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Metabolic responses to cold in subterranean crustaceans

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Summary

Changes in polyol, sugar and free amino acid (FAA) body contents were investigated in the aquatic, subterranean (i.e. hypogean) crustaceans *Niphargus rhenorhodanensis* and *Niphargus virei* and in a morphologically close aquatic, surface-dwelling (i.e. epigean) crustacean *Gammarus fossarum* acclimated to 12°C, 3°C and –2°C. With decreasing temperature, *G. fossarum* significantly increased its alanine and glutamine levels, while trehalose body content was found to increase above control levels only at –2°C. *N. virei* showed moderate increases of alanine and glycine, and no change in trehalose level was observed in this species. By contrast, *N. rhenorhodanensis* was the only species showing a significant rise in its total FAA pool, mainly explained by alanine, glycine, arginine and glutamine accumulations. This species also gradually increased its trehalose body content with decreasing temperature. Several cold-hardy ectotherms show metabolic responses to cold that are identical to those observed in *N. rhenorhodanensis*. A previous comparative study showed that the hypogean *N. rhenorhodanensis* exhibited a survival time (Lt_{50}) at –2°C that was 26.3 times and 2.6 times higher than the hypogean *N. virei* and the epigean *G. fossarum*, respectively. Thus, crustacean levels of FAA and trehalose were correlated with their respective cold tolerances. Such differences in metabolic responses to cold in both hypogean organisms were unexpected since they both live in thermally buffered biotopes. Considering the current distribution areas of the two subterranean crustaceans studied, we assume that the cold hardiness found in the hypogean *N. rhenorhodanensis* could be correlated with its biogeography history during the quaternary glaciations.

Key words: hypogean crustacean, cold hardiness, free amino acid, trehalose, life history.

Introduction

The capacity to survive a temporary or permanent cold environment is a crucial challenge for ectotherms. Many physiological and biochemical mechanisms are known to extend the survival of freeze-susceptible species at subzero temperatures by avoiding injuries induced by the formation of ice crystals in tissues (Zachariassen, 2000). However, low but positive temperatures may also induce severe damage (Turnock, 1991, 1993; Tanaka and Udagawa, 1993; Ramløv, 2000; Hochachka and Somero, 2002; Renault et al., 2004). The mechanism of chill injury is not well understood, although it may be related to protein denaturation (Carpenter and Crowe, 1988; Ramløv, 2000; Hochachka and Somero, 2002), phase changes in membrane lipids and/or a complex metabolic disorder (Grout and Morris, 1987; Ramløv, 2000; Hochachka and Somero, 2002). Such injuries may induce chill coma and even death (Vannier, 1987; Renault et al., 1999; Ramløv, 2000). There are several low-molecular-mass sugars and polyols that may be accumulated and may prevent lethal injuries (Kostal et al., 2001; Slachta et al., 2002). The importance of glycerol, mannitol, trehalose and sucrose is widely recognized in insects during thermal acclimation and cold exposure (Salt, 1961; Ring and Danks, 1998). Although the importance of free amino acids (FAA) during cold exposure has been less investigated, authors found a positive correlation between the increase in content of a few FAAs (i.e. proline, alanine, leucine) in insects' body fluids and their acclimation to cold (Storey, 1984, 1997; Fields et al., 1998).

Although the literature on insect cold hardiness is overwhelming, very few studies deal with cold adaptations in crustaceans. An accumulation of trehalose and myo-inositol was found in the terrestrial isopod *Porcellio scaber* (Tanaka and Udagawa, 1993). Moreover, the FAA composition is known to widely vary with season in this taxon (Graney and Giesey, 1986). Alanine, arginine, leucine and glycine are the

major FAAs found in crustacean hemolymph and, together with other amino acids, are involved in several metabolic processes including protein synthesis/catabolism, gluconeogenesis and oxidative pathways (Graney and Giesy, 1986). However, their roles in crustacean cold tolerance are still obscure.

In a previous study, Issartel et al. (2005) undertook a comparative study on the behavioural, ventilatory and respiratory responses in hypogean (i.e. subterranean) and epigean (i.e. surface-dwelling) crustaceans when exposed to different temperatures. Subterranean environments (mainly porous and karstic aquifers) are basically energy- and oxygen-poor habitats, and several complex behavioural and physiological adaptations to hypoxia (Hervant et al., 1995, 1996, 1997b, 1999a; Malard and Hervant, 1999) and food shortage (Hervant et al., 1997a; 1999b; Hervant and Renault, 2002) were previously found in hypogean crustaceans. Moreover, such biotopes are also characterised by an extreme thermal stability (Ginet and Mathieu, 1968), and hypogean organisms should theoretically be classified as stenothermal species (Huey and Kingsolver, 1989; Angilleta et al., 2002). Unexpectedly, an opposite conclusion was found in the aquatic subterranean crustacean *Niphargus rhenorhodanensis*. Indeed, this species exhibited eurythermal characteristics (from –2 to 28°C), with particularly high survival times and a large capacity to maintain its metabolism at cold temperatures (Issartel et al., 2005).

In the present study, we focused on the cold hardiness of *N. rhenorhodanensis*. We investigated the changes in polyol, sugar and free amino acid contents in the aquatic hypogean crustaceans *N. rhenorhodanensis* and *N. virei* and in a morphologically close aquatic epigean aquatic crustacean *Gammarus fossarum* acclimated at 12°C, 3°C and –2°C. We thus tried to determine whether biochemical mechanisms are involved in *N. rhenorhodanensis* cold-hardiness and if such mechanisms may also be found in another aquatic subterranean species.

Materials and methods

Animals, rearing and acclimation conditions

Specimens of *Niphargus rhenorhodanensis* (Shellenberg) (Amphipoda, 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. *Niphargus virei* (Chevreux) (Amphipoda, 97–101 mg fresh mass) was collected using a net placed at the emergence spring of a karst system at Geux, near Dijon, France (47°26.4′ N, 5°5.7′ E). *Gammarus fossarum* (Koch) (epigean aquatic Amphipoda, 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 thermally regulated chambers as described by Hervant et al. (1997a,b). The tanks with *N. rhenorhodanensis* and *N. virei* contained clay and stones; these organisms were fed with minced meat every week. Tanks with *G. fossarum* contained leaves; these organisms were fed with carrots once a week. All animals were maintained at 12°C for 15 days. Then they were separated into three acclimation groups: the first group was kept at 12°C for 6 months. The second group was acclimated to 3°C for 6 months. Except for *N. virei*, which showed too low survival at –2°C (Issartel et al., 2005), the third group was first acclimated to 3°C for 6 months and then acclimated to –2°C for 2 weeks. 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 all physico-chemical parameters did not change during acclimation.

Sample preparation

For each experimental condition, 10–15 groups, each of three animals, were weighed before being lyophilised for 6 h. Food was removed from experimental tanks one week before sampling the animals to ensure that the presence of food in the gut would not affect the results.

Metabolite extraction

Amino acids, sugars and polyols were extracted from dry material. Groups of three animals were homogenised in 1.5 ml of 70% ethanol and Fontainebleau sand, before adding 1.5 ml of 40% ethanol. The homogenate was centrifuged for 10 min at 4500 ***g*** and 4°C, and the supernatant collected. The first pellet was re-suspended in 1.5 ml of 70% ethanol and centrifuged for 10 min at 4500 ***g*** and 4°C, and the supernatant collected. The second pellet was re-suspended in 1.5 ml ultrapure water and centrifuged for 10 min at 4500 ***g*** and 4°C. The combined supernatant (N=3) was pooled in a balloon flask and dried by evaporation using a rota-vapor system. The insoluble residue was re-suspended in 1 ml of ultrapure water. Samples were stored at –80°C until needed for metabolite assays.

Analytical procedure

Free amino acids assay

Free amino acids were assayed as described by Bouchereau et al. (1999). Amino acids were characterized and quantified by HPLC after pre-column derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate (AQC) (using a Waters Accq-Tag amino acid analysis system; Waters Corporation, Milford, USA) and reversed-phase liquid chromatographic separation (see Bouchereau et al., 1999 for a full description of the method). 20 µl aliquots of the crude aqueous extracts were assayed using the procedure optimised by Cohen and Michaud (1993).

Polyols and sugars assay

Derivatization was achieved according to Adams et al. (1999). A known volume of supernatant was transferred in a capped vial and lyophilised (12 Pa, –40°C). The dried residue was resuspended in pyridine containing hydroxylamine (30 mg ml^{-1}). The solution was then heated at 75°C for 30 min, which allows

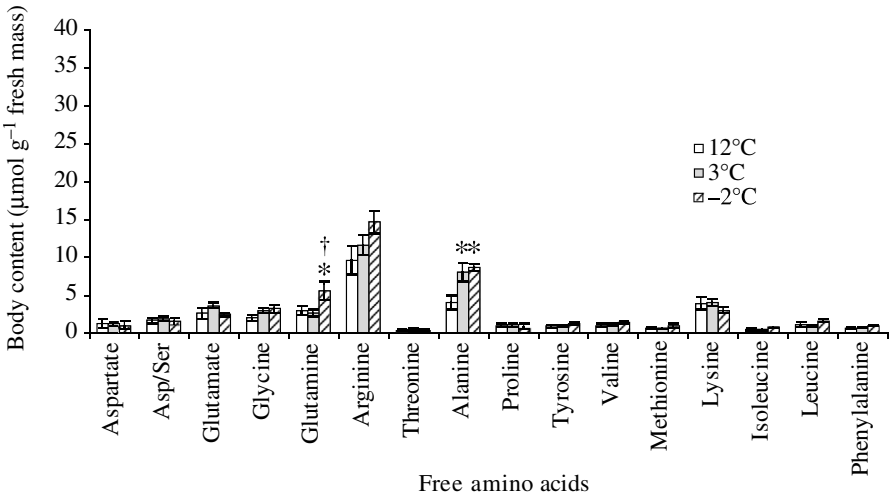

Fig. 1. Free amino acid body contents in the epigean aquatic crustacean *Gammarus fossarum* acclimated at 12°C (control), 3°C and –2°C. Values are means ± S.E.M. (N=6–8). Significant differences between FAA means are expressed as: *significant difference (P<0.05) between cold-acclimated and control groups; †significant difference (P<0.05) between groups acclimated at 3°C and groups acclimated at –2°C.

conversion of sugars to their oximes. Sugar oximes and polyols were converted to their TMS (trimethylsilane) derivatives by addition of a mixture of HDMS (hexamethyldisilazane) containing trifluoroacetic acid (10:1, v/v) and then sonicated at 50°C for 30 min before being heated at 100°C for 60 min. 1 µl of this solution was then injected into a gas chromatograph (model; Thermo Quest Trace GC 2000 series; Milan, Italy) equipped with a 30 m HP-1 capillary column with 0.25 µm film thickness. Injection was performed in the split mode (30:1) at 260°C. Hydrogen was used as the carrier gas at a flow rate of 1 ml min^{-1}. The HP-1 column was held in an oven at an initial temperature of 60°C for 2 min and then heated to 150°C at a rate of 20 deg. min^{-1} and to 300°C at 6 deg. min^{-1} and finally held at this temperature for 20 min. The temperatures of the injector and detector were kept at 260°C and 300°C, respectively. Calibration plots were constructed using external standards, and compounds were identified on the basis of retention time.

Statistical analyses

Values are presented as means ± S.E.M. The statistical differences in metabolite concentrations were investigated by a Student's *t*-test for two-sample comparisons and a one-way analysis of variance (ANOVA) with a Tukey *post-hoc* test for comparisons between three samples. Data were log- or square-root-transformed to homogenize variances when homoscedasticity was not observed. Statistical analyses were performed with Minitab software (version 13.32; Minitab Inc., State College, PA, USA).

Results

Free amino acids

In all three species, 17 free amino acids (FAAs) were found using the described analytical method (Figs 1–3). Total FAA contents were significantly different among the three species, regardless of acclimation temperature (P<0.01).

In the epigean crustacean *G. fossarum* maintained at 12°C (control temperature), three FAAs predominated and constituted 52% of the total FAA pool (Fig. 1): arginine (9.56±1.88 µmol g^{-1} fresh mass), alanine (4.02±0.98 µmol g^{-1} fresh mass) and lysine (3.90±0.84 µmol g^{-1} fresh mass). *G. fossarum* total FAA content was not statistically different when it was acclimated at 3°C and –2°C (Table 1). Alanine content was double control values after an acclimation at 3°C (P<0.01)

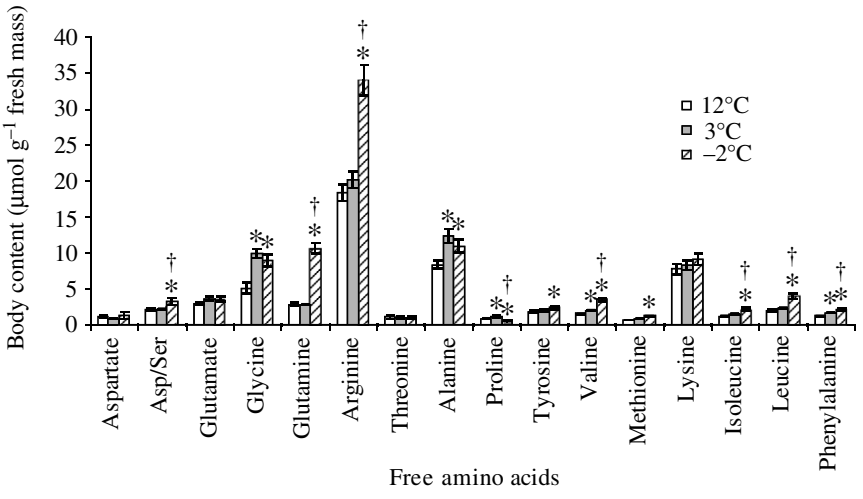

Fig. 2. Free amino acid body contents in the hypogean aquatic crustacean *Niphargus rhenorhodanensis* acclimated at 12°C (control), 3°C and –2°C. Values are means ± S.E.M. (N=6–8). Significant differences between FAA means are expressed as: *significant difference (P<0.05) between cold-acclimated and control groups; †significant difference (P<0.05) between groups acclimated at 3°C and groups acclimated at –2°C.

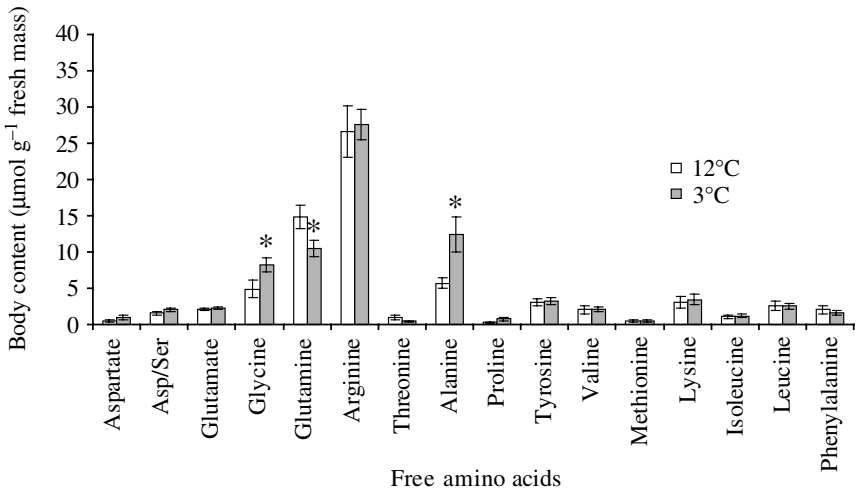

Fig. 3. Free amino acid body contents in the hypogean aquatic crustacean *Niphargus virei* acclimated at 12°C and 3°C. Values are means ± S.E.M. (N=6–8). Significant differences between FAA means are expressed as: *significant difference (P<0.05) between both groups.

Table 1. *Effect of cold acclimation on the total FAA body content in* G. fossarum, N. rhenorhodanensis *and* N. virei

	Total FAA content (µmol g^{-1} fresh mass)			
	12°C	3°C	–2°C	Significance
G. fossarum	33.83±6.79	41.87±4.71	47.02±3.63	ns
N. rhenorhodanensis	58.83±3.88	72.61±3.94	98.63±6.89	*
N. virei	71.85±9.64	79.55±7.02	†	ns

Values are means ± S.E.M. (N=6–8). P-values of statistical analysis between total FAA contents were expressed as: ns = not significant; *P<0.01.

and then remained at a constant level until –2°C. Glutamine was the only FAA found to significantly increase (+90%) after an acclimation at –2°C.

In the subterranean crustacean *N. rhenorhodanensis* (Fig. 2), arginine (18.36±1.11 µmol g^{-1} fresh mass), alanine (8.32±0.56 µmol g^{-1} fresh mass) and lysine (7.75±0.72 µmol g^{-1} fresh mass) also constitute the three major FAAs in control animals. A decrease in the acclimation temperature induced a significant increase of the total FAA content by 23% at 3°C and 68% at –2°C (Table 1). At 3°C, six of the 17 FAAs detected in *N. rhenorhonanensis* increased: glycine and alanine rose significantly by 95% and 48% (P<0.01), respectively, while proline, valine and phenylalanine content also increased significantly, albeit less markedly (P<0.05). When *N. rhenorhodanensis* was acclimated at –2°C, all FAA body contents, with the exception of aspartate, glutamate, proline, threonine and lysine, were significantly higher (P<0.05) than in the controls and/or 3°C-acclimated individuals.

At the control temperature, the subterranean crustacean *N. virei* (Fig. 3) showed high body levels of arginine (26.63±3.50 µmol g^{-1} fresh mass), glutamine (14.77±1.62 µmol g^{-1} fresh mass) and alanine (5.68±0.76 µmol g^{-1} fresh mass) compared with the other FAAs. No significant change in the total FAA content was observed after an acclimation at 3°C (Table 1). Glycine and alanine contents increased consistently (P<0.05), by 68% and 119%, respectively, with the decrease in acclimation temperature to 3°C.

Sugars and polyols

Among all sugars and polyols quantified by the method described, trehalose was the only one that accumulated in crustaceans that had been cold-acclimated (see Table 2). In *G. fossarum*, no difference in the trehalose body content was observed between groups acclimated at 12 and 3°C. However, the trehalose level was found to be five times higher at –2°C than in control organisms (P<0.01).

In *N. rhenorhodanensis* acclimated at 3°C and –2°C, trehalose values were 6- (P<0.01) and 12-fold higher, respectively, than in control individuals.

Finally, no variation in trehalose was observed in the

Table 2. *Effect of cold acclimation on trehalose body content in* G. fossarum, N. rhenorhodanensis *and* N. virei

	Trehalose content (µmol g^{-1} fresh mass)		
	12°C	3°C	–2°C
G. fossarum	1.19±0.20	1.05±0.35	5.65±1.69*,†
N. rhenorhodanensis	1.54±0.20	6.31±0.51*	19.66±5.17*,†
N. virei	0.61±0.36	2.00±0.66	–

Values are means ± S.E.M. (N=4–6). *Significant difference (P<0.05) between cold-acclimated and control groups; †significant difference (P<0.05) between groups acclimated at 3°C and groups acclimated at –2°C.

hypogean *N. virei* between the control and 3°C acclimation temperature.

Discussion

For several years, groundwater biologists considered subterranean crustaceans to be strictly stenothermal (Leclerq, 1943; Dresco-Derouet, 1959), as has recently been reported in *Niphargus virei* (Issartel et al., 2005). The low survival times noticed in *N. virei* as soon as it was out of its temperature optimum are concomitant with its ecology: subterranean ecosystems are characterized by greatly reduced temperature variations throughout the year (Ginet and Mathieu, 1968). However, Issartel et al. (2005) highlighted an unexpected tolerance to low temperatures in another hypogean crustacean, *N. rhenorhodanensis*. The significantly higher cold tolerance found in this species is ambiguous since it also never experiences temperature variation during its life cycle (Ginet and Mathieu, 1968) and this may be related to different capacities to cope with chill injuries.

The three species investigated responded differently after being cold acclimated: the subterranean crustacean *N. rhenorhodanensis* was the only one showing a significant rise in the total FAA pool (see Table 1). Such an accumulation of FAAs during cold acclimation has previously been shown in insects (Zachariassen, 1985; Fields et al., 1998) and is believed to play a major role in cold hardening. Increasing levels of proline, alanine and glycine seem to be the common feature accompanying insect acclimation to cold (Hanzal and Jegorov, 1991; Storey et al., 1993). Interestingly, alanine and glycine were largely accumulated in the hypogeans *N. rhenorhodanensis* and *N. virei* during cold exposure, whereas glycine level did not change in the surface-dwelling *G. fossarum*. *In vitro* experiments have demonstrated that glycine and alanine act as cryoprotectants by stabilizing enzymes and by preserving their activity at cold temperatures (Carpenter and Crowe, 1988; Carpenter et al., 1990). Thus, alanine and glycine may play a similar cryoprotective function in both hypogean crustaceans during cold acclimation. Moreover, glutamine and arginine were also significantly accumulated at –2°C in *N. rhenorhodanensis*; as a result, we hypothesise that they play a possible role in the cold hardening of this crustacean. Although the implicated role of such FAAs has never been shown in cold-hardy ectotherms before, Anchordoguy et al. (1988) suggested that amino acids containing positively charged amine groups in their side chain, such as arginine and glutamine, minimize membrane disruption by interacting directly with negatively charged membrane phospholipids. Regarding these data, obtained *in vitro* with artificial membranes, the authors suggest that these two FAAs prevent the close apposition of two bilayers during low-temperature exposures. However, the cryoprotective role of such complex side-chain amino acids in *in vivo* conditions remains to be explored more deeply. Furthermore, FAAs such as arginine play an important role in metabolism. As a result, the accumulation of such molecules could also result from an alteration (induced by low temperatures) of metabolic pathways (Fields et al., 1998).

Proline, which is generally found in large amounts in cold-exposed insects, occurred at low levels in *N. rhenorhodanensis*, *N. virei* and *G. fossarum* regardless of temperature. This suggests that this FAA is not as essential as it is in insects for energy metabolism (Auerswald and Gäde, 1999; Yi and Adams, 2000; Gäde and Auerswald, 2002) or cold hardiness: a positive correlation has been found between proline level and cold acclimation in insects (Hanzal and Jegorov, 1991; Fields et al., 1998). The constant low level of proline during temperature decrease is surprising, as this amino acid increased markedly in several stressed ectotherms (Danks, 2000; Ramlov, 2000).

Among all sugars detected, trehalose was the only one that accumulated in cold-acclimated crustaceans. Thus, with decreasing temperature, the hypogean *N. rhenorhodanensis* gradually increased its trehalose level, reaching the maximum concentration previously observed in cold-acclimated insects (Fields et al., 1998). The trehalose body content of the epigean *G. fossarum* was found to increase only at –2°C, and no variation was observed in the hypogean *N. virei*. An increasing trehalose content has already been detected in cold-acclimated terrestrial crustaceans (but never in aquatic or hypogean ones, as far as we know), as in the overwintering isopod *Porcellio scaber* (Tanaka and Udagawa, 1993). This organism inhabits a cold buffered environment below the ice during winter, and its trehalose level increases consistently when the temperature goes down to 0°C. Trehalose is widely recognized as a compatible solute: it has been identified as a membrane and protein protectant under desiccating conditions and thermal stress in a variety of organisms (Crowe, 1998; Ring and Danks, 1998; Fields et al., 1998). No variation in body water content was found in the studied animals during these experiments, indicating that the trehalose rise measured in cold-acclimated crustaceans is not due to desiccation stress.

In vitro experiments showed that this sugar appears to (1) interact with polar head groups of membrane lipids to stabilize the bilayer structure (Rudolph and Crowe, 1985) and (2) stabilize proteins by replacing the extensive shell of water molecules around them and thus maintain their tertiary structure (Carpenter and Crowe, 1988; Carpenter et al., 1990).

In a previous study, Issartel et al. (2005) found that *N. virei* showed survival times (Lt_{50}) of 2 days at –2°C whereas *G. fossarum* and *N. rhenorhodanensis* had Lt_{50} values of 21 and 55 days, respectively. The present study clearly demonstrated that the distinct physiological responses exhibited by the three crustaceans during cold exposure appeared to be correlated with their survival at cold temperatures. *N. rhenorhodanensis*, which combined high durations of survival at low temperatures and significant accumulations of alanine, glycine and trehalose, may therefore be classified as a cold-hardy crustacean, although it never experiences temperature variations in its natural environment. We should hypothesise that these elevations of FAAs and trehalose measured in cold-acclimated *N. rhenorhodanensis* probably remain too low to involve a

depression of the supercooling point (Storey, 1997). *N. virei*, which showed a very low survival time at –2°C, moderate levels of glycine and alanine, and no change in its trehalose content, may be classified as a cold-susceptible crustacean. The epigean *G. fossarum* showed an intermediate pattern, with significant accumulations of FAA (mainly alanine) and trehalose (only at –2°C).

In subterranean biotopes, temperature is strongly buffered and generally shows an annual variation of less than 1°C (Ginet, 1960). Consequently, the *N. rhenorhodanesis* cold hardiness does not make any ecological sense in the present climatic conditions. However, the mechanisms pointed out in our study may result from the biogeographic history of this species. It is well established that glaciations represent one of the most important factors explaining present hypogean species distribution (Ginet, 1988). In France, *N. rhenorhodanensis* and *N. virei* are presently found inside and outside the Pleistocene glaciation areas, respectively (Ginet and Juberthie, 1987). Some biogeography studies provide proof of a sub-glacial survival and/or a post-glacial recolonization of some subterranean amphipods in Europe and North America (for a review, see Proudlove et al., 2003). Thus, we assume that *N. rhenorhodanensis* may have survived the Pleistocene glaciations in refugium-habitats at the outskirt of the glaciers, in a mixture of cold glacial water and cool groundwater, and may have subsequently recolonized subterranean biotopes that were formerly covered by the ice using river corridors. Such survival and recolonization could have selected a eurythermal profile with high efficiency at cold temperatures. After the last quaternary deglaciation, *N. virei* might have shown a lower individual variability than *N. rhenorhodanensis*, and thus no selection of any eurythermal profile occurred in this species. As a result, *N. virei*'s current distribution outside glaciation areas may be explained by its inability to cope with cold temperatures.

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