The Journal of Experimental Biology 209, 3469-3475
Published by The Company of Biologists 2006
doi:10.1242/jeb.02387

Freezing or supercooling: how does an aquatic subterranean crustacean survive exposures at subzero temperatures?

Julien Issartel[1,*], Yann Voituron[2], Valentina Odagescu[3], Anne Baudot[3], Geneviève Guillot[4], Jean-Pierre Ruaud[4], David Renault[5], Philippe Vernon[5] and Frédéric Hervant[1]

[1]Ecologie des Hydrosystèmes Fluviaux, UMR CNRS 5023, Université Claude Bernard Lyon 1, 69622 Villeurbanne cedex, France, [2]Laboratoire Souterrain de Moulis (CNRS), 09200 Moulis, France, [3]Centre de Recherches sur les Très Basses Températures, CNRS, BP 166, 38042, Grenoble cedex 9, France, [4]Unité de Recherche en Résonance Magnétique Médicale, UMR 8081 CNRS-Université Paris-Sud 91405 Orsay, France and [5]Ecosystèmes – Biodiversité – Evolution, UMR CNRS 6553, Université Rennes 1, 35042 Rennes cedex, France

*Author for correspondence (e-mail: julien.issartel@univ-lyon1.fr)

Accepted 15 June 2006

Summary

Crystallization temperature (T_c), resistance to inoculative freezing (IF), ice contents, bound water, protein and glycogen body contents were measured in the aquatic subterranean crustacean *Niphargus rhenorhodanensis* and in the morphologically close surface-dwelling aquatic crustacean *Gammarus fossarum*, both acclimated at 12°C, 3°C and –2°C. Cold acclimation induced an increase in the T_c values in both species but no survival was observed after thawing. However, after inoculation at high sub-zero temperatures, cold-acclimated *N. rhenorhodanensis* survived whereas all others, including the 3°C and –2°C acclimated *G. fossarum* died. In its aquatic environment, *N. rhenorhodanensis* is likely to encounter inoculative freezing before reaching the T_c and IF tolerance appears as a highly adaptive trait in this species. Bound water and glycogen were found to increase in the 3°C and –2°C acclimated *N. rhenorhodanensis*, whereas no variation was observed in *G. fossarum*. Considering the hydrophilic properties of glycogen, such a rise may be correlated with the increased bound water measured in cold-acclimated *N. rhenorhodanensis*, and may be linked to the survival of this species when it was inoculated. The ecological significance of the survival of the aquatic subterranean crustacean to inoculative freezing is paradoxical, as temperature is currently highly buffered in its habitat. However, we assume that past geographical distribution and resulting life history traits of *N. rhenorhodanensis* are key parameters in the current cold-hardiness of the species.

Key words: crustaceans, subterranean, epigean, freezing tolerance, bound water, crystallisation temperature, inoculative freezing, ice content, cold acclimation, glycogen.

Introduction

Groundwater ecosystems are generally described as very poor in nutrients and oxygen (Spicer, 1998; Malard and Hervant, 1999), and are also characterized by cool but highly buffered temperatures throughout the year [11±1°C in French plains (Ginet and Mathieu, 1968)]. Thus, most of the aquatic crustacean species living in these environments should exhibit stenothermal characteristics (Huey and Kingsolver, 1989). However, we recently found that the hypogean amphipod *Niphargus rhenorhodanensis* paradoxically survived –2°C for 60 days, whereas they never normally endure such thermal conditions (Issartel et al., 2005a). This ability to survive low-temperature exposure may correspond to a relict adaptation dating back to the European quaternary glaciations (Issartel et al., 2005a; Issartel et al., 2005b; Lefébure, 2005). During that cold palaeoclimate period, sub-zero temperatures presumably occurred, perhaps even in sub-surface groundwater habitats (Tweed et al., 2005). Thus if organisms such as *N. rhenorhodanensis* did face sub-zero temperatures, they had to avoid freezing by extensive supercooling or tolerate ice formation in their tissues (Salt, 1961).

In freeze-avoiding species, the freezing temperature of body fluids, i.e. the lowest temperature they can endure, is depressed (supercooled state) particularly by accumulating large amounts of cryoprotectants [e.g. polyols, sugars, free amino acids or antifreeze proteins (for reviews, see Salt, 1961; Storey, 1997; Ramløv, 2000)]. By their hydrophilic nature, cryoprotectants bind water molecules and reduce the probability of them forming an ice embryo (Ramløv, 2000). In freezing-tolerant ectotherms, ice-nucleating agents are synthesized and trigger nucleation at high sub-zero temperatures (Lee and Costanzo, 1998). This mechanism prevents the extensive supercooling of

cells and thus reduces the probability of lethal intracellular freezing (Holmstrup and Zachariassen, 1996). Moreover, ice progression results in a strong increase of the extracellular fluid concentration and causes a water loss from the cell to the extracellular compartment (Zachariassen and Kristiansen, 2000). In order to prevent the deleterious effects of 'freezing' dehydration (causing membrane and protein denaturation) in cells, cryoprotective substances, such as glycerol, trehalose and free amino acids may also be accumulated (Ramløv, 2000). In *N. rhenorhodanensis*, large accumulations of trehalose and amino acids were found during low-temperature acclimation (Issartel et al., 2005b); but until now no studies have accurately investigated whether this subterranean species is freeze tolerant or freeze avoiding.

In addition, some authors have studied the changes in the free water/bound water ratio during low temperature acclimation in cold-hardy ectotherms. The bound water is the water that is so closely associated with cellular or other components in an organism that it is not available to participate in the freezing processes (Hazelwood, 1977). The bound water content of the freeze-tolerant larvae of *Eurosta solidaginis* increased with cold acclimation (Storey et al., 1981), and this was due to changes in water binding by cryoprotectants and macromolecules (mainly glycogen and proteins). In freeze-tolerant species, bound water will not participate in ice formation and this results in non-freezable shells of water surrounding cellular components, protecting them from the denaturation due to freezing dehydration (Storey et al., 1981).

The data dealing with freezing survival in invertebrates are overwhelming, but very few studies have investigated the problem of freezing in aquatic invertebrates (Moore and Lee, 1991; Frisbie and Lee, 1997; Lencioni, 2004). When water from aquatic environments freezes, the physical constraints differ significantly from terrestrial ones: aquatic invertebrates may be subjected to anoxia or mechanical stress due to external ice (Frisbie and Lee, 1997; Lencioni, 2004). Moreover, contact with external ice may trigger ice growth inside the body, which strongly increases the probability of freezing occurring. As a result, supercooling in aquatic invertebrates may not be a likely strategy (Frisbie and Lee, 1997).

In this study, we compared the responses of two freshwater amphipod crustaceans, the hypogean (i.e. subterranean) *N. rhenorhodanensis* and the morphologically close epigean (i.e. surface-dwelling) *Gammarus fossarum* when exposed to subzero temperatures. Thus, in both species we investigated the influence of cold acclimation on (1) the supercooling point, (2) the freezing resistance by inoculation, (3) the ice contents and (4) biophysical parameters such as the bound water content determined by a non-invasive method.

Materials and methods

Animals, rearing and acclimation conditions

Specimens of *Niphargus rhenorhodanensis* (Shellenberg) (subterranean aquatic amphipod, 12–13 mg fresh mass) were collected from an interstitial aquatic environment (Chalamont, Dombes Forest, France, 46°0.4′N, 5°10′E), with traps sunk into the sediment. *Gammarus fossarum* (Koch) (epigean aquatic amphipod, 32–34 mg fresh mass) was collected from a swiftly flowing river (La Verna, Hyères-sur-Amby, France, 45°48′N, 5°17′E) with a net. All species were placed in the dark, in separate tanks kept in thermostated chambers as described elsewhere (Hervant et al., 1997a; Hervant et al., 1997b). The tanks with *N. rhenorhodanensis* contained clay and stones. Tanks with *G. fossarum* contained leaves. Both sets of organisms were fed once a week. They were first maintained at 12°C for 15 days. Then they were separated into three groups: the first group was kept at 12°C for 6 months, the second group was acclimated to 3°C for 6 months. The third group was first acclimated to 3°C for 6 months and next acclimated to –2°C for 2 weeks as described by Issartel et al. (Issartel et al., 2005b). For acclimation at –2°C, crustaceans were individually put in 6 ml plastic tubes containing 3 ml of filtered rearing tank water. For all acclimation groups, water was changed twice a week and no physico-chemical parameters changed during acclimation. Food was removed from experimental tanks 1 week before the measurements to ensure that the presence of food in the gut would not affect the results.

Glycogen and protein assays

The glycogen content was determined by standard enzymatic methods as described by Hervant et al. (Hervant et al., 1995; Hervant et al., 1996). Total proteins were extracted according to published methods (Elendt, 1989; Barclay et al., 1983), and then determined using specific test-combinations. All assays were performed in a recording spectrophotometer (Beckman DU-6) at 25°C. Enzymes, coenzymes and test-combination substrates used for enzymatic assays were purchased from Boehringer (Mannheim, Germany) and Sigma Co. (St Louis, USA).

Cryobiological experiments

Supercooling point measurement

To determine the crystallisation temperature (T_c) of body fluids, we used a differential scanning calorimeter DSC_7 system (Perkin–Elmer). The experiments were conducted using standard hermetically sealed aluminium pans (Perkin-Elmer, 0219-0062) designed for volatile samples. In order to check the insulation of the sealed pans, their masses were measured at the end of the experiments and compared with the masses obtained before the DSC_7 measurements. We used a microbalance (Sartorius, type 1712 001; accuracy ±0.1 mg). A single individual was removed from the rearing tanks and placed briefly on a filter paper to remove excess water as described elsewhere (McAllen and Block, 1997). It was then placed in the pan before sealing and weighing. The pan was placed in the DSC_7 oven manually. Each sample was run against an empty sealed aluminium pan for reference. Temperature and heat flow DSC_7 calibration were evaluated from the melting of the ice of deionized water (T=0°C and latent heat of fusion of ice ΔH=333.88 J g^{-1}) and from the crystallographic transition of cyclohexane to its solid state (T=–87.1°C). Temperature values

were found to be reproducible within ±0.5°C. The samples were cooled from 20°C to –15°C at a rate of 1°C min^{-1}. Thermograms were recorded on a computer (Pentium II), and T_c was obtained using the Pyris 3.7.A software.

Inoculative freezing

To emphasize the possible role of acclimation, the experiment was run on both crustacean species acclimated to 12°C, 3°C and –2°C. The animals were tested out of the water because of the unnatural mechanical stress that ice-filled containers can produce on the organisms (Frisbie and Lee, 1997). Thus, according to the methods described by Frisbie and Lee, individuals were placed in contact with a thermocouple connected to a Consort data logger. Then the animal and the thermocouple were wrapped in a water-saturated strip of paper towel. The wrapped animal was closely fitted into a 5 ml pipette cone. The cone was then lowered into a 15 ml plastic tube immersed in an alcohol-filled low temperature bath the temperature of which was adjusted to –2°C. Ice crystal formation was initiated in the wet paper towel by contact with a metal rod cooled in liquid nitrogen. The inoculative freezing of the water in the paper was verified by observing an exotherm (heat release during the freezing process). Once the temperature had again reached the set temperature (i.e. 2 h after the onset of the exotherm), the structure containing the animal was heated to 3°C. To check the effect of inoculative freezing, control structures were not inoculated, organisms were cooled and reheated in the low-temperature bath at the same time as the inoculated structures. Survival of the organisms was noted 24 h after the end of the experiment.

Ice content

To determine the ice content of frozen individuals, we used the whole-body calorimetry technique (Layne and Lee, 1987; Layne and Lee, 1991). The calorimeter consisted of an insulated flask that was imbedded in a block of Styrofoam insulation and fitted with a Styrofoam plug that fitted down into the flask, leaving a space of only about 200 μl at the bottom of the flask. Thawing was done in a volume of 200 μl water for all the animals. A thermocouple was positioned below the water surface and connected to a digital thermometer. The change in water temperature caused by thawing the crustacean was recorded. Calculations of body ice content used experimentally determined values for our system which were: F factor for the calorimeter=1.17, the percentage of body mass that is water for *N. rhenorodanensis* and *G. fossarum* is 73.44±0.36% and 76.10±0.38%, respectively (values do not vary with acclimation), specific heat of the dry mass measured by calorimetry (± s.d.)=0.18±0.04 and the melting point of body fluids as estimated from osmolality determinations= –0.54°C for both species. Ice content was expressed in percentage of total body water.

Bound water contents using nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) measurements were performed on a 4.7 Tesla horizontal bore MR scanner, controlled with a TECMAG sequencer (Apollo, Tecmag, Houston, TX, USA), using a 16 mm-diameter parallel plate resonator (Gonord and Kan, 1994) built in the laboratory. All NMR measurements were performed at room temperature (20°C), at the proton NMR frequency of 200 MHz. Each specimen was transferred into a 5 mm-diameter Plexiglas sample holder placed at the centre of the NMR probe. For each sample, the whole NMR measurement lasted about 10 min: first the radiofrequency (RF) power was adjusted within 1 dB to obtain the required flip angles; then a multi-echo Carr Purcell Meiboom Gill sequence (CPMG) (Meiboom and Gill, 1958) was run to obtain the transverse relaxation curve, with an interecho time of 5 ms, a repetition time of 8 s, 100 to 400 echoes and 16 averages. The specimens were also weighed with an accuracy of ±0.1 mg. The relaxation $S(t)$ curves systematically diverged from monoexponentials, and they were analysed as sums of exponential decays with a Laplace inversion algorithm, following:

$$S(t) = \sum_{i=1 \text{ to } N} A_i e^{-t/T_i},$$

where A_i represents the relative weight of the exponential decay with the time constant T_i. Choosing 12 components with times equally spaced in log scale between 2 and 500 ms gave excellent fits of the data (χ^2 lower than 2×10^{-4}) and relatively robust distribution curves with a bimodal shape. The relative weight p of the peak of the short-time constant thus reflects the relative amount of bound water in the specimen. It was checked on one specimen that the interecho time tcp chosen in the experiment did not influence the bimodal aspect of the decay curve and the resulting value of P, as compared to inter-individual variations.

Statistical analysis

All results are presented as mean ± s.e.m. The intra- and inter-specific differences in metabolite concentrations, T_c, and bound water contents were investigated by a two-way ANOVA. When significant differences were found, the Tukey's HSD *post-hoc* test was performed. Data were log or square-root transformed to homogenize variances when homoscedasticity was not observed. Statistical analyses were performed with Statitistica 6 (StatSoft Inc., Tulsa, USA).

Results

Protein and glycogen contents

The hypogean *N. rhenorhodanensis* had body protein content of 0.134±0.008, 0.133±0.005 and 0.128±0.005 g g^{-1} FM (fresh mass) when acclimated at 12, 3, and –2°C, respectively (Fig. 1). The surface-dwelling *G. fossarum* showed quite similar values with protein amounts of 0.139±0.003, 0.139±0.007 and 0.129±0.004 g g^{-1} FM after being acclimated at 12, 3, and –2°C. No intra- and inter-specific differences in protein content were found (P>0.3).

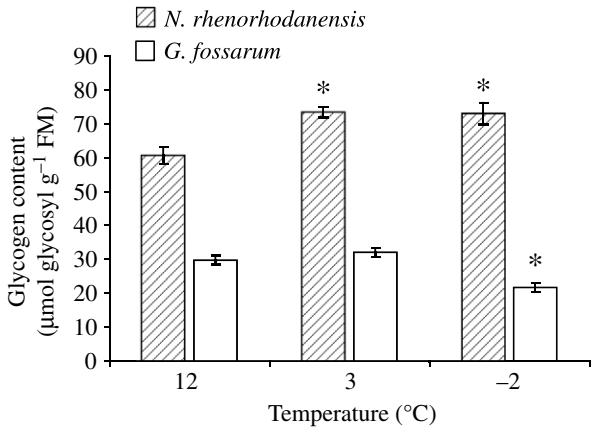

Fig. 1. Glycogen content in *N. rhenorhodanensis* and *G. fossarum* after acclimations at 12°C, 3°C and –2°C. Values are means ± s.e.m. (*N*=10). FM, fresh mass. *Significant differences (*P*<0.05) between the acclimated and the control (12°C) groups.

Whatever the acclimation temperature, body content of glycogen was statistically higher in the hypogean *N. rhenorhodanensis* than in the epigean *G. fossarum* (*P*<0.001).

In *N. rhenorhodanensis*, the amount of glycogen in the body rose significantly by 21% (*P*<0.001), changing from 60.8±2.4 μmol glycosyl g^{-1} FM at 12°C to 73.5±1.5 and 73.3±3.2 μmol glycosyl g^{-1} FM when acclimated at 3°C and –2°C, respectively (Fig. 1). In *G. fossarum*, acclimation at 3°C did not affect the glycogen level (29.8±1.3 μmol glycosyl g^{-1} FM at 12°C, and 32.1±1.4 μmol glycosyl g^{-1} FM at 3°C), but a significant decrease of 32% (*P*<0.001) was measured at –2°C (21.8±1.3 μmol glycosyl g^{-1} FM; Fig. 1).

Table 1. *Mean crystallization temperature of N. rhenorhodanensis and G. fossarum after acclimation to 12°C, 3°C and –2°C*

	Acclimation temperature (°C)	T_c (°C)	*N*
G. fossarum	12	–8.4±0.31	6
	3	–5.09±0.46*	10
	–2	–3.57±0.20*	7
N. rhenorhodanensis	12	–12.88±0.42	12
	3	–8.17±0.44*	14
	–2	–7.07±0.48*	11

T_c, crystallization temperature.
Values are means ± s.e.m.

Crystallisation temperature

The T_c values are presented in Table 1. The mean crystallisation temperature was statistically lower in *N. rhenorhodanensis* than in *G. fossarum*, whatever the acclimation temperature (*P*<0.05).

Cold acclimation induced a significant increase in the T_c in both species: the T_c rose by 37% (*P*<0.001) in *N. rhenorhodanensis* and by 40% (*P*<0.01) in *G. fossarum*.

No survival was observed in either species after thawing.

Inoculative freezing

Survival values are presented in Table 2. In *G. fossarum*, no survival was observed after inoculation, whatever the acclimation temperature. In *N. rhenorhodanensis*, no survival was observed after inoculation in 12°C-acclimated individuals. After acclimation at 3°C and –2°C survival rose to 90% and

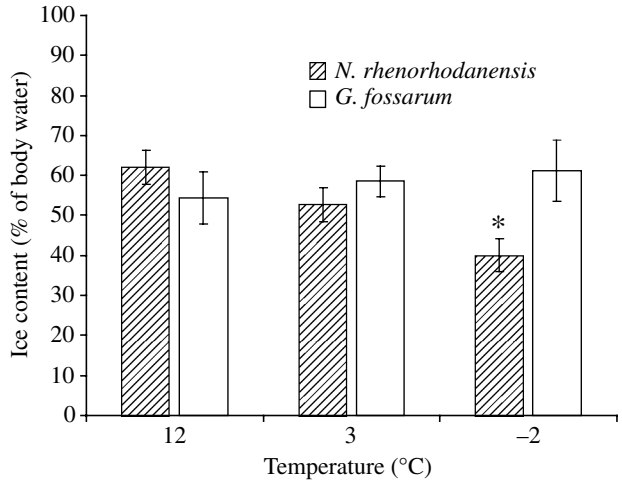

Fig. 2. Ice content (% of body water) after being inoculated at –2°C and completion of the exotherm in *N. rhenorhodanensis* and *G. fossarum* acclimated at 12°C, 3°C and –2°C. Values are means ± s.e.m. (*N*=10). *Significant differences (*P*<0.05) between the acclimated and the control (12°C) groups.

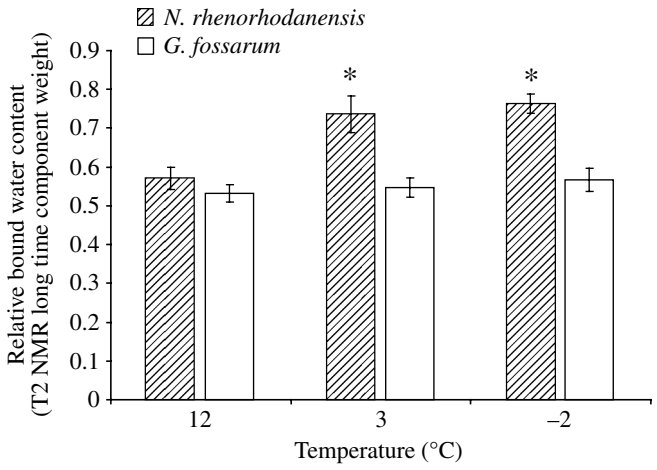

Fig. 3. Relative amounts of bound water according to the nuclear magnetic resonance measurements in *N. rhenorhodanensis* and *G. fossarum* after acclimation at 12°C, 3°C and –2°C. Values are means ± s.e.m. (*N*=7). *Significant differences (*P*<0.05) between the acclimated and the control (12°C) groups.

Table 2. *Survival of inoculative freezing by N. rhenorhodanensis and G. fossarum after acclimation to 12°C, 3°C and –2°C*

	Acclimation temperature (°C)					
	12		3		–2	
	Control	IF	Control	IF	Control	IF
G. fossarum	9/10	0/10	10/10	0/10	10/10	0/10
N. rhenorhodanensis	10/10	0/10	10/10	9/10	10/10	10/10

IF, inoculative freezing.
Values are the number of live individuals/total.

100%, respectively. No mortality was recorded in control (non inoculated) individuals of either species.

Ice content

The percentages of crustaceans' body water transformed into ice after inoculation are presented in the Fig. 2. No variation of the ice content was apparent in *G. fossarum* whatever the temperature (54.40±6.41, 58.52±3.80 and 61.25±7.59% at 12°C, 3°C and –2°C, respectively). *N. rhenorhodanensis* had ice contents of 62.07±4.30, 52.72±4.32 when acclimated at 12°C and 3°C, respectively. The percentage of ice is significantly lower in specimens acclimated at –2°C (40.07±4.1%; $P<0.05$) than in the control group.

Bound water content

The relative bound water contents in *N. rhenorhodanensis* and *G. fossarum* when acclimated to 12°C, 3°C and –2°C is shown in Fig. 3. No variation in bound water was observed in the epigean *G. fossarum* whatever the acclimation temperature. After being acclimated to 3°C and –2°C, the subterranean *N. rhenorhodanensis* showed a significant increase in its bound water content ($P<0.001$).

Discussion

Traditionally, two main strategies of cold-hardiness are used in ectotherms that are exposed to temperatures below the freezing point of their body fluids (Salt, 1961; Vernon and Vannier, 2002). Ectotherms are either freeze-tolerant or freeze-avoiding species, depending on their ability to survive the formation of extracellular ice (Bale, 1987; Lee, 1989). In freeze-avoiding species, temperature of crystallisation (T_c) is relatively low, often below –10°C, whereas many freeze-tolerant animals have limited abilities to supercool, i.e. ice formation occurs at relatively high temperatures [T_c above –10°C (Bale, 1996; Sinclair, 1999; Sømme, 1999)]. Moreover, cold acclimation generally leads to a T_c decrease in freeze-avoiding species (Danks, 1978; Duman et al., 1991). In our study, amphipods exhibited a relatively high T_c, and cold acclimation induced a T_c increase in both *G. fossarum* and *N. rhenorhodanensis*, but no survival was observed after thawing whatever the acclimation temperature. A higher T_c was also reported in animals acclimated at the lowest temperature in the intertidal copepod *Tigriopus brevicornis* (McAllen and Block, 1997). In addition, although it was increased, the T_c remained very low (about –20°C) when the animals were acclimated at 0 or 10°C. Such a T_c elevation in *G. fossarum and N. rhenorhodanensis* when cold acclimated is thus probably non-adaptive but may result from endogenous or exogenous ice nucleating agents.

If temperature falls below 0°C, *N. rhenorhodanensis* and *G. fossarum* probably encounter external ice crystals and thus become vulnerable to inoculative freezing (IF). In such cases, supercooling is not likely to work as a strategy (Frisbie and Lee, 1997). We observed distinct patterns in the two crustaceans: after they were in contact with ice crystals, only the 3°C and –2°C acclimated *N. rhenorhodanensis* survived whereas all others, including the 3°C and –2°C acclimated *G. fossarum*, died. Specimens that survived IF showed a recovery time of a few hours whereas all control organisms (exposed at –2°C but non-inoculated) were immediately active when reheated at 3°C. This survival is probably linked to the lower ice contents endured by cold-acclimated *N. rhenorhodanensis* that does not exceed 53% ice within body, unlike the other groups. Contact with external ice induced inoculative freezing of body fluids as the external ice lattice can propagate through a body orifice or directly through the cuticle (Salt, 1963; Lee and Hankinson, 2003). The very thin cuticle characteristic of amphipod gills (allowing gas diffusion) may be the preferential sites from which ice will propagate through the body.

A number of terrestrial arthropods that live in wet habitats require IF in order to survive extracellular ice formation, since if contact with external ice is prevented, they will supercool and die when spontaneous freezing occurs (Lee et al., 1996). Cold-acclimated *N. rhenorhodanensis* survived freezing if nucleation occurred after an inoculation at high subzero temperature. A similar feature was previously reported in the centipede *Lithobius forficatus*: it survived freezing only when nucleation was initiated at temperatures of almost –1°C by inoculative freezing (Tursman et al., 1994). In nature, if temperature drops to almost –1°C, *N. rhenorhodanensis* will experience inoculative freezing before reaching its T_c, as it will be surrounded by ice. Consequently, the tolerance to inoculative freezing seems to be an adaptive trait in these organisms (Tursman et al., 1994).

The physiology of cold tolerance of many arthropods is based on water and its activity at low temperatures. Water

content influences the supercooling capacity of freezing-susceptible species, and in freezing tolerant ones a proportion of body water remains unfrozen in order to allow a low level basal metabolism (Block, 2003). Thus, one of the key features that has been rarely studied in arthropods is the capacity to bind water molecules (Storey et al., 1981; Storey, 1983). In our study, we used an original non-invasive protocol to determine the relative bound water content in crustacean bodies by proton NMR transverse relaxation measurements performed on the whole live organisms. The hypogean *N. rhenorhodanensis* contained 25% more bound water when cold-acclimated, whereas no changes occurred in the epigean *G. fossarum*. Adaptations that increase the amount of bound water are used to ensure that the lethal limit is not exceeded (Storey and Storey, 1989). Our results are in agreement with these findings, as ice contents decrease with increasing bound water in *N. rhenorhodanensis*. Furthermore, the present results confirm previous work showing that both low-molecular weight compounds (LMWs; mainly polyols and sugars) and high-molecular weight compounds (mainly glycogen and proteins) participate in this phenomenon (Storey et al., 1981; Storey, 1983). Indeed, cold-acclimated *N. rhenorhodanensis* accumulate both glycogen (this study) and amino-acids and trehalose (Issartel et al., 2005b). An increase in glycogen is rather paradoxical as numerous studies have reported a decrease in glycogen during cold acclimation of ectotherms: glycogen being generally used as a fuel for synthesis of polyols and sugars. In *G. fossarum*, which exhibited no changes in the amount of bound water, glycogen remained stable. Furthermore, glycogen levels are twice as high in *N. rhenorhodanensis* as in *G. fossarum*, which may partly explain the larger bound water content found in the former. However, even if the increased glycogen in cold-acclimated *N. rhenorhodanensis* may be partly responsible for the increased bound water (together with increase of amino acids and trehalose), its function in the freeze tolerance adaptation still remains unclear and needs further investigations.

Storey and Storey (Storey and Storey, 1989) reported that the most important mechanism for controlling the freezing process is the accumulation of LMWs. Moreover, total levels of polyols and sugars are usually significantly lower in freezing tolerant species than in freeze-avoiding ones. In *N. rhenorhodanensis*, we found a significant accumulation of the total free amino acids (from 58.93±3.88 to 98.63±6.89 μmol g^{-1} FM at 12°C and –2°C, respectively) and trehalose [from 1.19±1.2 to 19.66±5.2 μmol g^{-1} FM at 12°C and –2°C, respectively (Issartel et al., 2005b)]. These findings may also explain the increased bound water found in *N. rhenorhodanensis* and we may hypothesize that accumulated LMWs are used for controlling the amount of ice in the body rather than for supercooling. On the other hand, LMW concentrations measured in both species are probably too small to involve a decrease of the glycogen content as it is usually observed in cold hardy invertebrates. From an adaptive standpoint, the amount of bound water has been found to vary in direct proportion to cold hardiness (Danks, 1978; Storey et al., 1981, Ring, 1981). By increasing the amount of water-binding micro and macromolecules, a greater fraction of intracellular water can exist as bound water, and therefore the probability of intracellular freezing (which is lethal for organisms) is strongly decreased (Storey et al., 1981; Ramløv, 2000). However, the osmotic water loss from the cell during extracellular freezing exposes the intracellular components to a dramatic dehydration stress (Zachariassen and Kristiansen, 2000). It is hypothesised that unfrozen water shells surrounding sub-cellular components could prevent irreversible protein denaturation due to freezing desiccation and cold temperatures (Hazelwood, 1977; Storey et al., 1981).

Thus, the presence of such adaptations in the subterranean *N. rhenorhodanensis* may explain its survival capacity when exposed to inoculative freezing.

According to the data in the literature, Sinclair (Sinclair, 1999) proposed that freezing tolerance is divisible into four groups according to T_c and lower lethal temperature: partially freeze-tolerant, moderately freeze-tolerant, strongly freeze-tolerant and freeze-tolerant. Partially-freezing tolerant species survive the conversion of a small proportion of their body water into ice, but do not survive if ice formation reaches an equilibrium at or above the T_c, which is visually represented by the total completion of the exotherm at a given temperature (Sinclair, 1999). The epigean crustacean *G. fossarum* that does not survive nucleation whatever its acclimation, is a freezing intolerant species; it belongs to the chill-susceptible species (species that die after brief chilling to high sub-zero temperatures). The subterranean crustacean *N. rhenorhodanensis* exhibited responses to subzero temperatures similar to those found in freeze-tolerant species that survive IF. However, it appears from our results that *N. rhenorhodanensis* can neither be classified as a partially freeze-tolerant nor as a moderately freeze-tolerant species since: (i) survival was observed after the total completion of the exotherm which is lethal in partially freeze-tolerant individuals (Sinclair, 1999), and (ii) no survival was observed after the animals reached the T_c, whereas moderately freeze-tolerant species survive after T_c is reached. Thus, like numerous arthropods showing similar characteristics (see Lee et al., 1996), *N. rhenorhodanensis* seems to belong to a still indeterminate category.

The presence of such complex adaptations in an organism that currently never endures cold during its life cycle seems at first very paradoxical. However, from recent biogeographical and phylogenetic studies, there are now several proofs that the subterranean amphipod *N. rhenorhodanensis* survived the quaternary glaciations at the limit or within the nunataks, i.e. the mountain tops surrounded by ice never covered by the glaciers (Lefébure, 2005). Thus, in such palaeo-environments, freshly-melted water coming from the glacier [at temperatures near, or even just below 0°C (Tweed et al., 2005)] may have infiltrated the sediment and considerably influenced the subterranean temperatures. As a result, the hypogean crustacean *N. rhenorhodanensis* may have encountered sub-zero temperatures and ice, and may thus have been subjected to inoculative freezing.

To conclude, our results agree with this evolutionary scenario, and the possible 'near-glacial' survival of *N. rhenorhodanensis* during that period may explain (i) the cold-induced accumulation of cryoprotectants (Issartel et al., 2005b), (ii) the bound water increase, and the resulting inoculative freezing tolerance (this study).

This research was supported by funds from University Claude Bernard-Lyon I and the National Centre for French Scientific Research (CNRS). The authors thank Dr C. Romestaing for her valuable assistance in protein assays, M. D. Hürlimann, for providing access to a fast Laplace inversion routine and Prof. E. Pattee for his assistance in checking a first version of the manuscript.

References

Bale, J. S. (1987). Insect cold hardiness: freezing and supercooling: an ecophysiological perspective. *J. Insect Physiol.* **33**, 899-908.

Bale, J. S. (1996). Insect cold hardiness: a matter of life and death. *Eur. J. Entomol.* **93**, 369-382.

Barclay, M. C., Dall, W. and Smith, D. M. (1983). Changes in lipid and protein during starvation and the moulting cycle in the tiger prawn, *Penaeus esculentus* Haswell. *J. Exp. Mar. Biol. Ecol.* **68**, 229-244.

Block, W. (2003). Water or ice? The challenge for invertebrate cold survival. *Sci. Prog.* **86**, 77-101.

Danks, H. V. (1978). Modes of seasonal adaptation in the insects. *Can. J. Entomol.* **110**, 1167-1205.

Duman, J. G., Wu, D. W., Xu, L., Tursman, D. and Olsen, T. M. (1991). Adaptations of insects to subzero temperatures. *Q. Rev. Biol.* **66**, 387-410.

Elendt, B. P. (1989). Effects of starvation on growth, reproduction, survival and biochemical composition of *Daphnia magna*. *Arch. Hydrobiol.* **116**, 415-433.

Frisbie, M. P. and Lee, R. E. (1997). Inoculative freezing and problem of winter survival for freshwater macroinvertebrates. *J. N. Am. Benthol. Soc.* **16**, 635-650.

Ginet, R. and Mathieu, J. (1968). Comparaison des températures létales supérieures de *Niphargus longicaudatus* (Crust. Amphipodes) hypogés et épigés. *Annal. Biospéléol.* **23**, 425-440.

Gonord, P. and Kan, S. (1994). Multigap parallel-plate bracelet resonator frequency determination and applications. *Rev. Sci. Instrum.* **65**, 3363-3366.

Hazelwood, C. (1977). Bound water in biology. *Acta Biochim. Biophys. Acad. Sci. Hung.* **12**, 263-273.

Hervant, F., Mathieu, J., Garin, D. and Freminet, A. (1995). Behavioral, ventilatory and metabolic responses to severe hypoxia and subsequent recovery of the hypogean *Niphargus rhenorhodanensis* and the epigean *Gammarus fossarum*. *Physiol. Zool.* **68**, 223-244.

Hervant, F., Mathieu, J., Garin, D. and Freminet, A. (1996). Behavioral, ventilatory and metabolic responses of the hypogean amphipod *Niphargus virei* and the epigean isopod *Asellus aquaticus* to severe hypoxia and subsequent recovery. *Physiol. Zool.* **69**, 1277-1300.

Hervant, F., Mathieu, J., Barré, H., Simon, K. and Pinon, C. (1997a). Comparative study on the behavioral, ventilatory and respiratory responses of hypogean and epigean crustacean to long-term starvation and subsequent feeding. *Comp. Biochem. Physiol.* **118A**, 1277-1283.

Hervant, F., Mathieu, J. and Messana, G. (1997b). Locomotory, ventilatory and metabolic responses of the subterranean *Stenasellus virei* (Crustacea, Isopoda) to severe hypoxia and subsequent recovery. *C. R. Acad. Sci.* **320**, 139-148.

Holmstrup, M. and Zachariassen, K. E. (1996). Physiology of cold hardiness in earthworms. *Comp. Biochem. Physiol.* **115A**, 91-101.

Huey, R. B. and Kingsolver, J. G. (1989). Evolution of ectotherm performance. *Trends Ecol. Evol.* **4**, 131-135.

Issartel, J., Hervant, F., Voituron, Y., Renault, D. and Vernon, P. (2005a). Behavioural, ventilatory and respiratory responses of epigean and hypogean crustaceans to different temperatures. *Comp. Biochem. Physiol.* **141A**, 1-7.

Issartel, J., Renault, D., Voituron, Y., Bouchereau, A., Vernon, P. and Hervant, F. (2005b). Metabolic responses to cold in subterranean crustaceans. *J. Exp. Biol.* **208**, 2923-2929.

Layne, J. R. and Lee, R. E. (1987). Freeze tolerance and the dynamics of ice formation in wood frogs (*Rana sylvatica*) from southern Ohio. *Can. J. Zool.* **65**, 2062-2065.

Layne, J. R. and Lee, R. E. (1991). The importance of body tissue composition to calorimetric assessments of ice content in whole animals. *J. Therm. Biol.* **16**, 61-64.

Lee, R. E. (1989). Insect cold hardiness: to freeze or not to freeze. How insects survive low temperatures. *BioScience* **39**, 308-312.

Lee, R. E. and Costanzo, J. P. (1998). Biological ice nucleation and ice distribution in cold-hardy ectothermic animals. *Annu. Rev. Physiol.* **60**, 55-72.

Lee, R. E. and Hankison, S. J. (2003). Acquisition of freezing tolerance in early autumn and seasonal changes in gall water content influence inoculative freezing of gall fly larvae, *Eurosta solidaginis* (Diptera, Tephritidae). *J. Insect Physiol.* **49**, 385-393.

Lee, R. E., Costanzo, J. P. and Mugnano, J. A. (1996). Regulation of supercooling and ice nucleation in insects. *Eur. J. Entomol.* **93**, 405-418.

Lefébure, T. (2005). Origine, évolution et mesure de la biodiversité des eaux souterraines: analyse moléculaire du genre *Niphargus* (Crustacea). PhD thesis, Université Claude Bernard, Lyon, France.

Lencioni, V. (2004). Survival strategies of freshwater insects in cold environments. *J. Limnol.* **63**, 45-55.

Malard, F. and Hervant, F. (1999). Oxygen supply and the adaptations of animals in groudwater. *Freshw. Biol.* **41**, 1-30.

McAllen, R. and Block, W. (1997). Aspects of the cryobiology of the intertidal harpacticoid copepod *Tigriopus brevicornis* (O. F. Muller). *Cryobiology* **35**, 309-317.

Meiboom, S. and Gill, D. (1958). Modified spin-echo method for measuring nuclear relaxation rates. *Rev. Sci. Instrum.* **29**, 688-691.

Moore, M. V. and Lee, R. E. (1991). Surviving the big chill: overwintering strategies of aquatic and terrestrial insects. *Am. Entomol.* **37**, 111-118.

Ramløv, H. (2000). Aspects of cold tolerance in ectothermic animals. *Hum. Reprod.* **15**, 26-46.

Ring, R. A. (1981). Adaptations to cold in Canadian Arctic insects. *Cryobiology* **18**, 199-211.

Salt, R. W. (1961). Principles of insect cold-hardiness. *Annu. Rev. Entomol.* **6**, 55-74.

Salt, R. W. (1963). Delayed inoculative freezing of insects. *Can. J. Entomol.* **95**, 1190-1202.

Sinclair, B. J. (1999). Insect cold tolerance: how many kinds of frozen? *Eur. J. Entomol.* **96**, 157-164.

Sømme, L. (1999). The physiology of cold hardiness in terrestrial arthropods. *Eur. J. Entomol.* **96**, 1-10.

Spicer, J. I. (1998). Is the reduced metabolism of hypogean amphipods solely a result of food limitation? *Hydrobiologia* **377**, 201-204.

Storey, K. (1983). Metabolism and bound water in overwintering insects. *Cryobiology* **20**, 365-379.

Storey, K. (1997). Organic solutes in freezing tolerance. *Comp. Biochem. Physiol.* **117A**, 319-326.

Storey, K., Baust, J. G. and Buescher, P. (1981). Determination of water 'bound' by soluble subcellular components during low-temperature acclimation in the gall fly larva, *Eurosta solidagensis*. *Cryobiology* **18**, 315-321.

Storey, K. B. and Storey, J. M. (1989). Freeze tolerance and freeze avoidance in ectotherms. In *Animal Adaptation to Cold (Advances in Comparative and Environmental Physiology, Vol. 4)* (ed. L. C. H. Wang), pp. 51-82. Berlin, Heidelberg: Springer Verlag.

Tursman, D., Duman, J. and Knight, C. A. (1994). Freeze tolerance adaptations in the centipede, *Lithobius forficatus*. *J. Exp. Zool.* **268**, 347-353.

Tweed, F. S., Roberts, M. J. and Russell, A. J. (2005). Hydrologic monitoring of supercooled meltwater from Icelandic glaciers. *Quaternary Sci. Rev.* **24**, 2308-2318.

Vernon, P. and Vannier, G. (2002). Evolution of freezing susceptibility and freezing tolerance in terrestrial arthropods. *C. R. Biol.* **352**, 1185-1190.

Zachariassen, K. E. and Kristiansen, E. (2000). Ice nucleation and antinucleation in nature. *Cryobiology* **41**, 257-279.