Corrigendum

Do current environmental conditions explain physiological and metabolic responses of subterranean crustaceans to cold?

Céline Colson-Proch, David Renault, Antoine Gravot, Christophe J. Douady and Frédéric Hervant

10.1242/jeb.047019

The authors would like to correct several errors published in *J. Exp. Biol.* **212**, 1859-1868.

In Table 2, the reported values of oxygen consumption rate at 10°C and 3°C were too high by a factor of 60. The correct values are presented below in a corrected Table 2. These errors did not affect either the statistical results or the Q_{10} values presented in Table 2. As the differences in oxygen consumption rates between 10°C and 3°C are discussed only from the Q_{10} values, these errors do not have consequences for the results and discussion presented in the paper.

Table 2. Mean oxygen consumption rate and Q_{10} values for the seven populations of the subterranean crustacean *N. rhenorhodanensis* for a temperature range of 10°C to 3°C

Population	Oxygen consumption rate at 10°C (μmol O_2 h^{-1} g^{-1} fresh mass)	Oxygen consumption rate at 3°C (μmol O_2 h^{-1} g^{-1} fresh mass)	Q_{10} values
Kpi	4.74±0.18	2.75±0.21*	1.98
Kco	4.57±0.17	2.42±0.17*	2.21
Kvol	5.70±0.36	1.91±0.24*	3.93
Kch1	4.87±0.08	2.86±0.13*	1.95
Kch2	5.41±0.32	2.91±0.08*	2.18
Kfr	5.89±0.06	3.19±0.23*	2.15
Kalex	7.55±0.29	1.71±0.17*	6.4

Values are means ± s.d.

*Significant difference (*t*-test, P<0.001) between cold-acclimated and control groups.

Q_{10} values reflect the capacity of organisms to change their metabolic rate relative to changes in temperature (between 10 and 3°C here).

The authors apologize for these errors but assure readers that the results and conclusions of the original paper remain unchanged.

© 2010. Published by The Company of Biologists Ltd

The Journal of Experimental Biology 212, 1859-1868
Published by The Company of Biologists 2009
doi:10.1242/jeb.027987

Do current environmental conditions explain physiological and metabolic responses of subterranean crustaceans to cold?

Céline Colson-Proch[1,*], David Renault[2], Antoine Gravot[3], Christophe J. Douady[1,4] and Frédéric Hervant[1]

[1]Equipe 'Hydrobiologie et Ecologie Souterraine', CNRS, UMR5023, 'Ecologie des Hydrosystèmes Fluviaux', Université de Lyon, Université Lyon 1, Villeurbanne, F-69622, France, [2]Equipe 'Paysages-Changements climatiques-Biodiversité', CNRS, UMR6553, 'Ecosystèmes-Biodiversité-Evolution', Université de Rennes 1, Rennes, F-35042, France, [3]INRA, UMR118, 'Amélioration des Plantes et Biotechnologies Végétales', Le Rheu, F-35653, France and [4]Institut Universitaire de France, Paris, F-75005, France

*Author for correspondence (e-mail: celine.colson@ens-lyon.org)

Accepted 26 March 2009

SUMMARY

Subterranean environments are characterized by the quasi absence of thermal variations (±1°C within a year), and organisms living in these biotopes for several millions of years, such as hypogean crustaceans, can be expected to have adapted to this very stable habitat. As hypogean organisms experience minimal thermal variation in their native biotopes, they should not be able to develop any particular cold adaptations to cope with thermal fluctuations. Indeed, physiological responses of organisms to an environmental stress are proportional to the amplitude of the stress they endure in their habitats. Surprisingly, previous studies have shown that a population of an aquatic hypogean crustacean, *Niphargus rhenorhodanensis*, exhibited a high level of cold hardiness. Subterranean environments thus appeared not to be following the classical above-mentioned theory. To confirm this counter-example, we studied seven karstic populations of *N. rhenorhodanensis* living in aquifers at approximately 10°C all year round and we analysed their behavioural, metabolic and biochemical responses during cold exposure (3°C). These seven populations showed reduced activities, and some cryoprotective molecules were accumulated. More surprisingly, the amplitude of the response varied greatly among the seven populations, despite their exposure to similar thermal conditions. Thus, the overall relationship that can be established between the amplitude of thermal variations and cold-hardiness abilities of ectotherm species may be more complex in subterranean crustaceans than in other arthropods.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/12/1859/DC1

Key words: cold hardiness, free amino acid, hypogean crustacean, thermal variation, trehalose.

INTRODUCTION

Subterranean environments are composed of a great diversity of habitats (Juberthie and Decu, 1994b), in which 97% of the worlds liquid freshwater can be found (Castany, 1998). Depending on the geological substratum, water can percolate through the limestone, producing karsts with caves; it can also run out from faults in volcanic or metamorphic systems (fissured habitats) or circulate in alluvial sediments (porous habitat) (Gibert, 2001). Despite their diversity, all these subterranean biotopes share several properties, highly reduced indigenous primary production being one of the most important from a biological standpoint. Indeed, as sunlight does not enter these environments, primary production through the process of photosynthesis is absolutely impossible and thus all supplies of oxygen and most supplies of organic matter and nutrients come from the surface. These supplies are unpredictable (Malard and Hervant, 1999). The lack of oxygen production and the scarcity of nutrient supplies (Malard and Hervant, 1999) restrict the development of life and lead to the characterisation of the subterranean biotopes as extreme environments (Culver et al., 2004). Nearly constant temperature is another characteristic of these environments with, in most systems, groundwater temperature varying by only one degree around the annual average temperature of the region (Freeze and Cherry, 1979; Ginet and Decou, 1977). In these unusual environments, 7000 species are described as obligatory groundwater inhabitants (also called stygobionts) (Botosaneanu, 1986; Juberthie and Decu, 1994a; Juberthie and Decu, 1998; Juberthie and Decu, 2001). All of them are ectotherms, and among them crustaceans account for the most diverse taxa, representing more than 43% of these stygobionts (Gibert and Deharveng, 2002). Subterranean organisms are characterized by limited dispersal abilities and significant geographic isolation (Gibert et al., 1994; Humphreys, 2000). As many hypogean organisms have inhabited underground ecosystems for several million years, we expect them to be well adapted to their biotope, their physiology reflecting selective pressure exercised by environmental conditions.

The thermal characteristics of the habitats are of prime importance when working on ectotherm species because they are one of the major abiotic parameters that influence their physiology (Addo-Bediako et al., 2000; Chown, 2001). Several studies have shown that the level of thermal tolerance in many organisms is proportional to the magnitude of the temperature variation they experience in their natural habitat (Addo-Bediako et al., 2000; Farrell et al., 2008; Ghalambor et al., 2006; Pörtner, 2006; Stevens, 1989). For example, Gaston and Chown studied the thermal tolerance range of 26 species of dung beetle along an elevation transect (approximately 2500 m) in Southern Africa (Gaston and Chown, 1999). They showed evidence for an increase in the temperature tolerance range of these scarabs with elevation: individuals living at 500 m of altitude had a thermal tolerance range of 28°C whereas organisms living at 2865 m exhibited a 38°C thermal tolerance range, the minimum

critical temperature decreasing steeply with altitude. These functional differences can even be found between populations of the same species, as shown for two salmon populations from the Fraser River in British Columbia, Canada (Farell et al., 2008). Both populations displayed a large thermal tolerance range but their optimal and upper critical temperatures differed by 2°C and 3°C, respectively. Moreover, Peck et al. showed that Antarctic marine species cannot survive a temperature increase of 2°C, corroborating once again the model outlined above (Peck et al., 2004). In groundwater habitats, where temperature is highly buffered, hypogean crustaceans experience minimal to null thermal fluctuations. Thus, we hypothesised that they would not exhibit any particular thermal adaptations to cold or heat, or any particular thermal plasticity.

Yet surprisingly, Issartel et al. (Issartel et al., 2005a; Issartel et al., 2005b) showed that a population of the subterranean aquatic amphipod *Niphargus rhenorhodanensis* survived low-temperature exposures by developing adaptations to cold (such as accumulation of cryoprotectants) and even displayed an eurythermal profile as defined by Huey and Kingsolver (Huey and Kingsolver, 1989). These observations indicate that subterranean environments do not always follow the above-mentioned theory, i.e. the thermal plasticity of some subterranean species does not match the thermal characteristics of their current natural environments. To address this hypothesis and corroborate this counter-example, we focused on the cold-hardiness ability of seven populations of *N. rhenorhodanensis* inhabiting karst environments (of all groundwater bodies, karstic ones are the most thermally buffered) within aquifers of similar altitude and latitude. We then investigated whether these organisms, which usually do not endure thermal variations, can acclimate to cold exposures and present adaptive features to low temperatures. As a side issue, previous studies on *N. rhenorhodanensis* (Issartel et al., 2005a; Issartel et al., 2005b) were conducted on a population inhabiting a porous environment for which the exact yearly thermal variations are unknown (Ginet and Mathieu, 1968; Issartel et al., 2007). Therefore, we here investigated whether this population suitably represents the physiological responses of all populations of *N. rhenorhodanensis.*

The three levels used to characterize a biological response to stressful conditions, i.e. behaviour, metabolism and biochemistry (Hochachka and Somero, 2002), were studied at two temperatures: the mean temperature of the subterranean aquifers considered here (10°C) and a cold temperature (3°C). The first expected response of an organism under stress is for it to flee. As subterranean biotopes are very fragmented (Gibert et al., 1994), *N. rhenorhodanensis* is unlikely to be able to cope efficiently with stress using this strategy, but monitoring locomotory activity may be a good indicator in determining whether this is an intrinsic response or not. Second, organisms have to develop metabolic responses, by modifying their catabolism and anabolism, to cope with new thermal conditions. This can be measured by examining both ventilatory activity and oxygen consumption. Finally, biochemical responses such as production and accumulation of cryoprotectants (specific carbohydrates, polyols, amino acids) help organisms to survive cold stress. Recently, Issartel et al. showed that trehalose was the only carbohydrate that was accumulated in cold-acclimated crustaceans, with glucose and glycerol being key molecules for its metabolism (Issartel et al., 2005a; Issartel et al., 2005b). Here, we will thus focus our study on these three compounds, as well as on free amino acids that Issartel et al. found to be involved in cold-acclimation in *N. rhenorhodanensis* (Issartel et al., 2005b). Moreover, since cold exposure can induce a switch from aerobic metabolism to anaerobic metabolism (Hochachka and Somero, 2002), we measured lactate body content.

MATERIALS AND METHODS

Sampling sites

Locations were chosen in order to minimize the variance among environmental parameters such as altitude, latitude and distance to glacier edges from the last glacial maximum. We concentrated our sampling in the Jura Mountains (France), which were completely covered by ice during the last glacial maximum (20,000 years ago) on an area situated between 45 deg. 50′ and 46 deg. 50′ of latitude and between 5 deg. 24′ and 5 deg. 37′ of longitude. As the parameter studied between populations was temperature, thermally buffered sites were searched for, and we sampled only strictly karstic populations. Using the general relationship between temperature and altitude adapted for the Jura Mountains (Foulquier et al., 2008), we were able to choose springs or caves between 300 and 600 m of altitude, with a mean yearly temperature of approximately 10±1°C. To ensure the validity of the model, we measured temperature several times in the chosen sites. Fifty organisms were collected in each location in order to conduct all the experiments. All known locations for the *Niphargus rhenorhodanensis* species were considered and we only retained the seven locations that allowed us to collect enough living material in a maximum of four sampling days (Table 1).

Animal rearing and acclimation conditions

Niphargus rhenorhodanensis Schellenberg (hypogean amphipods) were collected from seven different subterranean areas, as described in Table 1. In all locations, *N. rhenorhodanensis* were collected by filtering water of the resurgence spring; except for Cormoran, where individuals were caught directly using baited traps in small pools within the cave. Individuals were kept in aquaria placed in the dark, in thermostatted chambers as described previously (Hervant et al., 1997). Tanks contained mesh and leaves in order to provide the organisms with a support to hang onto and under which to hide.

Table 1. Locations sampled and their characteristics

Location	Code	Habitat	Latitude	Longitude	Altitude (m)	Mean annual temperature of the resurgence spring (°C)	Individual fresh mass, mean ± s.d. (mg)
Pissoir spring	Kpi	Karstic	45 deg.55′N	5 deg.24′E	309	10.7±1	26.9±20.2
Cormoran cave	Kco	Karstic	45 deg.54′N	5 deg.24′E	523	9.4±1	32.3±13.1
Volognat spring	Kvol	Karstic	46 deg.10′N	5 deg.30′E	610	8.8±1	5.6±3
Froidières spring	Kfr	Karstic	45 deg.57′N	5 deg.32′E	435	9.9±1	8.9±5.3
Charabotte 1 spring	Kch1	Karstic	45 deg.57′N	5 deg.33′E	472	9.7±1	7.5±3.8
Charabotte 2 spring	Kch2	Karstic	45 deg.57′N	5 deg.33′E	468	9.7±1	7.4±4.2
Alex spring	Kalex	Karstic	46 deg.12′N	5 deg.36′E	521	9.4±1	6.8±4.7

Animals were fed with food for aquarium fishes once a week (TetraRubin, Tetra, Melle, Germany). All the animals were acclimated to laboratory conditions at 10°C (±0.3°C) for one month before the experiments. They were then separated into two groups: the first group was acclimated at a cold temperature (3°C) and the second group was kept at 10°C. These two groups were acclimated at their respective temperatures for another month. Water was changed once a week and all physico-chemical parameters (pH, oxygen concentration, temperature) were kept the same during acclimation and experiments. We chose to concentrate our multi-population study on cold responses rather than on both cold and heat responses. The rational for this was first to be able to increase the number of replicates (subterranean crustaceans are very difficult to catch in large quantities, and testing both responses would have meant less replicates) and secondly to be able to compare our results to those of previously published works. The cold temperature was determined following results obtained by Issartel et al. (Issartel et al., 2005b), where the authors demonstrated the freeze-tolerance of a population of *N. rhenorhodanensis* (experiment at –2°C) but also recorded clear evidence of cold response (experiment at 3°C). For the present study, we chose the latter temperature since it enabled us to avoid the difficulties involved in working at sub-zero temperatures.

Measurement of oxygen consumption, ventilatory and locomotory activities

Food was removed from the experimental tanks one week before sampling the animals, to ensure that the digestive tract was empty and that an overshoot in O_2 consumption due to digestive metabolism would not affect the results. It is noteworthy that these organisms have been shown to be highly tolerant to fasting (Hervant et al., 1997), which suggests that a fasting period of one week should not affect the biological significance of our results. In order to evaluate the whole-body metabolic response, oxygen consumption was measured on four replicates for each population with an oxygen sensor (Oximeter Oxi 330i; WTW, Weilheim, Germany) in special plastic respirometers of 65 ml that can be hermetically closed. Measurements of oxygen concentration were taken at the beginning of the experiment and 24 h later, without agitation because it stresses *N. rhenorhodanensis* individuals (Hervant et al., 1995; Hervant et al., 1996). Four to nine organisms were pooled together to give the same total fresh mass in each respirometer. The temperature sensitivity of oxygen consumption was determined using the Arrhenius relationship for the decrease in physiological rate with temperature (Q_{10}). This rate typically decreases two- or threefold every 10°C for ectotherms (Q_{10}=2–3) (Schmidt-Nielsen, 1997). Q_{10} values were calculated between 3°C and 10°C using the Van't Hoff equation: $Q_{10}=(R_2/R_1)^{10/(T_2-T_1)}$, where R_1 and T_1 represent the average low-temperature oxygen consumption and the low temperature (3°C), respectively; R_2 and T_2 are the average high-temperature oxygen consumption and the high temperature (10°C), respectively (Hochachka and Somero, 2002; Schmidt-Nielsen, 1997).

Ventilatory activity was determined by recording the frequency of pleopod (ventilatory appendages of malacostracean crustaceans) beats during 1 min as described previously (Hervant et al., 1997).

Locomotory activity was assessed visually by counting the number of moving animals in 1 litre tanks containing 10 individuals. During 45 min, the experimenter entered the dark thermostatted chamber every 5 min and used a very low energy red light to count moving individuals. The mean value of these 10 measurements was calculated in order to determine the percentage of individuals in movement. Great care was taken to not disturb animals (no vibration and no light stress).

Sample preparation for metabolic analysis

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. For each experimental condition, 10 pools of three organisms were weighed before and after being lyophilised, and then stored at –80°C. We pooled organisms in order to suppress individual variability and maintain interpopulation variability.

Metabolite extraction

Free amino acids, sugars, lactate and polyol were extracted from lyophilized organisms according to Renault et al. (Renault et al., 2006). Pools of three animals were homogenized 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 was collected. The remaining pellet was re-suspended in 1.5 ml of 70% ethanol and centrifuged for 10 min at 4500 ***g*** at 4°C, and the supernatant was collected. The second pellet was re-suspended in 1.5 ml of ultrapure water and centrifuged for 10 min at 4500 ***g*** at 4°C. The combined supernatants (*N*=3) were pooled in a balloon flask and dried by evaporation using a rota-vapour system (Speed Vac Concentrator, Savant^{TM}, Ramsey, MN, USA) whereas the last pellet was discarded. The solid residue obtained after evaporation of the three supernatants was re-suspended in 1 ml of ultrapure water and used in the following analytical procedure.

Analytical procedure

Free amino acids assay

Samples (5 µl) of the crude aqueous extracts were assayed [see Bouchereau et al. (Bouchereau et al., 1999) for a full description of the method]. Free amino acids were characterized and quantified by ultra-performance liquid chromatography (UPLC; Waters, Waters Corporation, Milford, MA, USA) after pre-column derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate (using a Waters Accq-Tag amino acid analysis system) and reversed-phase liquid chromatographic separation.

Lactate, glycerol and sugars assays

Lactate, glycerol, trehalose and glucose were quantified using specific kits (K-GCROL for glycerol and lactate, K-TREH for trehalose and glucose; Megazyme International, Bray, Co. Wicklow, Ireland). Analyses were performed with a spectrophotometer (Versamax, Molecular Devices, Sunnyvale, CA, USA) using the software SoftMax Pro 4.8 (Molecular Devices) at 340 nm.

Statistical analyses

All values are presented on graphs as means ± 95% confidence intervals. A canonical discriminant analysis was performed to examine the deviation of each mean of the replicate samples by temperature and population from the mean of each metabolite. In this analysis, our primary matrix was the metabolites, as columns, and replicate samples by temperature and populations, as rows. This analysis enabled us to determine the main metabolites that discriminate populations at each temperature. The multivariate analysis was run with Tanagra 1.4.17 (Rakotomalala, 2005), and graphs were displayed with ADE-4 2001 (Thioulouse et al., 1997). The statistical differences in metabolite concentrations observed between the control temperature and 3°C were investigated using a Student's *t*-test for two-sample comparisons. Significance levels

were adjusted using the Bonferroni correction. The degree of significance of the results was represented with one, two or three asterisks, which correspond to significance levels of 0.05, 0.01 and 0.001, respectively, for data that do not require Bonferroni adjustment. Data were log- or square-root-transformed to homogenize variances when homoscedasticity was not observed. Statistical analyses were performed with Statistica software (version 7) (Statsoft, Tulsa, OK, USA).

RESULTS

Oxygen consumption, ventilatory and locomotory activities after cold exposure

All populations showed a significant decrease of their mean oxygen consumption rate between 10 and 3°C (Table 2). The thermal sensitivity of oxygen consumption was determined by calculating Q_{10} values for this temperature range. Except for the Volognat and Alex populations, Q_{10} values appear to be fairly similar among the populations and ranged from 1.95 to 2.21 (2.09±0.12, mean ± s.d.) (Table 2). Q_{10} values were two to three times higher for the Volognat and Alex populations, reaching 3.93 and 6.4, respectively (2.97±1.66, mean considering all populations ± s.d.) (Table 2).

Temperature decrease had a strong effect on ventilatory activity (Fig. 1A); values were significantly lower at 3°C in all the populations (*P*-values ranged between 0.001026 and 0.000023, which corresponds to significance levels of ** and ***, respectively, after Bonferroni correction; see Materials and methods for definition of asterisks). During cold exposure, ventilatory activity showed a minimum decrease of 20% (Volognat population) and a maximum decrease of 48% for the Alex population (–34.0±8.7%, mean of decrease ± s.d.).

Locomotory activity also decreased when the temperature was reduced, as shown by the percentage of moving individuals (Fig. 1B). A minimum decrease of 50% was observed for the Froidières population, with a maximum decrease of 77% recorded in the Charabotte 2 population (–63.5±8.5%, mean of decrease ± s.d.).

Lactate body levels

No lactate was detected in the seven populations acclimated at either temperature (3°C and 10°C), suggesting that this compound was either absent or present at an undetectable level for the enzymatic method used (under 0.003 g l^{-1} according to manufacturer information).

Table 2. Mean oxygen consumption rate and Q_{10} values for the seven populations of the subterranean crustacean *N. rhenorhodanensis* for a temperature range of 10°C to 3°C

Population	Oxygen consumption rate at 10°C (μmol O$_2$ h^{-1} g^{-1} fresh mass)	Oxygen consumption rate at 3°C (μmol O$_2$ h^{-1} g^{-1} fresh mass)	Q_{10} values
Kpi	284.6±11.1	165.1±12.3*	1.98
Kco	274.0±10.3	145.0±10.1*	2.21
Kvol	342.1±21.4	114.4±14.4*	3.93
Kch1	292.5±5.0	171.4±7.8*	1.95
Kch2	324.8±19.4	174.3±5.0*	2.18
Kfr	353.5±3.3	191.7±13.6*	2.15
Kalex	453.0±17.4	102.6±10.4*	6.4

Values are means ± s.d.

* Significant difference (*t*-test, *P*<0.001) between cold-acclimated and control groups.

Q_{10} values reflect the capacity of organisms to change their metabolic rate relative to changes in temperature (between 10 and 3°C here).

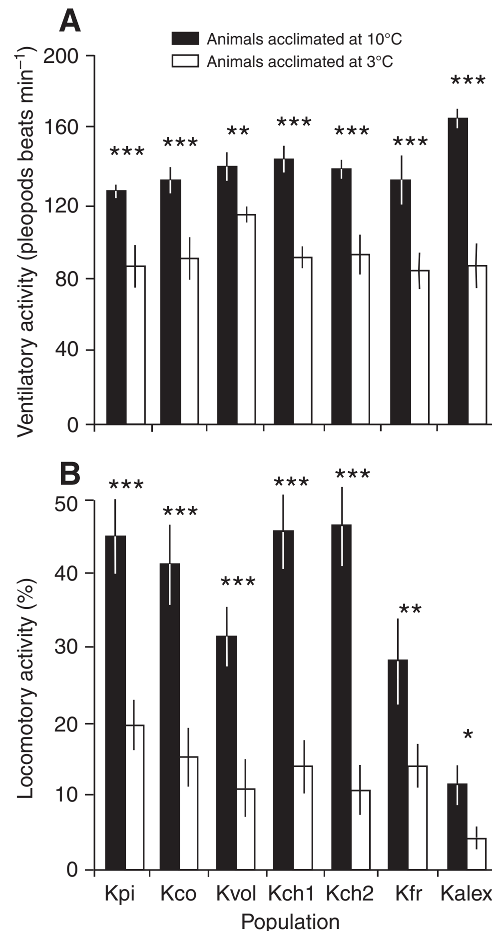

Fig. 1. Effect of cold temperature on (A) ventilatory and (B) locomotory activities in the subterranean crustacean *Niphargus rhenorhodanensis*. Values are means ± CI, *N*=10. *t*-tests after Bonferroni correction of the significance levels (**P*<0.008, ***P*<0.002 and ****P*<0.0002) indicate significant differences between control and cold-acclimated individuals for each population. Abbreviations represent the codes given in Table 1.

Influence of the temperature treatment on metabolite body contents

The F1 and F2 axes of the canonical discriminant analysis (Fig. 2A) explained 71.5% of the variability contained in the data matrix, with 40.5% on the first axis. This analysis allowed us to better characterize apparent differences observed among populations during cold acclimation and to define the main metabolites that were affected by cold exposure. Populations at a temperature of 10°C were regrouped and had positive scores on both F1 and F2 axes (Fig. 2A) whereas cold-acclimated populations had negative scores on F1 and/or F2. All cold-acclimated populations were characterized by reduced concentrations of glycerol and glucose and increased concentrations of trehalose, proline and alanine (Fig. 2A,B). At 10°C, overlap between populations occurred and reflected a low variability in metabolite compositions within the seven populations. By contrast, at 3°C, ellipses were highly dispersed (Fig. 2A) and, for all populations, the ellipses' surfaces were larger than the corresponding ones at 10°C (Fig. 2A). This pattern is the graphical consequence of both inter-population and inter-individual heterogeneous responses. Cold exposure seems to have a stronger effect on eight metabolites: glycerol, glucose, arginine, alanine, proline, trehalose, glutamine and lysine.

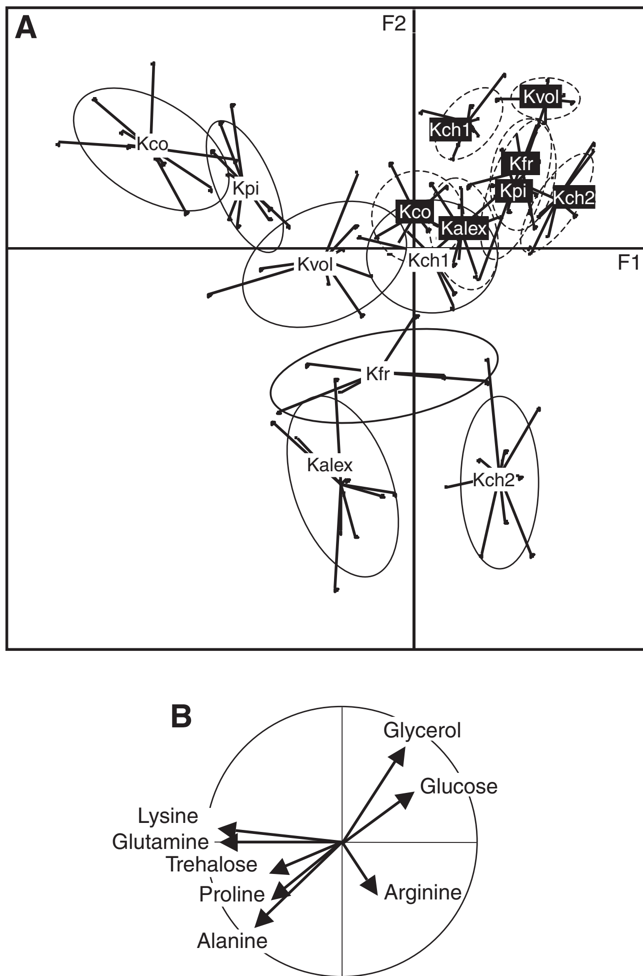

Fig. 2. Canonical discriminant analysis of the seven populations exposed to cold temperature. (A) Factorial scores of means «population–temperature» replicate samples on the first two canonical variables (F1 and F2) extracted from the canonical discriminant analyses, based on metabolite composition: individuals at 10°C (black background) and individuals at 3°C (white background). The position of points relative to the direction of vectors defined in B approximates correlations between populations and the concentration gradient of particular metabolites. (B) Two-dimensional representation of the correlation circle for metabolites. Metabolite vectors representing the underlying molecule structure of the canonical variables. Vector length indicates the overall contribution of the metabolites to the analysis. Vector direction indicates metabolite correlation with each axis. Angles between vectors represent correlations among metabolites. Abbreviations represent the codes given in Table 1.

Glycerol and glucose levels were significantly reduced in cold-acclimated organisms, except for the Volognat population (Fig. 3A,B). These decreases ranged from 34% in Cormoran to 85% in Charabotte 2 populations (–62±20%, mean of decrease ± s.d.) for glycerol (Fig. 3A) and from 30% in Charabotte 1 to 56% in Alex populations (–46±9%, mean of decrease ± s.d.) for glucose (Fig. 3B). By contrast, trehalose concentrations significantly increased (Fig. 3C). This increase was the least pronounced for the Charabotte 1 population (71%) and was the highest (up to 151%) for the Cormoran population (+110±26%, mean of increase ± s.d.).

Twenty amino acids could be discretized by UPLC: 19 proteinogenic amino acids plus ornithine. Quantification of β-alanine body content by this method was not reliable because of its extremely low level in each sample. In the present work, we use alanine to refer to α-alanine. Total free amino acid content (FAA) increased when temperature decreased from 10°C to 3°C but it was only significant for four of the seven studied populations (Fig. 4): Cormoran (FAA increased by 55%, P=0.000139***), Alex (FAA increased by 57%, P=0.000143***), Pissoir (FAA increased by 147%, P=0.000138***) and Volognat (FAA increased by 243%, P=0.000138***). Glutamine, alanine and lysine predominated over the free amino acid pool and, together, their levels presented a statistically significant increase during cold exposure for five of the seven populations (P-values were equal to 0.000138*** or 0.000192***). In the Cormoran population, these three amino acids accounted for 46% and 50% of the total FAA pool at 10°C and 3°C, respectively, 40% and 53% in the Pissoir population, 44% and 45% in the Volognat population (all amino acids increased drastically at 3°C for this population), 37% and 45% in the Alex population, and 28% and 42% in the Froidières population (Fig. 5; supplementary material Fig. S1).

Glutamine content was two or three times higher than that of the control values after the subterranean crustaceans were acclimated at 3°C (169±131%, mean of increase ± s.d.), except for Charabotte 1 (Fig. 5A). Alanine levels were increased by at least 32% (cold-exposed animals from the Charabotte 1 station compared with the control animals at 10°C), with a maximum increase of 272% recorded in cold-exposed crustaceans from the Volognat station (97±81%, mean of increase ± s.d.) (Fig. 5B). The increase of lysine amounts ranged between 0.9% (Charabotte 1) and 288% (Volognat) during the cold acclimation process (115±114%, mean of increase ± s.d.) (Fig. 5C).

The variability observed between populations at 3°C (Fig. 2A) seemed to be predominantly linked to three amino acids: lysine and glutamine on the one hand and arginine on the other hand (Fig. 2B). The most notable effect is the differential accumulation between the Charabotte 2 population situated on the right end of the first axis and the Cormoran population situated on the left end of this axis (Fig. 2A). In the Charabotte 2 population, arginine was found in high amounts at 3°C, and overall this amino acid presented very heterogeneous fluctuations between populations (supplementary material Fig. S1). Arginine concentrations increased for six populations between 10°C and 3°C but it was significant for only two of them (the Volognat and Charabotte 1 populations) (supplementary material Fig. S1). It is interesting to note that for both the Charabotte populations, arginine was the predominant amino acid at 3°C whereas lysine body content was very low compared with all the other populations.

To summarize, the Cormoran population was characterized by the lowest decrease of glycerol and by the highest increase of trehalose during cold acclimation. Volognat exhibited one of the highest Q_{10}, the lowest reduction of ventilatory activity and the highest increases of FAA, arginine, alanine and lysine amounts. Except for glycerol and trehalose, which were highly significantly decreased and increased, respectively, the Charabotte 1 population tended to have similar characteristics between the cold-exposed organisms (3°C) and the control organisms (10°C).

DISCUSSION

Physiological responses of organisms to stress are generally proportional to the stress amplitude they undergo in their living environment (i.e. Gaston and Chown, 1999; Farrell et al., 2008). However, in the present study, we demonstrated that such a conclusion does not apply to populations of the subterranean crustacean *Niphargus rhenorhodanensis*. Moreover, we also proved

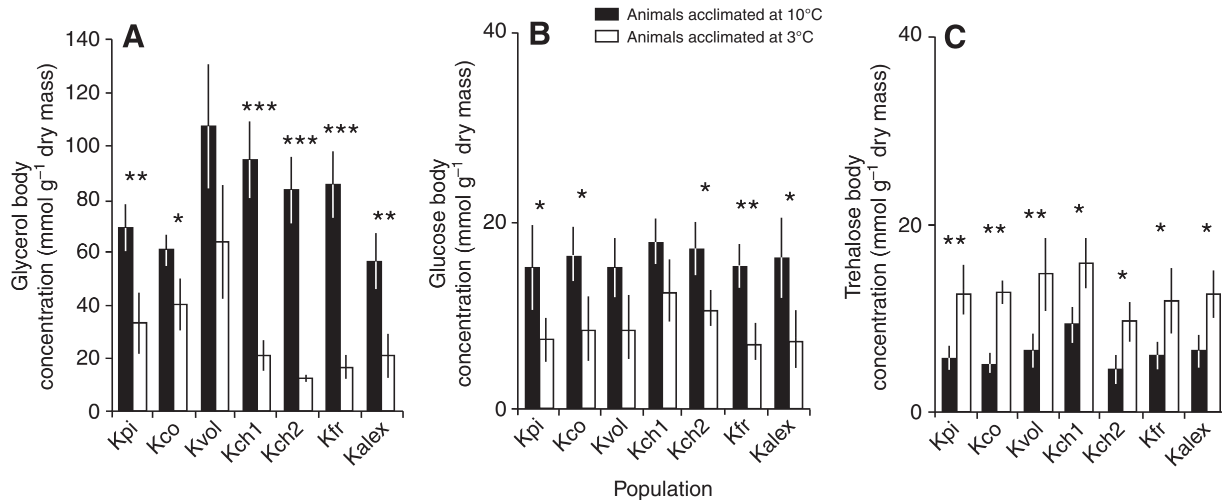

Fig. 3. Effect of cold temperature on (A) glycerol, (B) glucose and (C) trehalose concentrations in the subterranean crustacean *Niphargus rhenorhodanensis*. Values are means ± CI, N=10 replicates of three individuals. t-tests after Bonferroni correction of the significance levels (*P<0.008, **P<0.002 and ***P<0.0002) indicate significant differences between control and cold-acclimated individuals for each population. Abbreviations represent the codes given in Table 1.

that the thermal response is not restricted to subterranean populations living in the phreatic system (Issartel et al., 2005a; Issartel et al., 2005b) but is more widely distributed, including in the most typical and extremely thermally buffered karstic environments.

We characterized cold-hardiness in seven populations of *N. rhenorhodanensis* at three levels: behaviour, metabolism and biochemistry. Most studies have analyzed only one or two of these aspects and, to date, we have only found two studies dealing with these three components of the response to cold in crustacean species (Issartel et al., 2005a; Issartel et al., 2005b).

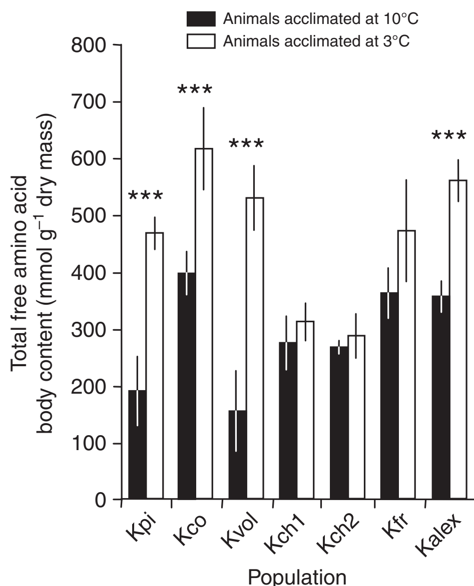

Fig. 4. Effect of cold temperature on total free amino acid body content in the subterranean crustacean *Niphargus rhenorhodanensis*. Values are means ± CI, N=10 replicates of three individuals. t-tests after Bonferroni correction of the significance levels (***P<0.0002) indicate significant differences between control and cold-acclimated individuals for each population. Abbreviations represent the codes given in Table 1.

In the seven populations we studied, locomotory activity was drastically reduced at 3°C, but no organisms were motionless during cold exposure. By contrast, Issartel et al. showed that individuals of another subterranean crustacean, *Niphargus virei*, were motionless at 3°C (Issartel et al., 2005a). Thus, the ability to maintain a minimal locomotory activity at cold temperature is not a general trend for subterranean organisms. As these organisms lack visual organs, they need to move permanently to find trophic resources. The minimal activity maintained even at cold temperatures probably enables *N. rhenorhodanensis* species to continue to feed, which represents an important functional advantage. Interestingly, Issartel et al. found a similar response in a surface-dwelling crustacean adapted to large thermal variations, *Gammarus fossarum* (Issartel et al., 2005a). Further experiments should examine whether the non-zero locomotory activity displayed by *N. rhenorhodanensis* at 3°C is adaptive or reflects nonadaptive physicochemical effects of the decrease in temperature.

Most biological processes are slowed down when temperature decreases. Oxygen consumption follows this rule, and expected Q_{10} values range between 2 and 3 (Schmidt-Nielsen, 1997; Daoud et al., 2007; Irwin et al., 2007; Jimenez and Bennett, 2007). For all populations, except Alex and Volognat, *N. rhenorhodanensis* populations responded as expected (Q_{10}=2.09±0.12, mean ± s.d.) (Table 2). The very high reduction of oxygen consumption (and the associated high Q_{10} value) measured in the Alex population highlighted that the metabolism was more highly depressed in those crustaceans than in the other populations. It leads us to think that this atypical decrease in oxygen consumption may be the expression of physiological changes other than standard thermal-induced changes occurring on physicochemical processes. As for the Volognat population, our results are rather surprising. Indeed, the Volognat population displayed a highly depressed oxygen consumption (Q_{10}=3.93) (Table 2) but the weakest decrease in ventilatory activity. While further experiments are needed, this suggests that a decoupling exists between the ventilatory activity and the metabolic rate in these organisms. Keeping in mind that all the populations studied inhabit similar environments where the temperature is highly buffered all year long, these heterogeneous responses are particularly interesting. We can thus hypothesize that

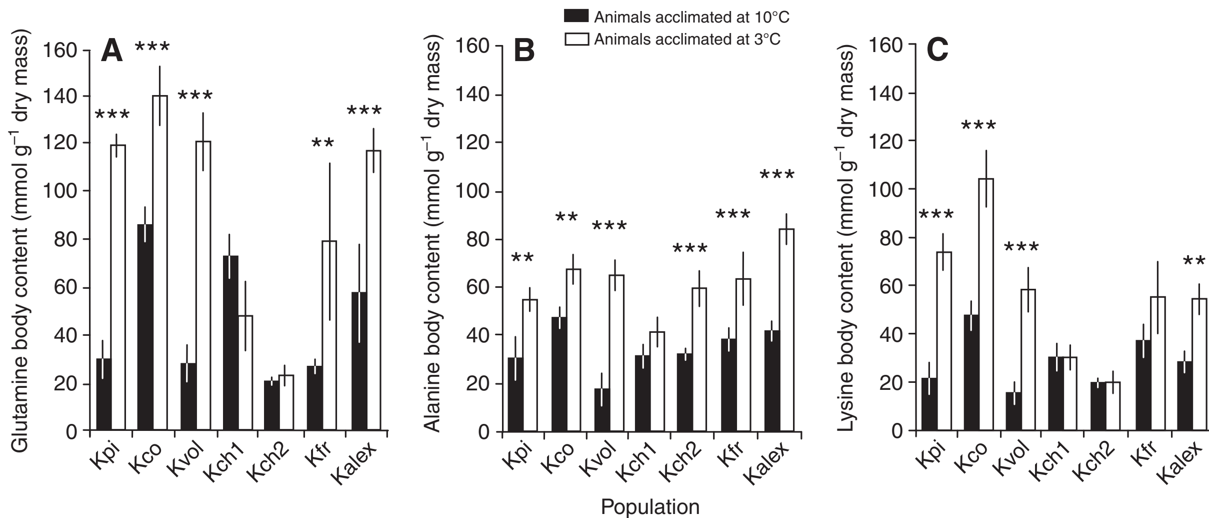

Fig. 5. Effect of cold temperature on the three amino acids most accumulated in the subterranean crustacean *Niphargus rhenorhodanensis*: (A) glutamine, (B) alanine and (C) lysine body contents. Values are means ± CI, N=10 replicates of three individuals each. t-tests after Bonferroni correction of the significance levels (** P<0.002 and *** P<0.0002) indicate significant differences between control and cold-acclimated individuals for each population. Abbreviations represent the codes given in Table 1.

the current environmental conditions experienced by the animals are not the only parameters driving the amplitude of the stress response of the organisms we studied.

Despite the large decrease in oxygen consumption observed during cold exposure, crustaceans did not switch to anaerobic metabolism. Indeed, no lactate was detected, this compound being a major end-product of anaerobic metabolism in *N. rhenorhodanensis* (Hervant et al., 1995; Hervant et al., 1996). Cold exposure is well known to induce aerobic metabolism perturbations (Hochachka and Somero, 2002), resulting in a decrease in ATP production. Thus, some organisms have to use anaerobic means for some portion of their energy needs (Costanzo et al., 2004; Hartley et al., 2000; Packard and Packard, 2004). However, the energetic yield of aerobic metabolism is largely superior to that of anaerobic metabolism (Hochachka and Somero, 2002). Consequently, organisms such as *N. rhenorhodanensis*, which are able to maintain their whole metabolism as aerobic, present a functional advantage that enhances their survival time during cold exposure.

From a biochemical point of view, several molecules were accumulated or degraded at 3°C in the seven populations we examined. Organisms were not able to maintain their glucose body contents. Since stabilising the glucose level in the haemolymph is essential for the regular functioning of the nervous, muscle and reproductive systems of the organisms (Buckup et al., 2008), one might expect that, even if glucose body content decreased, haemolymph glucose content would remain more stable. Unfortunately, our analytical procedure did not allow us to differentiate glucose in the haemolymph from cellular glucose. Glycerol amounts were also reduced during cold exposure, indicating that this metabolite, well known as a colligative antifreeze compound accumulated in large amounts in insects during cold acclimation (Bennett et al., 2005; Kostal et al., 2001; Michaud and Denlinger, 2007; Renault et al., 2002), did not play any cold acclimation role in the seven studied populations of *N. rhenorhodanensis.* As far as we are aware, only two studies have dealt with cold responses in Crustacea. Issartel et al. studied three species – *Gammarus fossarum*, *Niphargus virei* and *Niphargus rhenorhodanensis* – (Issartel et al., 2005b), and Karanova and Gakhova studied a cryotolerant crustacean, *Gammarus lacustris* (Karanova and Gakhova, 2002). These works showed that glycerol is not accumulated in response to cold in these crustacean species. Thus, the absence of glycerol accumulation in the populations studied here is not surprising and cannot be considered as evidence for nonadaptive features to cold. Glycerol in *N. rhenorhodanensis* can be used either as a glucose precursor to maintain a stable haemolymph glucose level using the neoglucogenesis pathway or as an intermediate to generate trehalose. This idea is supported by the significant rise in trehalose concentration during cold exposure, in particular in the Cormoran and Pissoir populations. Similar results have been reported in the overwintering isopod *Porcellio scaber* (Tanaka and Udagawa, 1993) and in a population of *N. rhenorhodanensis* living in a porous aquifer (Issartel et al., 2005a; Issartel et al., 2005b). Accumulation of trehalose probably helped in protecting protein and membrane integrity during the exposure at 3°C (Fields et al., 1998; Ramløv, 2000; Ring and Danks, 1998) and suggests that this response could be adaptive.

Cold stress was very often found to be associated with an increase in the level of several free amino acids (FAA) during the first days in most species tested to date, resulting in an increase in the total FAA pool (Fields et al., 1998; Renault et al., 2006). In the present study, the FAA pool of the subterranean crustacean *N. rhenorhodanensis* was significantly altered by thermal stress, and the highest increase was found in the Volognat population. This cold-induced accumulation of several amino acids possibly resulted from a nonadaptive change in gene and protein expression (Colinet et al., 2007; Lalouette et al., 2007), i.e. a change in the balance between protein anabolism/catabolism. And yet, we would like to stress that four amino acids were significantly accumulated: alanine, glutamine, lysine and arginine, representing more than 50% of the total amino acid body content for each population. This point is particularly interesting because these amino acids are known to play an important role during cold exposure, conferring cryoprotection. *In vitro* experiments have demonstrated that alanine acts as a cryoprotectant, maintaining enzyme activities at low temperatures (Carpenter and Crowe, 1988; Carpenter et al., 1990), and several studies have observed an alanine upregulation in insects during cold acclimation (Fields et al., 1998; Goto et al., 2001; Michaud and

Denlinger, 2007). Alanine seems to have the same colligative effect as glycerol when it is used as a cryoprotectant in ectothermic organisms (Michaud and Denlinger, 2007); it protects proteins and membrane integrity and promotes supercooling of body fluids (Lee, 1989; Ramløv, 2000).

Glutamine variations are of interest because this amino acid is involved in several physiological processes. Catabolism of both amino acids and proteins during exposure to low temperatures leads to the production of ammonia (here we use ammonia to refer to NH_3 or NH_4^+, or a combination of the two). Ammonia can be fixed on glutamate to yield glutamine, which is accumulated in large amounts in cold-exposed subterranean crustaceans. Moreover, as glutamine contains positively charged amine groups, Anchordoguy et al. suggested that it could minimise membrane disruption at cold temperatures by interacting with phospholipids (Anchordoguy et al., 1988). Numerous studies dealing with cold-acclimated insects (Fields et al., 1998; Hanzal and Jegorov, 1991; Michaud and Denlinger, 2007; Renault et al., 2006) have shown an increase in the glutamine content similar to what we found here in five populations of *N. rhenorhodanensis*.

The third amino acid to be significantly accumulated during cold exposure by four populations is lysine, an essential amino acid in arthropods (Ramsay and Houston, 2003). Such an accumulation of lysine was previously found in two species of Coleoptera acclimated to cold temperatures (Fields et al., 1998) and in larvae of the wax moth *Galleria mellonella* during cold acclimation (Hanzal and Jegorov, 1991). As lysine is an essential amino acid, the increase in lysine concentration is probably due to protein catabolism.

Arginine, another essential amino acid, presented highly heterogeneous variations among the seven populations of *N. rhenorhodanensis*. This highly variable tendency could reflect (1) protein catabolism at 3°C (which is highly individual and population dependent) or (2) the breakdown of arginine phosphate, a phosphagen largely accumulated by hypogean crustaceans (Hervant et al., 1995; Hervant et al., 1996), to produce and therefore maintain their ATP body content. We can point out that the Volognat population presented the highest increase of arginine at 3°C so it seems that this population displayed the highest disturbance in ATP production during cold exposure, which would be consistent with Q_{10} measurements.

Proline has very often been found to be accumulated in response to cold exposure in a wide range of arthropod species (Storey, 1997; Ramløv, 1999; Ramløv, 2000) and is usually the most accumulated amino acid in insects (Hanzal and Jegorov, 1991; Fields et al., 1998; Ramløv, 1999). *N. rhenorhodanensis* exhibited very low amounts of proline whatever the experimental conditions and, even if it was accumulated in four cold-exposed populations, its concentration remained at low levels. As previously suggested in other studies, this amino acid (and this holds true even if it had been accumulated) may not play a cold acclimation role in all arthropod species (Issartel et al., 2005b; Lalouette et al., 2007).

Since temperature in subterranean ecosystems is very stable throughout the year, the physiological responses and adaptations to cold exhibited by these crustaceans cannot be linked to the thermal variations they undergo in their living environment. Some of the responses we found, which typically occur in regularly cold-exposed arthropods (Lalouette et al., 2007; Ramløv, 2000), are also intriguing in view of the thermal characteristics of the habitats of *N. rhenorhodanensis*. In a recent study, Issartel et al. questioned the ecological relevance of the cold-hardiness of *N. rhenorhodanensis* and compared it to the response of another subterranean karstic species, *N. virei* (Issartel et al., 2005a). As expected, *N. virei* exhibited a typical stenothermal profile. Issartel et al. thus hypothesised that the observed responses may result from the past life history of these two species (Issartel et al., 2005a), i.e. this cold-hardiness would be a relict adaptation that enabled the survival of *N. rhenorhodanensis* within glaciers during the Pleistocene (Issartel et al., 2005b). Lefébure et al. supplied DNA evidence that several populations of *N. rhenorhodanensis* survived within the area covered by glaciers, possibly in small areas called nunataks (locations free of ice surrounded by glaciers), during the last glacial maximum (LGM) (Lefébure et al., 2007), whereas Foulquier et al. showed that all populations of *N. virei* survived the LGM outside of the glacier area (Foulquier et al., 2008). These two studies are thus in agreement with the hypothesis of a relict adaptation for the cold-hardiness found in *N. rhenorhodanensis*.

Keeping in mind that the seven populations of *N. rhenorhodanensis* live in very similar biotopes and have endured the same evolution process, another interesting point of this study is the distinct amplitudes of the response we found between cold-exposed populations (Fig. 2). Cormoran, Pissoir and Volognat organisms appeared to be the 'most cold-hardy' (i.e. exhibited the most pronounced metabolic and biochemical responses), whereas the 'least cold-hardy' individuals were found in both the Charabotte populations. Three hypotheses can be put forward to explain the heterogeneity observed here in the responses to cold.

(1) Differential paleoacclimation. As previously suggested, the level of cold hardiness found in *N. rhenorhodanensis* could be an adaptive relict to survival under or close to glaciers. However all seven studied populations are located in an area that was totally covered by ice 20,000 years ago. Thus, location alone is unlikely to explain the heterogeneity observed between populations.

(2) Genetic polymorphism. Lefébure et al. showed that *N. rhenorhodanensis* is composed of several lineages (or evolutionary significant units) that can likely be considered as independent species (Lefébure et al., 2007). Knowing whether or not the seven populations we used belong to distinct lineages would be helpful in testing the possible relationship between historical factors and the cold-hardiness heterogeneity we found here.

(3) Cryptic diversity of environments. Even if locations were chosen in order to best homogenize environmental parameters such as latitude, longitude and altitude, the temperature (in °C) was calculated and measured using the exit point of the aquifer. Indeed, for six populations, we had no access to the organisms' genuine biotope since the karst is closed and aquifers unreachable. Thus, and *a posteriori*, it appears possible that the heterogeneity we found in responses may be linked to the level of stress generated by the experiment, i.e. the difference between 3°C and the real habitat temperature. This does not apply to the Cormoran population, for which individuals were directly collected in the cave, so that the temperature calculated in Table 1 is more likely to be representative of the living-environment temperature of these animals. As a first attempt to test this hypothesis, and using geological characteristics of the region, we tried delineating catchment areas from where organisms could drift. For all areas and populations we then re-inferred temperatures based on calculated mean elevations in the hope that these temperatures would be more representative of the genuine biotopes. In this way, the Froidières population and both the Charabotte populations appeared as the most elevated stations, meaning the coldest habitats, and Pissoir as the lowest elevated station, i.e. the warmest one, like Cormoran. The strongest physiological responses found in the Pissoir and Cormoran populations during cold exposure and the lowest one found in both the Charabotte populations and Froidières population seemed to be

consistent with this hypothesis, even if more studies on other populations are needed to confirm it.

To conclude, we found that the overall relationship that can be established between the amplitude of thermal variations in the biotope and cold-hardiness abilities of the species may be more complex in subterranean crustaceans than in other arthropods. Indeed, we found that populations of *N. rhenorhodanensis* surprisingly displayed likely adaptive strategies to the cold even if they live in strongly thermally buffered environments: they accumulated cryoprotective molecules, they maintained a locomotory activity and managed to conserve an aerobic metabolism even at cold temperatures. Thus, subterranean environments seem to constitute a counter-example to the theory generally accepted. Moreover, we found a high heterogeneity in the cold responses between populations inhabiting similar and geographically close biotopes. The thermal plasticity of theses amphipods may result from (1) their past life history or (2) the intensity of the stress to which they are subjected. Thus, present environmental conditions (approximately 10°C) cannot explain the cold-adaptation abilities of the subterranean crustacean *N. rhenorhodanensis* but could partly explain the heterogeneity found in physiological and metabolic responses to cold exposure among different populations.

This research was supported by funds from Universities Claude Bernard – Lyon 1 and Beaulieu – Rennes 1 and from the IFR41 of the National Centre of French Scientific Research (CNRS). The authors thank Dr F. Malard for his valuable assistance in collecting individuals of *Niphargus rhenorhodanensis* and his helpful suggestions for this manuscript. We are also grateful to M. Laparie, who helped us with the statistical analysis. We also wish to thank Christina Richardson and two anonymous reviewers, who helped us to improve this article.

REFERENCES

Addo-Bediako, A., Chown, S. L. and Gaston, K. J. (2000). Thermal tolerance, climatic variability and latitude. *Proc. Biol. Sci.* **267**, 739-745.
Anchordoguy, T., Carpenter, J. F., Loomis, S. H. and Crowe, J. H. (1988). Mechanisms of interaction of amino acids with phospholipid bilayers during freezing. *Biochim. Biophys. Acta* **946**, 299-306.
Bennett, V. A., Sformo, T., Walters, K., Toienn, O., Jeannet, K., Hochstrasser, R., Pan, Q., Serianni, A. S., Barnes, B. M. and Duman, J. G. (2005). Comparative overwintering physiology of Alaska and Indiana populations of the beetle *Cucujus clavipes* (Fabricius): roles of antifreeze proteins, polyols, dehydration and diapause. *J. Exp. Biol.* **208**, 4467-4477.
Botosaneanu, L. (1986). *Stygofauna Mundi: A Faunistic, Distributional, and Ecological Synthesis of the World Fauna Inhabiting Subterranean Waters* (ed. L. Botosaneanu), pp. 740. Leiden: Brill Academic Publishers.
Bouchereau, A., Duhazé, C., Martin-Tanguy, J., Guégan, J. P. and Larher, F. (1999). Improved analytical methods for determination of nitrogenous stress metabolites occurring in *Limonium* species. *J. Chromatogr. A* **836**, 209-221.
Buckup, L., Dutra, B. K., Ribarcki, F. P., Fernandes, F. A., Noro, C. K., Oliveira, G. T. and Vinagre, A. S. (2008). Seasonal variations in the biochemical composition of the crayfish *Parastacus defossus* (Crustacea, Decapoda) in its natural environment. *Comp. Biochem. Physiol.* **149A**, 59-67.
Carpenter, J. F. and Crowe, J. H. (1988). The mecanism of cryoprotection of proteins by solutes. *Cryobiology* **25**, 244-255.
Carpenter, J. F., Crowe, J. H. and Arakawa, T. (1990). Comparison of solute-induced protein stabilization in aqueous solution in the frozen and dried states. *J. Dairy Sci.* **73**, 3627-3636.
Castany, G. (1998). *Hydrogéologie: Principes et Methodes*, pp. 236. Paris: Dunod.
Chown, S. L. (2001). Physiological variation in insects: hierarchical levels and implications. *J. Insect Physiol.* **47**, 649-660.
Colinet, H., Vernon, P. and Hance, T. (2007). Does thermal-related plasticity in size and fat reserves influence supercooling abilities and cold-tolerance in *Aphidius colemani* (Hymenoptera: Aphidiinae) mummies? *J. Therm. Biol.* **32**, 374-382.
Costanzo, J. P., Dinkelacker, S. A., Iverson, J. B. and Lee, R. E., Jr (2004). Physiological ecology of overwintering in the hatchling painted turtle: multiple-scale variation in response to environmental stress. *Physiol. Biochem. Zool.* **77**, 74-99.
Culver, D. C., Christman, M. C., Sket, B. and Trontelj, P. (2004). Sampling adequacy in an extreme environment: species richness patterns in Slovenian caves. *Biodivers. Conserv.* **13**, 1209-1229.
Daoud, D., Chabot, D., Audet, C. and Lambert, Y. (2007). Temperature induced variation in oxygen consumption of juvenile and adult stages of the northern shrimp, *Pandalus borealis*. *J. Exp. Mar. Biol. Ecol.* **347**, 30-40.
Farrell, A. P., Hinch, S. G., Cooke, S. J., Patterson, D. A., Crossin, G. T., Lapointe, M. and Mathes, M. T. (2008). Pacific Salmon in hot water: applying aerobic scope models and biotelemetry to predict the success of spawning migrations. *Physiol. Biochem. Zool.* **81**, 697-709.
Fields, P. G., Fleurat-Lessart, F., Lavenseau, L., Febvay, G., Peypelut, L. and Bonnot, G. (1998). The effect of cold acclimation and deacclimation on cold tolerance, trehalose and free amino acids levels in *Sitophilus granaries* and *Cryptolestes ferrugineus* (Coleoptera). *J. Insect Physiol.* **44**, 955-965.
Foulquier, A., Malard, F., Lefébure, T., Douady, C. J. and Gibert, J. (2008). The imprint of Quaternary glaciers on the present-day distribution of the obligate groundwater amphipod *Niphargus virei* (Niphargidae). *J. Biogeogr.* **35**, 552-564.
Freeze, R. A. and Cherry, J. A. (1979). *Groundwater,* pp. 604. Upper Saddle River, NJ: Prentice-Hall.
Gaston, K. J. and Chown, S. L. (1999). Elevation and climatic tolerance: a test using dung beetles. *Oikos* **86**, 584-590.
Ghalambor, C. K., Huey, R. B., Martin, P. R., Tewksbury, J. J. and Wang, G. (2006). Are mountain passes higher in the tropics? Janzen's hypothesis revisited. *Integr. Comp. Biol.* **46**, 5-17.
Gibert, J. (2001). Basic attributes of groundwater ecosystems. In *Groundwater Ecology: A Tool For Management of Water Resources* (ed. C. Griebler, D. L. Danielopol, J. Gibert, H. P. Nachtnebel and J. Notenboom), pp. 39-52. Luxembourg: Office for Official Publications of the European Communities.
Gibert, J. and Deharveng, L. (2002). Subterranean ecosystems: a truncated functional biodiversity. *Biosciences* **52**, 473-481.
Gibert, J., Stanford, J. A., Dole-Olivier, M. J. and Ward, J. V. (1994). Basic attributes of groundwater ecosystems and prospects for research. In *Groundwater Ecology* (ed. J. Gibert, D. L. Danielopol and J. A. Stanford), pp. 7-40. San Diego, CA: Academic Press.
Ginet, R. and Decou, V. (1977). *Initiation à la Biologie et à l'Écologie Souterraines*, pp. 345. Paris: Delarge.
Ginet, R. and Mathieu, J. (1968). Comparaison des temperatures létales supérieures de *Niphargus longicaudatus* (Crust. Amphipodes) hypogés et épigés. *Ann. Spéléo.* **23**, 425-440.
Goto, M., Sekine, Y., Outa, H., Hujikura, M. and Suzuki, K. (2001). Relationships between cold hardiness and diapause, and between glycerol and free amino acid contents in overwintering larvae of the oriental corn borer, *Ostrinia furnacalis. J. Insect Physiol.* **47**, 157-165.
Hanzal, R. and Jegorov, A. (1991). Changes in free amino acid composition in haemolymph of larvae of the wax moth, *Galleria mellon ella* L., during cold acclimation. *Comp. Biochem. Physiol.* **100A**, 957-962.
Hartley, L. M., Packard, M. J. and Packard, G. C. (2000). Accumulation of lactate by supercooled hatchlings of the painted turtle (*Chrysemys picta*): implications for overwinter survival. *J. Comp. Physiol. B* **170**, 45-50.
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* (Crustacea: Amphipoda). *Physiol. Biochem. 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. Biochem. Zool.* **69**, 1277-1300.
Hervant, F., Mathieu, J., Barré, H., Simon, K. and Pinon, C. (1997). Comparative study on the behavioural, ventilatory and respiratory responses of hypogean and epigean crustacean to long-term starvation and subsequent feeding. *Comp. Biochem. Physiol.* **118A**, 1277-1283.
Hochachka, P. and Somero, G. (2002). *Biochemical Adaptation, Mechanism and Physiological Evolution*, pp. 480. New York: Oxford University Press.
Huey, R. B. and Kingsolver, J. G. (1989). Evolution of ectotherm performance. *Trends Ecol. Evol.* **4**, 131-135.
Humphreys, W. F. (2000). Relict faunas and their derivation. In *Subterranean Ecosystems* (ed. H. Wilkens, D. C. Culver and W. F. Humphreys), pp. 417-432. Amsterdam: Elsevier.
Irwin, S., Wall, V. and Davenport, J. (2007). Measurement of temperature and salinity effects on oxygen consumption of *Artemia franciscana* K., measured using fibre-optic oxygen microsensors. *Hydrobiologia* **575**, 109-115.
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.
Issartel, J., Voituron, Y. and Hervant, F. (2007). Impact of temperature on the survival, the activity and the metabolism of the cave-dwelling *Niphargus virei*, the ubiquitous stygobiotic *N. rhenorhodanensis* and the surface-dwelling *Gammarus fossarum* (Crustacea, Amphipoda). *Subterr. Biol.* **5**, 9-14.
Jimenez, A. G. and Bennett, W. A. (2007). Metabolic responses of sand fiddler crabs, *Uca pugilator*, in northwest Florida to seasonal temperature change. *J. Therm. Biol.* **32**, 308-313.
Juberthie, C. and Decu, V. (ed.) (1994a). *Encyclopaedia Biospeologica I*. Moulis-Bucarest: Société de Biospéléologie.
Juberthie, C. and Decu, V. (1994b). Structure et diversité du domaine souterrain: particularités des habitats et adaptations des espèces. In *Encyclopaedia Biospeologica I* (ed. C. Juberthie and V. Decu), pp. 5-22. Moulis-Bucarest: Société de Biospéléologie.
Juberthie, C. and Decu, V. (ed.) (1998). *Encyclopaedia Biospeologica II*. Moulis-Bucarest: Société de Biospéléologie.
Juberthie, C. and Decu, V. (ed.) (2001). *Encyclopaedia Biospeologica III*. Moulis-Bucarest: Société de Biospéléologie.
Karanova, M. V. and Gakhova, E. N. (2002). Molecular nature of the cryotolerant lake amphipod *Gammarus lacustris. Biofizika* **47**, 116-124.
Kostal, V., Slachta, M. and Simek, P. (2001). Cryoprotective role of polyols independent of the increase in supercooling capacity in diapausing adults of *Pyrrhocoris apterusi* (Heteroptera: Insecta). *Comp. Biochem. Physiol.* **130B**, 365-374.
Lalouette, L., Kostal, V., Colinet, H., Gagneul, D. and Renault, D. (2007). Cold exposure and associated metabolic changes in adult tropical beetles exposed to fluctuating thermal regimes. *FEBS J.* **274**, 1759-1767.

Lee, R. E. (1989). Insect cold-hardiness: to freeze or not to freeze? *BioScience* **39**, 308-313.

Lefébure, T., Douady, C. J., Malard, F. and Gibert, J. (2007). Testing dispersal and cryptic diversity in a widely distributed groundwater amphipod (*Niphargus rhenorhodanensis*). *Mol. Phylogenet. Evol.* **42**, 676-686.

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

Michaud, R. M. and Denlinger, D. L. (2007). Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold-hardening in flesh flies (*Sarcophaga crassipalpis*): a metabolomic comparison. *J. Comp. Physiol. B* **177**, 753-763.

Packard, G. C. and Packard, M. J. (2004). To freeze or not to freeze: adaptations for overwintering by hatchlings of the North American painted turtle. *J. Exp. Biol.* **207**, 2897-2906.

Peck, L. S., Webb, K. E. and Bailey, D. M. (2004). Extreme sensitivity of biological function to temperature in Antarctic marine species. *Funct. Ecol.* **18**, 625-630.

Pörtner, H. O. (2006). Climate-dependent evolution of Antarctic ectotherms: an integrative analysis. *Deep Sea Res. Part II Top. Stud. Oceanogr.* **53**, 1071-1104.

Rakotomalala, R. (2005). TANAGRA: a free software for research and academic purposes. In *Proceeding of EGC'2005, RNTI-E-3, vol. 2*, pp. 697-702. Paris: Cépaduès-Editions. (In French.)

Ramløv, H. (1999). Microclimate and variations in haemolymph composition in the freezing-tolerant New Zealand alpine weta *Hemideina maori* Hutton (Orthoptera: Stenopelmatidae). *J. Comp. Physiol. B* **169**, 224-235.

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

Ramsay, S. L. and Houston, D. C. (2003). Amino acid composition of some woodland arthropods and its implications for breeding tits and other passerines. *Ibis* **145**, 227-232.

Renault, D., Hervant, F. and Vernon, P. (2002). Comparative study of the metabolic responses during food shortage and subsequent recovery at different temperatures in the adult lesser mealworm, *Alphitobius* diaperinus (Coleoptera: Tenebrionidae). *Physiol. Entomol.* **27**, 291-301.

Renault, D., Bouchereau, A., Delettre, Y., Hervant, F. and Vernon, P. (2006). Changes in free amino acids amounts in *Alphitobius diaperinus* Panzer (Coleoptera: Tenebrionidae) during thermal and food stress. *Comp. Biochem. Physiol.* **143A**, 279-285.

Ring, R. A. and Danks, H. V. (1998). The role of trehalose in cold-hardiness and dessication. *Cryo Letters* **19**, 275-282.

Schmidt-Nielsen, K. (1997). *Animal Physiology: Adaptation and Environment*, 5th edn, pp. 217-238. Cambridge: Cambridge University Press.

Stevens, G. C. (1989). The latitudinal gradient in geographical range: how so many species coexist in the tropics. *Am. Nat.* **133**, 240-256.

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

Tanaka, K. and Udagawa, T. (1993). Cold adaptation of the terrestrial isopod, *Porcellio scaber,* to subnivean environments. *J. Comp. Physiol. B* **163**, 439-444.

Thioulouse, J., Chessel, D., Dolédec, S. and Olivier, J. M. (1997). ADE-4: a multivariate analysis and graphical display software. *Stat. Comput.* **7**, 75-83.