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Survival and metabolism of *Rana arvalis* during freezing

Yann Voituron, Louise Paaschburg,
Martin Holmstrup, Hervé Barré, Hans Ramløv

Abstract Freeze tolerance and changes in metabolism during freezing were investigated in the moor frog (*Rana arvalis*) under laboratory conditions. The data show for the first time a well-developed freeze tolerance in juveniles of a European frog capable of surviving a freezing exposure of about 72 h with a final body temperature of -3°C . A biochemical analysis showed an increase in liver and muscle glucose in response to freezing (respectively, 14-fold and 4-fold between 4 and -1°C). Lactate accumulation was only observed in the liver (4.1 ± 0.8 against $16.6 \pm 2.4 \mu\text{mol g}^{-1}$ fresh weight (FW) between 4 and -1°C). The quantification of the respiratory metabolism of frozen frogs

showed that the aerobic metabolism persists under freezing conditions ($1.4 \pm 0.7 \mu\text{l O}_2 \text{g}^{-1} \text{FW h}^{-1}$ at -4°C) and decreases with body temperature. After thawing, the oxygen consumption rose rapidly during the first hour (6-fold to 16-fold) and continued to increase for 24 h, but at a lower rate. In early winter, juvenile *R. arvalis* held in an outdoor enclosure were observed to emerge from ponds and hibernate in the upper soil and litter layers. Temperature recordings in the substratum of the enclosure suggested that the hibernacula of these juvenile frogs provided sheltering from sub-zero air temperatures and reduced the time spent in a frozen state corresponding well with the observed freeze tolerance of the juveniles. This study strongly suggests that freeze tolerance of *R. arvalis* is an adaptive trait necessary for winter survival.

Y. Voituron (✉)
Ecologie des Hydrosystème Fluviaux (U.M.R. CNRS 5023),
Université Claude Bernard Lyon1, Université de Lyon,
Bât. Darwin C, 69622 Villeurbanne Cedex, France
e-mail: yann.voituron@univ-lyon1.fr; voituron@univ-lyon1.fr

L. Paaschburg · H. Ramløv
Department of Science, Systems and Models,
Roskilde University, Universitetvej 1, P.O. Box 260,
4000 Roskilde, Denmark
e-mail: hr@ruc.dk

M. Holmstrup
Department of Terrestrial Ecology,
National Environmental Research Institute, University of Aarhus,
Vejløsvej 25, P.O. Box 314, 8600 Silkeborg, Denmark
e-mail: mho@dmu.dk

H. Barré
Laboratoire de Physiologie Intégrative,
Cellulaire et Moléculaire UMR 5123,
Université Claude Bernard Lyon 1, Université de Lyon,
43 bd du 11 Novembre 1918, 69622 Villeurbanne Cedex, France
e-mail: herve.barre@univ-lyon1.fr

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Hibernaculum · Metabolic rate · Ranidae · Winter survival

Introduction

Water, which is major constituent of living organisms, has the particularity of being present in various forms according to the temperature (gas, liquid, solid, vitreous). Upon cooling, water freezes to ice; this phase transition occurs widely in free water and within ectotherm animals whose body temperature follows the environmental temperatures. However, survival of the conversion of a significant part of the body water into ice requires a number of adaptations allowing a slow propagation of ice restricted to the extracellular spaces, and the avoidance of cell volume reduction and dehydration beyond an injurious limit (the minimum cellular volume) (Ramløv 2000). A number of complementary physiological processes are initiated upon the cooling

of freeze tolerant animals. These include the production of ice nucleators (e.g., in insects), which allow the initiation of freezing at high sub-zero temperatures and the accumulation of cryoprotectants that elevates body fluid osmolarity, decreasing the extent of cell volume reduction during extracellular ice formation, preventing cell shrinkage below a critical minimum cell volume and stabilizing membrane proteins (Storey and Storey 1988). Various invertebrates and vertebrates exhibit some degree of freeze tolerance (from a few hours at high sub-zero temperatures to several months at temperatures as low as -50°C). However, other cold tolerant ectotherms do not tolerate ice within their body and their survival during periods of sub-zero temperatures is then dependant on the ability to avoid freezing by reducing their crystallization temperature. This freeze avoidance strategy is characterized by various metabolic adaptations involving the release/masking of potent ice nucleators and the accumulation of low molecular weight substances such as carbohydrates and polyols and anti-freeze proteins that provide freezing point depression (Zachariassen 1985). Among vertebrates, the group most studied regarding freeze tolerance are the Anurans. Anurans are highly sensitive to inoculative freezing because of their water-permeable skin, which ensure freezing at high sub-zero temperatures (Costanzo et al. 1999). Ice may spread through the tissues during cooling below the melting point of the body fluids and can reach a maximum level of about 65% in freeze tolerant anurans. Ice content above this level is traditionally considered lethal in the anurans investigated to date (Storey and Storey 1992).

Despite a number of studies on freeze tolerance in Anurans, data on Eurasian species, even those distributed far North, are still scarce. With regard to their overwintering sites and the winter climate they endure, some species may have evolved significant freeze tolerance capacities. This may be the case in the moor frog, *Rana arvalis*, which is a lowland species with a broad Eurasiatic distribution, extending from the arctic tundra through forest to the steppe zone (Matz and Weber 1999). We have observed that *R. arvalis* inhabiting an outdoor enclosure hibernates on land, in shallow depressions few centimeters below the soil surface. The overwintering habitat is quite similar to the sites used by its vicariant species, the wood frog (*R. sylvatica*) which can endure 3 weeks of freezing (Storey and Storey 1996).

The aim of the present study was (1) to investigate the cold tolerance capacities of the moor frog *R. arvalis*, (2) to quantify its aerobic metabolism during cooling, freezing and thawing and (3) to detect freeze-induced metabolic changes, such as the accumulation of carbohydrates and lactate. Finally, these results are discussed in the light of the microclimate experienced by these terrestrially hibernating frogs during winter in Northern Europe.

Materials and methods

Animals

Juvenile frogs were caught from late September to mid October from ponds and streams around Roskilde, Denmark (latitude, $55^{\circ}35'N$; longitude $12^{\circ}07'E$; altitude 20 m). The juvenile frogs were kept in 15-m^2 outdoor enclosures until the freezing experiments were carried out from November to mid January. The enclosures simulated natural habitat conditions with small ponds surrounded by forest floor litter and mosses. Thus, the frogs were given the opportunity to create a terrestrial hibernaculum in the soil and litter layer. The hibernacula were slightly moist but not inundated at any time during the hibernation period. The *R. arvalis* ($n = 120$) used in these experiments had a mean body mass of 3.16 ± 0.66 g (mean \pm SD). During autumn, we observed all the juvenile frogs emerging from the ponds and digging themselves into soil and litter or hiding beneath the moss layer. Frogs used for experiments were collected from these hibernacula.

Microclimate

Measurements of the microclimate temperatures were carried out at two places in the enclosures representative of the hibernating microhabitats of the frogs. The dataloggers were placed in the immediate vicinity of two hibernating frogs. At one site (Station 1) temperatures at the soil surface beneath a moss layer were recorded. At another site (Station 2) temperatures were recorded 4 cm under the soil surface overlaid by mosses. Consequently, Station 1 represented a less sheltered overwintering habitat than Station 2. Temperature was recorded on an hourly basis from October 1998 to April 1999 using Tinytalk dataloggers (Gemini Data Loggers, UK) equipped with a Type-K probe. The accuracy of these dataloggers is 0.5°C in the measured temperature range and the resolution is $\pm 0.05^{\circ}\text{C}$.

Air temperature measurements (2 m) at the nearby Roskilde airport were obtained from the Danish Meteorological Institute.

Freezing protocol

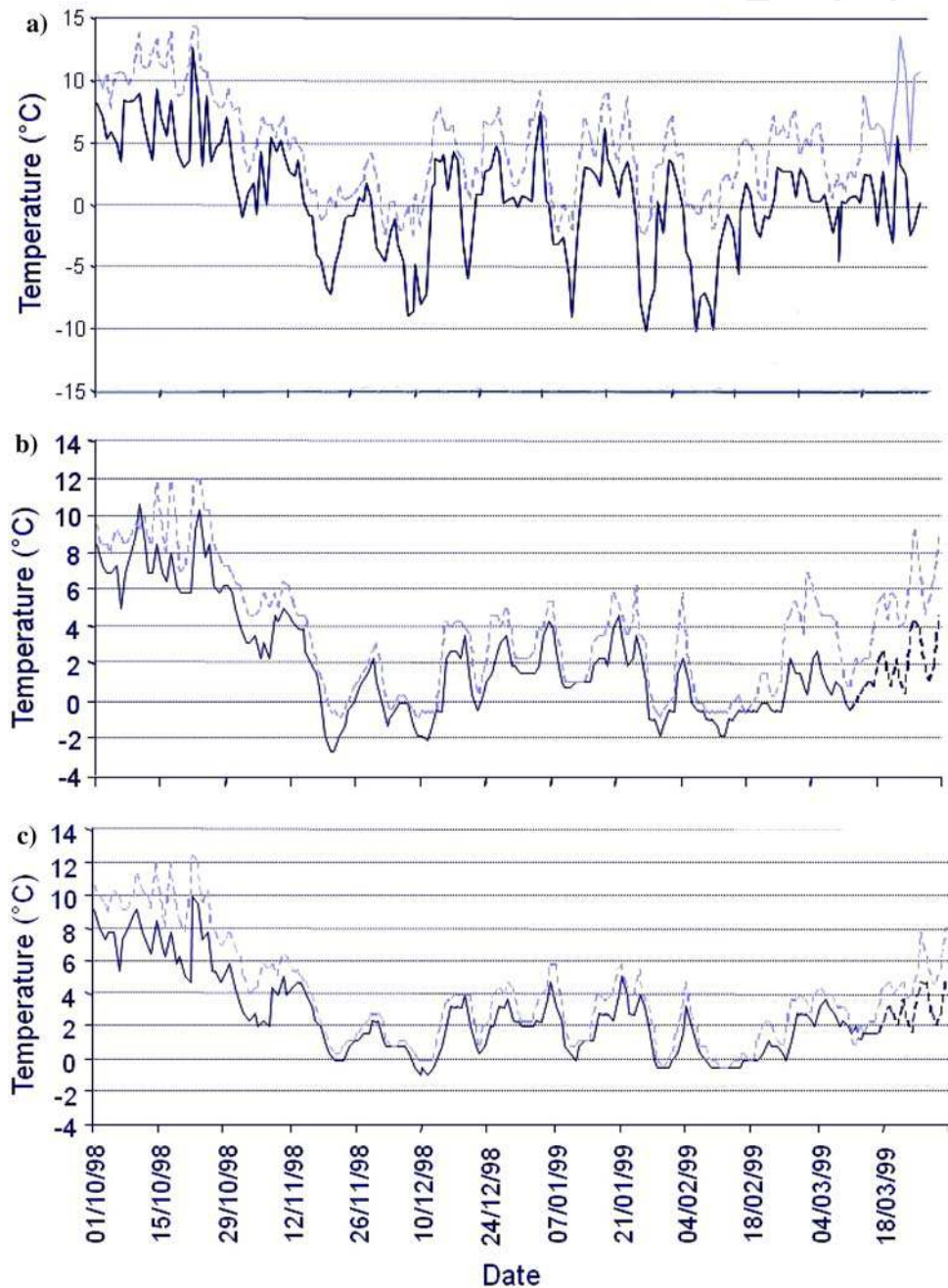
One day before the experiments, the frogs were removed from the outdoor enclosure, weighed and placed in small plastic boxes and kept at 3°C until the next day. The bottom of the plastic boxes was covered with moist filter paper to avoid desiccation of the frogs. The experiments were carried out in the period 11 November to 17 December, 1998. During this period, the temperature of the hibernacula in the enclosures varied between $+2$ and -2°C with an average

temperature at 0°C (Fig. 1). Thus no laboratory acclimation was used before the experiments.

All the preparations for the freezing experiments were carried out in a cold room at 3°C to avoid heating the animals before experiments. The frogs were placed in moist rubber foam tubes with a thermocouple in contact with the frogs ventral side. To ensure that the frogs froze in a natural position, their legs were tucked in against the body and tape was wrapped around the rubber foam tubes, to keep the frogs in place. The rubber foam tubes with the frogs were

individually placed in 50-ml plastic tubes. These tubes were plugged with cotton wool and immersed in a cooling bath (HetoFrig baths filled with ethylene glycol/water mixture) without the cooling mixture entering the tubes. Cooling was commenced at 1°C and the cooling rate was 0.063°C/h. The temperature of the thermocouples was logged every minute using a DigiSense Scanning Thermocouple thermometer (Barnant Co., Barrington, IL, USA) connected to a computer. Inoculation of the frogs was done with spray freeze at -0.1°C. Spray freeze was applied onto

Fig. 1 Winter variations in temperatures in **a** air **b** at the soil surface covered by moss (Station 1) and **c** 4 cm under the soil surface covered by moss (Station 2). Data shown are daily minima and maxima



the rubber foam tubes, so that the water in these froze, hence inoculating the frogs slightly below the melting point of their body fluids. The initiation of freezing was detected by an increase in body temperature from the released heat of crystallization. When the frogs had reached the desired temperature (from -0.5 to -5.5°C), they were moved to a second cooling bath set at this temperature and kept there for 24 h.

Assessment of survival after freezing

After 24 h at the final temperature to which the frogs were cooled, the thermocouple and the rubber foam surrounding the frogs was carefully removed and the frogs were placed in small plastic boxes with moist filter paper in the bottom, in a controlled temperature room at $3 \pm 1^{\circ}\text{C}$ in darkness. All frogs were observed daily for the next 14 days.

Frogs were scored as having survived the freezing if they showed normal positioning movements, reacted towards physical stimulation and were able to show spontaneous movement. Some of the frogs had damages due to freezing and thus only frogs which exhibited normal nervous responses without damages to the nerve system controlling movements of the limbs were counted as survivors.

Respirometric protocol

Respiration was measured at the following temperatures: 4, 1, 0, -0.5 , -1 , -2 , -3 , -4°C . Four animals were measured individually at each experimental temperature. The animals were all acclimated to winter conditions and were starving at the time of the experiment.

The animals which were tested at temperatures below 0°C were all cooled using the same protocol as described above. The respiration was then measured for 2 h at the experimental temperature. During the first hour before the experiment, the valves were closed to ensure that no leaks were present in the experimental setup. The tests were run in darkness to avoid stressing the animals. To ensure temperature equilibration and to minimize stress at the time of measuring, the animals were acclimated in the test chamber for 1 h with open valves before each experiment.

Oxygen consumption was measured differentially using two glass chambers (one test and one reference chamber) which were both connected to a pressure gauge (Micro Switch 160PC01D36 low pressure sensor, Honeywell) via two Masterflex hoses of equal length. The glass chambers were fitted with a glass filter in one end separating the animal from a piece of filter paper soaked with a 40% NaOH solution absorbing the released CO_2 . The output from the pressure gauge was sampled via a MacLab/4 (AD Instruments Pty Ltd.) connected to a classic Macintosh running

the software Chart v. 3.2.6dl. Before each run, a stable baseline was observed with the setup assembled but without animals ensuring that the setup was leak proof. The setup was calibrated with a U-manometer so that all pressure measurements were related to cm water column. There was a linear relation between cm water column and the voltage output from the pressure gauge. The temperature was kept at preset temperatures by immersing the test and reference chamber into a cooling bath with stirring. The valves, hoses and pressure gauges were kept at room temperature during the experiment.

After reaching the experimental temperature, the frogs were kept for a predetermined time; the temperature was then increased to 4°C . The frogs were kept at this temperature for 1 h after which the valves were closed again and respiration was measured for 1–2 h. At 4°C the maximal time respiration measured using this setup was 8 h after which the pressure difference exceeded the range of the pressure gauge. Further it was calculated that 10 h at 4°C was the maximal time the frogs could spend in the respirometer without reaching a critical limit where the anoxic conditions would begin to influence the metabolism of the frogs. The oxygen content of the chamber air did not in any case decrease more than 0.4% during the experiment.

The frogs were then weighed and placed at 3°C to record the survival. Frogs were considered as survivors if they could move about without any motor abnormalities and if they did not show any cryo-damage in the form of haematoma.

Glucose and lactate analysis

Metabolic responses to freezing were measured as changes in glucose and lactate concentrations. Once frozen, frogs were allowed to reach temperatures of -1 , -2 , -3 , and -4°C and kept at this temperature for 24 h before the liver and gastrocnemius muscles of the frogs were rapidly removed. After perchloric acid extraction, tissues were analyzed spectrophotometrically using enzymatic kits for glucose and lactate (Boehringer, Mannheim, Deutschland).

Statistical analysis

Mean values are presented as means \pm SD where appropriate. Statistical analysis was performed with the Statistica statistical package (StatSoft, Tulsa, USA). Analysis of the data included one-way and two-way ANOVAs followed by Tukey multiple comparison tests. A 5% ($P < 0.05$) level of significance was used in all tests. The temperature causing 50% lethality (LT_{50}) was determined using probit analysis.

Results

Winter macro and microclimate

Daily minimum air temperatures at a weather station 10 km from the study site gradually decreased from early October to mid-December (Fig. 1). Temperatures decreased below 0°C for the first time on 2 November. The minimum daily temperature was consistently below 0°C from mid-November to late February. The minimum prevailing soil temperatures under moss and/or few centimeters under the soil surface was always lower for Station 1 than for Station 2. During this period, microhabitat temperatures never fell below -2.8°C in the exposed hibernaculum (Station 1). In the more sheltered hibernaculum temperature did not fall below -0.6°C.

Freezing survival

Upon removal from the cooling chamber, the frogs showed clear evidence of internal freezing such as dark skin, rigid limbs, opaque eyes and lack of responsiveness to mechanical stimuli. Of the *R. arvalis* that ultimately recovered, the lowest body temperature and longest freezing episode was -4°C and 81 h, respectively (Table 1). However, only 14.3% *R. arvalis* survived this treatment. From the data of the Table 1, the probit analysis gives a 50% survival at $-3.4 \pm 0.6^\circ\text{C}$ corresponding to 72-h freezing.

Metabolic rate during cooling, freezing and thawing

Oxygen consumption of *R. arvalis* was strongly influenced by the animal's body temperature (Fig. 2; $F_{(7,35)} = 46.9$; $P < 0.0001$). A shift from 4 to 0°C induced a significant decrease of the metabolic rate (Tukey, $P < 0.0001$). The

Table 1 The number of surviving, juvenile *Rana arvalis* after freezing for various periods of time and estimated ice content at various freezing temperatures

Freezing trial		No. of survivors/ no. of tested specimens	Theoretical ice content (% of body water frozen)
Min Tb (°C)	Duration (h)		
-0.5	25.6	4/4	7.1
-1	33.5	10/10	29
-2	49.4	7/7	54.5
-3	65.3	3/6	63
-3.5	73.2	3/4	65.5
-4	81.1	2/14	67.2
-5	97.0	0/6	69.8
-5.5	105.0	0/3	70.7

The frogs were sampled from enclosures in the period 18 November to 14 December, 1998

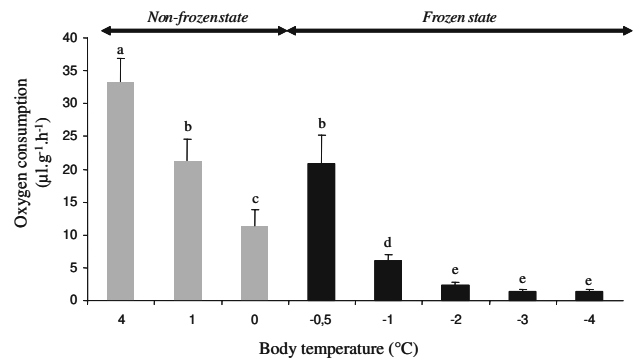


Fig. 2 Oxygen consumption of juvenile *Rana arvalis* between 4 and -4°C. Values are means \pm SD ($n = 4$). The gray and the black shading of the bars refers to the physiological state of the animals (Gray shading cold but unfrozen animals, black shading frozen animals). The letters indicate significant differences between means

freezing onset seemed to induce a sharp increase; however, oxygen consumption at -0.5°C was not statistically different from those at 1°C (Tukey test, $P = 0.99$). At lower freezing temperatures, the oxygen consumption values rapidly decreased with body temperature reaching a minimum value of about $2 \mu\text{l O}_2 \text{ g}^{-1} \text{ h}^{-1}$.

Upon rewarming, metabolic rates (Fig. 3) were strongly influenced by both the initial body temperature under freezing and time after thawing. The interaction detected by the ANOVA between these two parameters ($F_{(9,56)} = 10.5$; $P < 0.0001$) was mainly explained by the group of animals frozen at -4°C as judged from an analysis where the -4°C group was omitted from the data set.

Already after 1 h of thawing, the metabolic rates were 6-fold to 16-folds higher than those under freezing (Fig. 3). After 12 h of thawing, the metabolic rate, except the group previously frozen at -4°C, was not significantly different

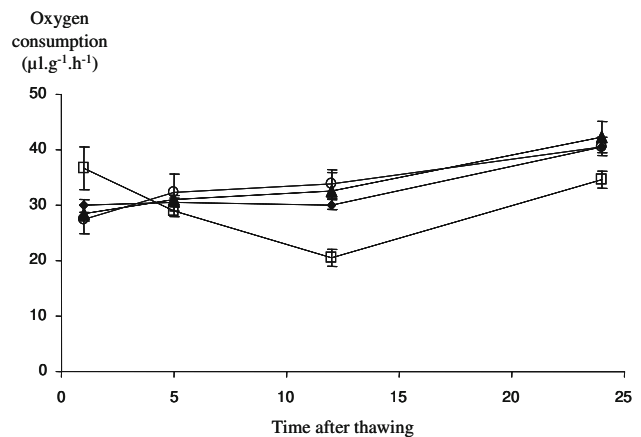


Fig. 3 Time course of oxygen consumption at 4°C of thawed juvenile *Rana arvalis* previously frozen at -1, -2, -3 and -4°C (represented by black diamonds, black triangles, open circles, and open squares, respectively). Values are means \pm SD ($n = 4$)

from values before freezing (Tukey, $P > 0.7$). After 24 h of thawing, the metabolic rates were significantly higher than those after 12 h thawing (ANOVA; $F_{(3,56)} = 84.2$, $P < 0.0001$ for time effect). However, the values for the group of animals frozen at -4°C were still significantly lower than the other groups (Tukey, $P < 0.001$).

Glucose and lactate

In liver tissue, a significant increase of glucose was observed when temperature fell from 4 to 0°C , i.e., without any tissue freezing (Table 2). However, the main increase in glucose was induced by freezing, with values increasing from 9.7 to about $40\ \mu\text{mol g}^{-1}$ FW. Since values were not corrected for decreases in water content of tissues, it was necessary to apply an appropriate correction factor taking into account that water content of various tissues usually decreases during extracellular freezing. It has been shown that water contents of liver and muscle tissue of *R. sylvatica* frozen at -2.5°C change from 75 to 60% in liver, and from 81 to 76% in muscle, respectively (Costanzo et al. 1992). According to these results, correction factors of 1.8 for liver values and 1.3 for muscle values can be applied. Even if a conservative correction factor of 2 is used (since *R. arvalis* were also frozen at temperatures lower than -2.5°C), it is evident that glucose increased significantly due to freezing in both tissues. However, glucose concentrations seemed not to differ between freezing temperatures. Lactate concentrations of liver also increased slightly due to freezing (when correcting for changes in tissue water content), but

lactate content in muscle tissue did not change as a function of body temperature ($F_{(5, 25)} = 20.7$, $P = 0.89$).

Discussion

The present study shows that *R. arvalis* juveniles, like some other freeze tolerant frogs, hibernate beneath the leaf litter, a few centimeters below the soil surface where subfreezing temperatures are frequently encountered (see Fig. 1b; Schmid 1982). Several workers suggested that Palearctic brown frogs with winter habitats similar to *R. sylvatica* could be also freeze tolerant (Layne and Lee 1995; Irwin et al. 1999), but no previous studies have demonstrated this. The present study confirms this hypothesis, especially by providing conclusive evidence of a well-developed capacity for freeze tolerance with 50% of *R. arvalis* juvenile frogs surviving 72 h of freezing with body temperatures of -3.4°C . This endurance represents the highest level of freeze tolerance reported to date for a European anuran. Some previous studies suggest that juveniles of freeze tolerant species may be less cold hardy than adults (Storey and Storey 1985b) and it would therefore be desirable in future investigations of this species to include adult *R. arvalis* in order to get a more comprehensive picture of the cold tolerance in this species.

A theoretical estimate derived from a model developed by Claussen and Costanzo (1990) suggests that *R. arvalis* specimens frozen at -3.4°C (50% survival) would have an ice content of about 65% of total body water (assuming that 20% of the body water is “unfreezable” or “bound” (Storey and Storey 1988) and that the osmotic pressure value is $267\ \text{mosmol kg}^{-1}$ (estimated from melting point values)). Such values are close to the conventional maximum ice content tolerated by other freeze tolerant vertebrates (Storey and Storey 1988, 1996). The rate of ice accumulation and the equilibrium ice content attained being dependent on the temperature of freezing exposure (Costanzo et al. 1991), the high mortality rate observed at -4°C is probably linked to a higher ice content (67.5% at -4°C and 69.8% at -5°C) (Storey and Storey 1988).

Freeze tolerant frogs generally mobilize glucose from hepatic glycogen stores as a direct response to tissue freezing (Storey and Storey 1986; Costanzo and Lee 1993). A similar mechanism seems to work in *R. arvalis*, since this species showed a significant increase of glucose in the liver and muscles during the freezing process. However, the data reveals three unusual points for a freeze tolerant frog during freezing that deserve attention and additional study. First, liver glucose rose with chilling to 0°C (and thus without freezing), which is in contrast to a number of earlier studies showing that freeze tolerance in anurans are associated with non-anticipatory freeze-induced glucose accumulation

Table 2 Glucose and lactate concentrations of organs ($\mu\text{mol g}^{-1}$ FW) of unfrozen (4 and 0°C) and frozen, juvenile *Rana arvalis*

Tissue	Body Temperature ($^{\circ}\text{C}$)	Glucose	Lactate
Liver	4	2.7 ± 0.4^a	4.1 ± 0.8^a
	0	9.7 ± 1.4^b	7.2 ± 1.0^b
	-1	39.1 ± 1.9^c	16.6 ± 2.4^c
	-2	39.9 ± 0.9^c	16.3 ± 1.0^c
	-3	42.7 ± 5.7^c	19.3 ± 1.4^c
	-4	38.0 ± 2.2^c	17.0 ± 1.0^c
Muscles	4	0.9 ± 0.3^a	33.0 ± 2.8^a
	0	0.9 ± 0.2^a	32.9 ± 12.2^a
	-1	3.5 ± 0.3^b	26.6 ± 4.8^a
	-2	2.5 ± 0.2^c	32.1 ± 7.3^a
	-3	2.8 ± 0.7^c	34.7 ± 4.1^a
	-4	2.7 ± 0.3^c	45.9 ± 9.6^b

Values are means \pm SD ($n = 5$). The letters indicate significant differences between means \pm SD. Values are not corrected for freeze-induced tissue dehydration (see text for further details). The frogs were sampled from enclosures in the period 14–17 December 1998

(Storey and Storey 1985a; Ramløv 2000). An interesting parallel to this unexpected result is found in a study on glucose accumulation in the freeze tolerant earthworm, *Dendrobaena octaedra*, that also accumulates some glucose by a non-freezing temperature decline (from +2 to 0°C) with a further glucose accumulation upon freezing of body water (Overgaard et al. 2007). Second, the highest glucose concentrations observed in *R. arvalis* were markedly lower than those measured in the freeze tolerant frog *R. sylvatica* and *Pseudacris triseriata*. The two latter species can accumulate up to 200 $\mu\text{mol g}^{-1}$ in liver tissue during the first 24 h of freezing (Storey and Storey 1986; Edwards et al. 2000), while *R. arvalis* only accumulated $\sim 25 \mu\text{mol g}^{-1}$ FW when correcting for freeze-induced tissue dehydration. Third, even if an earlier study has shown that tissue glucose level is a critical determinant of freeze tolerance (Costanzo et al. 1993), the relatively high freeze tolerance of juvenile *R. arvalis* with low liver glucose content may be explained by other low molecular weight molecules such as glycerol as found in other freeze tolerant frogs such as *Hyla versicolor* (Layne 1999; Layne and Jones 2001). It seems therefore worthwhile to undertake further studies of freeze tolerance and accumulation of alternative cryoprotectants.

As freezing proceeds over a course of days, the sequestration of cells by extracellular ice leads to an anoxic state, and survival thus depends upon fermentation of endogenous carbohydrate reserves (Storey and Storey 1988). The accumulation of lactate in the liver in *R. arvalis* was thus expected and in good agreement with earlier studies on *R. sylvatica* (Costanzo et al. 1997). On the other hand, muscle lactate levels in the cold acclimated but unfrozen frogs were already very high compared to wholly aerobic frogs. This suggests that anaerobiosis was unusually high in these animals during cold acclimation, perhaps due to a preceding freezing in the outdoor enclosure or handling stress, although it is not uncommon that lactate levels are relatively higher in muscle tissue than in other tissues such as liver (e.g., Costanzo et al. 1997). However, the constant lactate muscle value observed during freezing does not necessarily imply an absence of fermentation processes in this tissue since other anaerobic metabolism end-products such as alanine could also be accumulated. Such a phenomenon has been shown in freeze tolerant frogs before and may be linked to a physiologically less perturbing cost of using alanine as an anaerobic metabolic pathway (Churchill and Storey 1996; Storey and Storey 1986).

At 4°C we found that oxygen consumption was about 32 $\mu\text{l g}^{-1} \text{h}^{-1}$ in *R. arvalis* juveniles. These values are in good agreement with oxygen consumption rates of *R. sylvatica* reported by other authors (Layne 2000; Muir et al. 2007) suggesting that the method employed in the present study was appropriate. The oxygen consumption of *R. arvalis* during cooling and freezing showed two phases

which were easily detectable and probably linked to ice formation within the body. Indeed, the abrupt rise of oxygen consumption between 0 and -0.5°C can be explained by the near equality between the environmental temperature and the frogs melting point. Thus, a very small amount of ice was present within the frogs body at this temperature. So, according to the protocol used, it is probable that *R. arvalis* still exhibit a relatively high oxygen consumption to produce cryoprotectants.

Similar to previous studies on other organisms (Kanwisher 1959; Asahina 1969; Voituron et al. 2002a), the metabolic rate of frozen frogs was very low but still readily detectable (see Fig. 2). However, comparisons are difficult because of the scarcity of data available on oxygen consumption during freezing and thawing and especially for vertebrates. To our knowledge, only Voituron et al. (2002a) has studied the oxygen consumption of a vertebrate (the common lizard, *Lacerta vivipara*) during freezing and thawing periods. Even if data on frozen lizards and frozen frogs are hardly comparable because of the different protocols used, it seems that the former consumed less oxygen than the latter (around threefold less). This kind of comparison will be crucial for the understanding of the evolution of cold hardiness from an energetic point of view (Voituron et al. 2002b).

More data are available on the post-freeze oxygen consumption of freeze tolerant frogs. Indeed, the metabolism after being frozen has been quantified in *Pseudacris crucifer* (frozen at -1.5°C for 3 days, Layne and Kefauver 1997) and in *R. sylvatica* (frozen at -1.5°C for 1 and 7 days, Layne 2000). Similar to these two species, *R. arvalis* shows similar values between control and 5 h post-freeze metabolism. However, the increase in oxygen consumption over 24 h up to a significantly higher value than the control ones remains unclear and deserves further investigations. Only the previously frozen frogs with body temperature at -4°C showed a different temporal pattern in oxygen consumption post-freeze. An oxygen debt repayment appears and the post-freeze oxygen consumption remains lower than in the other groups after 24 h (see Fig. 3) suggesting a change in the bioenergetics of these recovering frogs.

The comparison between microclimate and air temperature data suggested that the overwintering sites under moss and in the soil significantly reduce the time period over which freezing may occur (36% for the moss and 77% for the moss plus few centimeters of soil) and thus may largely alleviate harsh conditions for overwintering animals (see Fig. 1b, c). However, the terrestrially hibernating *R. arvalis* would have experienced 22 days at Station 1 and 4 days at Station 2 at temperatures potentially inductive of freezing. However, owing to its freeze tolerance, only three periods in site 1 (and none in site 2) may have caused the death of

juvenile frogs (4 days in November with a minimum temperature of -2.7°C , 6 days in December with a minimum temperature of -2.2°C and 6 days in February with a minimum temperature of -1.8°C). Mortality due to freezing therefore appears not to be an important source of population loss during this particular winter.

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