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Behavioural, ventilatory and respiratory responses of epigean and hypogean crustaceans to different temperatures

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Impact of temperature (from -2 to 8 °C) on survival, oxygen consumption, locomotory and ventilatory activities was measured in two aquatic subterranean crustaceans (*Niphargus rhenorhodanensis* and *Niphargus virei*) and in a morphologically close surface-dwelling crustacean (*Gammarus fossarum*). The hypogean *N. virei* presented all characteristics of a stenothermal organism: it showed small thermal plasticity and optimised its performance on a narrow range of temperature. In contrast, the epigean *G. fossarum* and more surprisingly the hypogean *N. rhenorodanensis* can be both characterized as eurythermal organisms: they exhibited important survival times and conserved their performance optimum throughout a large range of temperature. Such differences of survival and performance patterns in two hypogean organisms were unexpected since they both live in very thermally buffered biotopes. Our data suggest fresh hypotheses about the role of glaciations in the history and adaptation of hypogean crustaceans.

Keywords: Subterranean crustacean; Temperature; Survival; Oxygen consumption; Ventilatory activity; Locomotory activity; Thermal plasticity; Eurytherm; Stenotherm

1. Introduction

Temperature may critically affect animal life and play a predominant role in establishing the distribution limits and the survival of ectotherm organisms (Addo-Bediako et al., 2000; Chown, 2001). Moreover, metabolic rates of ectotherms are highly temperature-dependent and all physiological processes slow down with decreasing temperatures (Hochachka and Somero, 2002). During exposure to a broad range of temperatures, the relationship between body temperature and metabolism can be described by an asymmetric function, where metabolic rate is maximized at an intermediate temperature: the thermal optimum (Huey and Kingsolver, 1989; Angilletta et al., 2002). From an ecological standpoint, this bell-shaped curve can be

explained by the Principle of Allocation (Huey and Slatkin, 1976): there is a trade-off between the maximum and the range of the performance. An individual that performs well (relatively to other individuals) in its optimal temperature zone should perform relatively poorly at non-optimum temperatures (Huey and Hertz, 1984). In stable thermal environments, natural selection should favour stenothermal organisms, i.e. organisms which maximize their performance along a very narrow range of temperature. Conversely, the performance of the same organisms in a fluctuating environment should evolve to a eurythermal profile, with a flatter shape, a smaller thermal optimum, but extended throughout a broader range of temperatures (Huey and Kingsolver, 1989). Ectotherms occupy various cold or warm habitats, but few of them show stable temperatures varying of only one or two degrees along the year (Eckert et al., 1997; Peck et al., 2004). This is the case in subterranean ecosystems, mainly because of the sedimentary and rocks layer that strongly buffer these habitats (Ginet and Mathieu,

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1968). Therefore, subterranean animals should exhibit reduced survival abilities as soon as the temperature leaves the optimum thermal zone. Consequently, such organisms should be classified as stenothermal organisms.

However, some biospeleologists noticed that the subterranean aquatic crustacean *Niphargus rhenorhodanensis* survived exposure at sub-zero temperatures (Hervant and Ginet, unpublished data). These observations raise three questions: (1) what is the thermobiological span of these organisms, (2) should we classify them as “stenotherms”, and (3) what is the ecological significance of such survival ability?

There are few experiments that deal with the impact of temperature on the biology in these species and no comparative studies on the behavioural, ventilatory and respiratory responses have been undertaken in hypogean (i.e. subterranean) and epigeal (i.e. surface-dwelling) crustaceans until now. Thus, to investigate the impact of temperature on the hypogean fauna, we compared the responses of aquatic subterranean crustaceans (*N. rhenorhodanensis* and *Niphargus virei*) to those of a morphologically close aquatic epigeal crustacean (*Gammarus fossarum*). *G. fossarum* regularly endures large temperature variations during its life cycle and therefore is likely to be considered as a eurythermal species (Rinderhagen et al., 2000; Wijnhoven et al., 2003). Measurements of survival, oxygen consumption, locomotory and ventilatory activities over a $-2/28$ °C gradient were performed in these three crustacean species.

2. Materials and methods

2.1. Animals, rearing and acclimation conditions

N. rhenorhodanensis (freshwater amphipod; fresh weight=13–14 mg) is an ubiquitous subterranean crustacean that lives in both porous and karstic aquifers. Specimens of *N. rhenorhodanensis* were collected from an interstitial system (Chalamont, Dombes Forest, France, 46°0.4' N, 5°10' E), with traps sunk into the sediment. *N. virei* (freshwater amphipod; fresh weight=90–100 mg) is a subterranean crustacean living only in karstic systems. The specimens of this species were 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). *G. fossarum* (freshwater amphipod; fresh weight=33–34 mg) is a common surface-dwelling crustacean. Specimens of this epigeal species were 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 aquaria kept in thermostated 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. They were directly afterwards separated into two groups: the first group was acclimated at 3 °C, and the second group was kept at 12 °C. These two groups were acclimated at their respective temperatures for 6 months. Water was changed twice a week and all physico-chemical parameters did not change during this acclimation.

2.2. Survival experiments

Survival was assessed at 9 temperatures (–2, 3, 7, 11, 14, 17, 21, 26 and 28 °C). For survival measurements at –2 °C, 30 individuals of each species were individually put in 6 mL plastic tubes containing 3 mL of tank water. For survival measurements from 3 to 28 °C, pools of 30 animals were transferred into tanks containing 100 mL of tank water. For each species, tanks were placed in the dark at each experimental temperature. Survival was measured (i) from –2 to 7 °C with the 3 °C acclimated organisms, and (ii) from 11 to 28 °C with the 12 °C acclimated organisms. The 3 and the 12 °C acclimated individuals were both exposed at –2 and 28 °C in order to emphasize the acclimation process. Survival was checked twice a day. When the survival reached 3 months, we considered that no mortality was observed at the experimental temperature.

2.3. Measurement of oxygen consumption, ventilatory and locomotory activities

Oxygen consumption, ventilatory and locomotory activities were investigated at all temperatures in all three species. Food was removed from experimental tanks 2 days before sampling the animals to ensure that the digestive tract was empty and that an overshoot in O₂ consumption due to digestive metabolism did not affect the results. Oxygen consumption was measured with a Warburg constant flow system (Hervant et al., 1995, 1996), which allows simultaneous measurement of 14 replicates. Small reaction vials of 12–14 mL were used for the experiment. The respirometers were not shaken, allowing the animals to respire without stress. The respirometer system was maintained at the experimental temperature in thermostated chamber for 12 h before the beginning of the measurements. The oxygen consumption was recorded every 15 min during 2 h (under a very low energy red light). At the end of the experiment, each animal was weighed (Scaltec SBC 31, accuracy 10^{-4} g).

Q_{10} values of all three species were calculated between –2 and 14 °C, and between 21 and 28 °C using the formula:

$$Q_{10} = (R_2/R_1)^{10/(T_2-T_1)}$$

where R_2 and R_1 are the oxygen consumptions at temperatures T_2 and T_1 (Hochachka and Somero, 2002).

Ventilatory activity was recorded by observation of the frequency of pleopod (ventilatory appendages of malacostracean crustaceans) beats during 1 min according to Hervant et al. (1997a,b). Locomotory activity was performed by measuring the number of moving animals in 800 mL tanks containing 10 individuals.

2.4. Performance breadth

Measurements of oxygen consumption, ventilatory and locomotory activities are all measurements of performance, an ecophysiological term including all physiological traits of a given organism (Huey and Kingsolver, 1989; Angilletta et al., 2002).

We calculated the performance breadth at 80% (B_{80}). B_{80} is the range of body temperature (equal to environmental temperature in ectotherms) over which performance is superior or equal to 80% of maximum performance value (Angilletta et al., 2002).

2.5. Statistical analysis

Lethal lapse of time for 50% of each sample (Lt_{50}) was determined using a Minitab probit analysis to compare survival at each temperature studied. If the fiducial limits ($\alpha=0.05$) of the two Lt_{50} values did not overlap, the Lt_{50} s were significantly different. Intra-specific differences in oxygen consumptions, locomotory and ventilatory activities were determined by an ANOVA. Inter-specific differences were measured using a two-way ANOVA. These statistical analyses were performed using Minitab software (Minitab Inc, version 13.32, State College, PA).

3. Results

3.1. Survival

All Lt_{50} s (Table 1) showed significant inter- and intra-specific differences ($P<0.05$). At -2 °C, Lt_{50} of all three species showed significant inter-specific and inter-acclimation group differences. Specimens acclimated at 3 °C

exhibited significantly higher Lt_{50} s than the specimens acclimated at 12 °C. Such acclimation effect was very low in *N. virei* that always presented the shortest survival times. Lt_{50} measured in *G. fossarum* were highly significantly shorter than those of *N. rhenorhodanensis*. The epigeic *G. fossarum* and the hypogean *N. rhenorhodanensis* did not die in the temperature range $3-17$ °C whereas a significant mortality was observed in *N. virei* at 17 °C. At 21 °C, similar durations of survival were found in *N. rhenorhodanensis* and *G. fossarum*, whereas the Lt_{50} of *N. virei* was 10 times lower. From 26 to 28 °C, *G. fossarum* showed significantly higher Lt_{50} 's than *N. rhenorhodanensis* and *N. virei*.

3.2. Oxygen consumption, ventilatory and locomotory activities

Oxygen consumption curves (Fig. 1a) of all three species followed a classical bell-shaped profile: aerobic metabolism was correlated with temperature. Mean values of oxygen consumption were 30% and 143% higher in the epigeic crustacean *G. fossarum* than in the hypogean *N. rhenorhodanensis* and *N. virei*, respectively ($P<0.001$). Q_{10} values were different among the three species (Table 2): Q_{10} s between -2 and 14 °C were greater than 1 and it was largely higher in *N. virei* than in the other two species. In contrast, Q_{10} values between 21 and 28 °C were smaller than 1 in all three species. *G. fossarum* and *N. rhenorhodanensis* had similar Q_{10} values, that are apparently twice higher of *N. virei*.

In all three crustaceans, temperature had a strong effect on ventilatory activity (Fig. 1b), and mean values were significantly different between species ($P<0.001$). Ventilatory activity values of *G. fossarum* were 16% and 190% higher than those of *N. rhenorhodanensis* and *N. virei*, respectively. The 14 °C temperature appeared to be an important threshold: ventilatory activity increased largely above this temperature in the three organisms. Ventilation doubled between 14 and 21 °C in all three animals.

Locomotory activity (Fig. 1c) presented contrasted profiles. Indeed, in *G. fossarum*, the percentage of individuals in movement varied largely with temperature

Table 1

Lethal times for 50% of the population (days)±SE in *G. fossarum*, *N. rhenorhodanensis* and *N. virei* exposed to different temperatures ($N=30$)

	-2 °C	3 °C	7 °C	11 °C	17 °C	21 °C	26 °C	28 °C
<i>G. fossarum</i>	2.70 ± 0.21^a 21.36 ± 0.51^b	*	*	*	*	137.28 ± 7.98^a	27.01 ± 0.51^a	8.23 ± 0.26^a 6.89 ± 0.25^b
<i>N. rhenorhodanensis</i>	8.72 ± 0.35^a 54.95 ± 1.17^b	*	*	*	*	111.58 ± 4.17^a	6.94 ± 0.26^a	1.60 ± 0.08^a 0.85 ± 0.03^b
<i>N. virei</i>	1.02 ± 0.03^a 2.09 ± 0.07^b	*	*	*	126.52 ± 8.20^a	8.89 ± 0.33^a	0.93 ± 0.03^a	0.42 ± 0.01^a 0.34 ± 0.01^b

*No mortality measured during the whole experience (3 months).

^a Individuals acclimated at 12 °C.

^b Individuals acclimated at 3 °C.

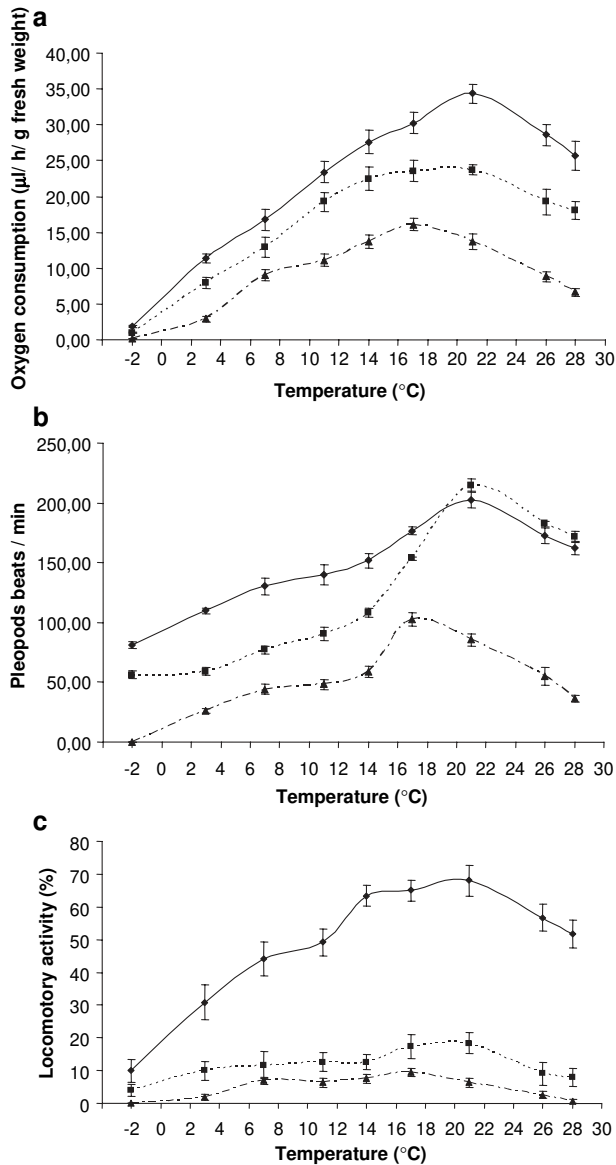


Fig. 1. Effect of temperature on (a) oxygen consumption, (b) ventilatory activity, and (c) locomotory activity in (◆) *G. fossarum*, (■) *N. rhenorhodanensis*, and (▲) *N. virei*. Values are means \pm SEM ($N=10$).

since values were 6.8 times higher at 21 °C than at –2 °C. Conversely, in both hypogean species, locomotory activity varied very little.

The performance breadths measured at 80% (B_{80}) of the maximal value are listed in Table 3. *N. virei* showed the smallest B_{80} s regardless of the physiological trait. Except for oxygen consumption, the epigean crustacean *G. fossarum* presented higher B_{80} s than the hypogean crustaceans. *N. rhenorhodanensis* maintained its oxygen consumption at

Table 2

Q_{10} calculated for *G. fossarum*, *N. rhenorhodanensis* and *N. virei*

	<i>G. fossarum</i>	<i>N. rhenorhodanensis</i>	<i>N. virei</i>
Q_{10} (–2 °C; 14 °C)	5.33	7.13	16.13
Q_{10} (21 °C; 26 °C)	0.66	0.68	0.36

Table 3

B_{80} s of oxygen consumption, ventilatory and locomotory activities in *G. fossarum*, *N. rhenorhodanensis* and *N. virei*

	Oxygen consumption (°C)	Locomotory activity (°C)	Ventilatory activity (°C)
<i>G. fossarum</i>	13.2	14	12.7
<i>N. rhenorhodanensis</i>	15.6	8	10
<i>N. virei</i>	9	5.5	6

80% of maximum on a broader range of temperature than the other species, showing a B_{80} of 15.6 vs. 13.2 °C in *G. fossarum* and 9 °C in *N. virei*.

4. Discussion

A majority of ecosystem is subjected to daily and/or seasonal temperature variations (Eckert et al., 1997), consequently, studies in ectotherm thermal tolerance were principally focused in organisms inhabiting these fluctuating environments. Few studies have investigated the importance of temperature variations in species living in thermally buffered biotopes (for a review see Somero et al., 1996) and even less in subterranean organisms until now.

In this study, the crustaceans *G. fossarum*, *N. rhenorhodanensis* and *N. virei* exhibited three distinct survival patterns: the epigean species *G. fossarum*, was characterized by the highest thermal plasticity, showing a significantly longer duration of survival than the hypogean *N. virei* and *N. rhenorhodanensis* in the thermal range 3–28 °C. This is relevant to the habitats of *G. fossarum*: in streams (or rivers), temperature may fluctuate from 2 to 28 °C along the seasons. The reduced survival ability noticed in *N. virei* as soon as it was out from its temperature optimum is concomitant with its ecology: subterranean ecosystems are characterized by highly reduced variations of the temperature throughout the year (Ginet and Mathieu, 1968). However, the large survival capacity found in *N. rhenorhodanensis* is unexpected. Both *N. rhenorhodanensis* and *N. virei* live in similar habitats, and therefore, never endure temperature variations. Thus, we may wonder the ecological significance of such survival ability.

As also found in many other species (Fields et al., 1998; Holmstrup et al., 1999; Bale et al., 2000; Renault et al., 2004), acclimation had a significant impact on the duration of survival in all three species. In crustaceans, thermal acclimation may induce several physiological changes in haemolymph (Tanaka and Udagawa, 1993) but also in enzyme properties (Mulkiewicz et al., 2000) or haemoglobin affinity (Paul et al., 2004). Such changes confer protection against injuries induced by temperature (Hochachka and Somero, 2002). In *N. virei*, which displays a very low thermal plasticity ecologically relevant with its habitat, acclimation had a much-reduced effect. Such results were also found in few stenothermal polar fishes inhabiting highly thermal stable environments (Wilson et al., 2001),

and we might hypothesize that the inability to thermally acclimate could be found in most of the stenotherm species.

Groundwater ecosystems are generally very poor in nutrients and oxygen (Spicer, 1998; Malard and Hervant, 1999). In order to reduce energetic expenditures, ectotherms living in these biotopes present very low metabolic rates when compared to epigean species (Hppop, 1985; Hervant et al., 1997a,b; Hervant and Renault, 2002). Our results are concomitant with these findings: the highest values of performance (oxygen consumption, ventilatory and locomotory activities) were found in the epigean amphipod *G. fossarum*. Moreover, visual predator–prey interactions are reduced in subterranean environments. As a result, hypogean organisms did not develop high capacities for locomotion (Hervant et al., 2001), which may explain the reduced impact of temperature on activity in *N. rhenorhodanensis* and *N. virei*. However, *N. rhenorhodanensis* preserved a basal level of locomotory activity even at extreme experimental temperatures, ensuring a basal predatory activity when its environment cools down, therefore avoiding starvation. In all three species, oxygen consumption and ventilatory activity increased with temperature, until a maximum value. This critical temperature is lower in *N. virei* than in *G. fossarum* and *N. rhenorhodanensis*. At $-2\text{ }^{\circ}\text{C}$, *N. virei* showed a very low oxygen consumption, and no ventilatory activity. We could hypothesize that the metabolism of this species was so low at this temperature that it no longer needed to create a water flow across the respiratory surface. Thus, $-2\text{ }^{\circ}\text{C}$ could be the threshold temperature of the chill coma in this species. Furthermore, a $10\text{ }^{\circ}\text{C}$ temperature variation (Q_{10} values) had a stronger impact on the metabolic rate of *N. virei* than in the other two crustaceans, suggesting a lower capacity to maintain optimal enzymatic activities. A very low or high Q_{10} signifies that temperature is damaging the properties of the underlying physiological and biochemical system, until the irreversible loss of function (Hochachka and Somero, 2002). Thus, these results may explain the limited survival of *N. virei* as soon as it was out of its optimal thermal range. The lower Q_{10} s and higher B_{80} s measured in *G. fossarum* and *N. rhenorhodanensis* (see Tables 2 and 3) indicated that these animals maximized their performance on broader ranges of temperature. The epigean *G. fossarum* and the hypogean *N. rhenorhodanensis* displayed similar physiological patterns on the temperature range we tested. These two crustaceans can be characterized as eurythermal species. Conversely, *N. virei*, exhibited a stenothermal profile.

From a physiological point of view, differences between stenotherms and eurytherms performance may reflect structural constraints resulting from a compromise between the flexibility and the stability of enzymes (Alexandrov, 1977). Moreover, in ectotherms, several studies pointed out that an exposure at non-optimum temperatures involves an increase in “metabolic key” enzymes expression or the induction of enzymes (isozymes) with different kinetic properties (Richmond and Zimmerman, 1978; Lesser and

Kruse, 2004; Zakhartsev et al., 2004; Somero, 2004). Further studies should determine whether “metabolic key” enzymes are quantitatively or qualitatively modulated during exposure to different temperatures in the eurythermal *G. fossarum* and *N. rhenorhodanensis* in comparison with the stenothermal *N. virei*.

From an evolutionary point of view, we may wonder why the two hypogean species, *N. rhenorhodanensis* and *N. virei*, exhibited such differences in their performance patterns. The strong selection pressures characterizing thermally buffered environments as subterranean ones (Edwards, 1986; Malard and Hervant, 1999) is likely to eliminate individuals keeping energetically costly mechanisms that may not be needed (Huey and Kingsolver, 1989). The eurythermal profile found in *N. rhenorhodanensis* might thus result from its life history. Glaciations represent one of the most important factors explaining current hypogean species distribution (Ginet, 1971, 1988). *N. rhenorhodanensis* and *N. virei* have both been identified in the massif mountain of Jura (France), but in distinct sites: *N. virei*'s current location exactly corresponds to the inferior limit of the Pleistocene glaciers (8000 years ago) whereas *N. rhenorhodanensis* can be found in sites that were entirely covered by these glaciers (Ginet, 1971). Thus, we may hypothesize that *N. rhenorhodanensis* endured and survived the Pleistocene glaciations below the ice, in phreatic and karstic refugium habitats. Furthermore, other works dealing with the hypogean species distribution areas revealed that some subterranean amphipods may survive below the ice during glaciations of the Pleistocene in Europe and North America (Holsinger et al., 1983; Strayer et al., 1995; Proudlove et al., 2003). The highest cold tolerance than that of *N. virei* and the eurythermal profile found in *N. rhenorhodanensis* might be a relict adaptation (as it has been observed in several invertebrates, Baust, 1980; Baust and Rojas, 1985; Mulkiewicz et al., 2000; Lee et al., 2003) which enabled the survival of these populations during glaciation periods. The survival of *N. rhenorhodanensis* under the ice refugiums during the last glaciation period cannot fully explain the current location of its populations. It has been suggested that few hypogean species have recolonised subterranean biotopes immediately after the last quaternary deglaciation using river corridor (Henry, 1976; Magniez, 1976, 1997). An alternative hypothesis would be that glaciations destructed most of *N. rhenorhodanensis* populations, but individuals living at the frontier of glaciers, in a mix of cold glacial water and cool groundwater, could have survived and recolonised subterranean biotopes that were formerly covered by glacier. This survival and recolonisation may have selected eurythermal individuals with strong efficiency at cold temperatures. As a result, *N. virei* and *N. rhenorhodanensis* may not possess the same phylogenetical baggage, which may partly explain the observed differences.

Our work shows rather atypical results if compare to theoretical expectations. The hypogean *N. rhenorhodanensis*

showed characteristics of eurythermal organisms whereas it lives in strongly buffered habitats. The differences in physiological patterns, regarding the temperature, between the two hypogean species clearly bring new elements allowing a better comprehension of their biogeographical history. Nevertheless, the greater cold tolerance found in *N. rhenorhodanensis* is not yet fully understood. Further studies should investigate the possible roles of cryoprotective substances in *N. rhenorhodanensis*, *N. virei* and *G. fossarum* for a better understanding of the cold hardiness of these species.

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