecular weight cryoprotectant (30).

Comparing our respirometric values to those obtained from the desert lizard *Uta stansburiana*, it appears that *L. vivipara* has a higher metabolic rate at 5°C but also a higher Q_{10} (from 2.2 to 3.73 compared with the 2.17–5.82 of *L. vivipara*) (11). This difference leads to similar values of oxygen consumption at sub-zero temperatures for both species. This high Q_{10} value may be interpreted as an adaptation of *L. vivipara* that reduces energetic costs at low temperatures. A reanalysis of the O_2 consumption of *Uta stansburiana* demonstrates the absence of a plateau at any temperature as the R^2 value is not improved by a finer analysis (0.96 by linear regression against 0.95 by the polynomial of degree 3). Thus it is reasonable to think that the physiological mechanisms underlying this plateau also constitute an adaptation of cold temperate ectotherms resulting in improved cold hardiness.

In contrast to the oxygen consumption curve, a higher-degree polynomial regression does not significantly improve the R^2 value ($R^2 = 0.57$ for polynomial and linear analyses) for the CO_2 curve. The absence of a plateau here may be explained by the high solubility of this gas at low temperature (7) in addition to the buffering capacity of the animal's blood. However, because this buffering capacity changes with temperature (7), the interpretation of the absence of the plateau will require further experiments involving the determination of the response of the pH/pCO_2 curve to

variations in temperature for overwintering supercooled and frozen *Lacerta vivipara*.

The freeze-tolerance capacity is supported by an ice-control mechanism, cell volume regulation, and a mechanism of anoxia/ischemia tolerance. During the freezing period, organisms exhibit no active signs of life, mainly due to the freezing of all organs except the brain, which maintains minimal activity (27). Since the studies of Salt (23) and Asahina (1), data on the respiration of frozen organisms have received little attention. Only Scholander et al. (25) assessed the aerobic metabolism in several plants and animals and found that "below freezing the oxygen consumption dropped steeply but still followed an exponential function with decreasing temperature." So, even if the metabolic rate is low during the frozen state, it is still measurable. The curve in Fig. 3 shows that the time course of the oxygen consumption of a frozen lizard drops steeply after 5–6 h of freezing. This corresponds to the time required to reach the ice equilibrium within the body (between 45 and 50% of body water converted into ice within 5 h) (30). Thereafter, oxygen consumption remains very low but constant for the duration of the freezing. These observations suggest that the internal organs such as the lungs are the last part of the body that freezes. A similar delay (15 h) has been observed for the cessation of the heart of frozen wood frogs *Rana sylvatica* (19). The 10-h difference observed between these two freeze-tolerant vertebrates may be explained by the large difference in body mass between the lizard and the frog.

On the basis of the RQ values, it is suggested that anaerobic metabolism occurs during the freezing period to maintain basal metabolism. However, it is important to note that gas diffusion (in particular CO_2) could occur in our experimental conditions. We noticed that a CO_2 -enhanced piece of ice can diffuse a part of the captured CO_2 toward the atmosphere [maximum value observed: $1.86 \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$ at -3°C with an ice cube obtained with 3 ml of water (initial $\text{PCO}_2 = 12.64$ Torr; molality = 0.5)]. Thus, although a frozen organism cannot be considered as a pure ice cube, principally due to the integument of the animal that constitutes a barrier, such diffusion may induce a slight overestimation of the RQ values observed in our experiment in particular due to the extremely low oxygen consumption in the frozen state (Fig. 4). Despite this possible diffusion effect, the nonoxidative pathways still represent the only energetic source in the frozen state and so end-product accumulation should occur. However, no "oxygen debts" were detected after thawing. The absence of an oxygen debt has already been observed in 1-day frozen *Rana sylvatica* (an oxygen debt was only detected for 7-day frozen *Rana sylvatica*) (16). The freezing duration of 24 h is thus probably insufficient to induce a metabolic compensation for freeze-induced disturbances in cells.

L. vivipara need 23.7 ± 2.5 h after the onset of thawing to reach a normal O_2 consumption at 3°C . It is noteworthy that the lizards return to an aerobic metabolism (RQ < 1) only when O_2 consumption values

reach their control values (see Fig. 4). Even if direct comparison with other freeze-tolerant vertebrates is difficult due to the different freezing durations and thawing temperatures (17), it has been established that this species takes a significantly greater time than *Rana sylvatica* and *Pseudacris crucifer* (16) to recover its total aerobic capacity. This important delay can be correlated with the long recovery time (44.8 ± 4.5 h) for the righting reflex in this species (30).

Perspectives

The present study confirms the fact that supercooled invertebrates and vertebrates respond differently to subzero temperatures in terms of aerobic metabolism. Such differences may derive from the nature of gas exchange and delivery in these two phyla. The tracheal system of insects avoids the energetic waste associated with the pulmonary and cardiovascular systems of vertebrates. Supercooled lizards obtain energy mostly through the oxidation of metabolic compounds, but anaerobic metabolism may play an important role especially at temperatures below -5°C (see Fig. 3). Because production of ATP under such conditions induces an acidosis (12), it would be interesting to study the buffering capacities of this lizard, which may constitute a key element of its winter survival. Metabolic activity under freezing conditions, even if very low, is still present. It would seem important now to conduct similar experiments on highly freeze-tolerant vertebrates such as *Rana sylvatica*, which can endure several weeks of freezing (27), and also poorly freeze-tolerant vertebrates such as *Podarcis sicula* (4). Such comparison between animals exhibiting different levels of freeze tolerance could provide deeper insight into the evolutionary physiology of temperate ectothermic species.

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