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Comparison of some epigean and troglobiotic animals regarding their metabolism intensity. Examination of a classical assertion

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Abstract: This study determines oxygen consumption (R), electron transport system (ETS) activity and R/ETS ratio in two pairs of epigean and hypogean crustacean species or subspecies. To date, metabolic characteristics among the phylogenetic distant epigean and hypogean species (i.e., species of different genera) or the epigean and hypogean populations of the same species have been studied due to little opportunity to compare closely related epigean and hypogean species. To fill this gap, we studied the epigean *Niphargus zagrebensis* and its troglobiotic relative *Niphargus stygius*, and the epigean subspecies *Asellus aquaticus carniolicus* in comparison to the troglobiotic subspecies *Asellus aquaticus cavernicolus*. We tested the previous findings of different metabolic rates obtained on less-appropriate pairs of species and provide additional information on thermal characteristics of metabolic enzymes in both species or subspecies types. Measurements were done at four temperatures. The values of studied traits, i.e., oxygen consumption, ETS activity, and ratio R/ETS, did not differ significantly between species or subspecies of the same genus from epigean and hypogean habitats, but they responded differently to temperature changes. Higher Q_{10}-values for oxygen consumption of *N. stygius* than *N. zagrebensis* in the temperature range 10-20°C and higher E_a indicated higher thermal sensitivity in the subterranean species. On the other hand, lower Q_{10} and E_a-values for ETS activity of *N. stygius* than *N. zagrebensis* indicated more thermally stable enzymatic machinery in *N. stygius* than *N. zagrebensis*. In *Asellus*, we observed a similar trend of lower E_a for oxygen consumption and higher E_a for ETS activity in epigean than the troglomorphic subspecies, but the values did not differ significantly between the two. Our most important conclusion is that the low metabolic rate is not a universal property of troglobiotic animals, and the degree of metabolic adaptation is not necessarily in agreement with the degree of morphological adaptation (troglomorphy).

Keywords: food scarcity, temperature, oxygen consumption, ETS activity, amphipod, isopod

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INTRODUCTION

One of the main characteristics of subterranean habitats is food scarcity (Sket, 1996; Hüppop, 2012) which intensifies how animals economize their use of resources. Consequently, one of the most distinctive non-morphological characteristics of subterranean animals was claimed to be a low metabolic rate. A number of studies compared standard metabolic rates (SMRs) in subterranean aquatic species and their epigean relatives. In crustaceans, the SMR in normoxia in epigean species was measured to be 1.6 to 4.5 times higher than in their hypogean relatives (Hervant et al., 1998).

Unfortunately, most comparative studies have been done on less-appropriate pairs of species, usually a hypogean *Niphargus* sp. and an epigean *Gammarus* sp., members of Niphargidae and Gammaridae, respectively. Another study compared the hypogean *Stenasellus* sp. and the epigean *Asellus aquaticus*, again members of different families, Stenasellidae and Asellidae. While the results are convincing, a small doubt exists, presenting the possibility of phylogenetic and historical reasons for physiological differences. A need to confirm and test this possibility has always been present, particularly since Culver & Poulson (1971) stated that in *Gammarus minus* Say, oxygen consumption of a slightly troglomorphic population

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from a well-aerated cave stream was not significantly different from that of epigean (spring) population. Additionally, responses to periodical hypoxia were little different in populations of *G. minus* from cave and surface habitats (Hervant et al., 1999b). Moreover, fasting experiment on the subterranean (*Astyanax fasciatus mexicanus*) and epigean (*Astyanax fasciatus fasciatus*) populations of the tropical fish showed that, despite a hypometabolism in cave fishes, they did not recover from the "food deprivation" stress in contrast to epigean ones after the refeeding period (Salin et al., 2010). Accordingly, the metabolic rate is most likely an adaptation to particular environmental conditions (e.g., food availability) rather than simply to the epigean/hypogean realm. Some additional comparisons are needed to clarify this question.

In ectotherm organisms, temperature is one of the most important environmental parameters influencing all biological process rates (Hochachka & Somero, 2002). Oxygen consumption as an estimator of metabolic activity, and the activities of respiratory enzymes are strongly affected by temperature and, therefore, frequently studied in terms of responses to temperature changes in different groups of organisms (e.g., Muskó et al., 1995; Simčič & Brancelj, 1997, 2000, 2001, 2004; Simčič, 2005; Simčič et al., 2014; Žagar et al., 2015). However, studies on the thermal physiology of subterranean invertebrates are scarce (Issartel et al., 2005, 2007; Mermillod-Blondin et al., 2013; Di Lorenzo & Galassi, 2017). In stable thermal environments, such as subterranean habitats, temperatures may vary a few degrees or even less throughout the year. It is therefore expected that natural selection would favour stenothermal organisms which maximize their performance along a very narrow temperature range compared to epigean animals. However, Issartel et al. (2005), who measured oxygen consumption as a physiological trait at different temperatures, reported that the hypogean *Niphargus virei* possessed all characteristics of stenothermal organisms, whereas epigean *Gammarus fossarum* Koch and hypogean *Niphargus rhenorodanensis* Schellenberg were characterised as eurythermal organisms. They suggested that further studies on "metabolic key" enzymes after exposure to different temperatures could provide additional information on thermal differences between the eurythermal and stenothermal species. Ectotherms can respond to chronic temperature changes by qualitative and/or quantitative adjustments in enzyme activity (Lannig et al., 2003; Simčič & Brancelj, 2004; Simčič et al., 2014). For example, the temperature-dependence of respiratory electron transport system (ETS) activity reflects changes in the concentration of ETS units resulting from acclimation to changed temperature (Bamstedt, 1980) and characteristics of the enzymatic complexes involved (Packard, 1971). ETS activity is a biochemical measure of potential metabolic activity which can be used for actual oxygen consumption in order to meet current energy demands. Previous studies have shown that the ratio between actual (i.e., oxygen consumption; R) and potential metabolic activities (R/ETS) is an important index of the organisms' metabolism. For example, in organisms with low ratios, the enzyme capacity for elevated metabolism is maintained and, thus, it is available for increased metabolic activity when needed (Muskó et al., 1995; Fanslow et al., 2001; Simčič et al., 2005; Simčič et al., 2017).

Slovenia is an ideal place for such a study as it abounds with troglobiotic fauna. Some species are present in both epigean and troglobiotic subspecies, while other genera are represented by related troglobiotic and epigean species. These include crustacean genera *Niphargus* (Amphipoda, Niphargidae) and *Asellus* (Isopoda, Asellidae).

Although all *Niphargus* spp. seem to be without eyes and feebly pigmented (Fišer, 2012), *Niphargus* is not an exclusively subterranean genus. Other than hundreds of ecologically diverse subterranean species (Fišer, 2012), more than ten epigean or eutroglophile species exist; more than five of these inhabit Slovenia (Sket, 1958, 1981). For our study, we selected the troglobiotic *N. stygius* (Schioedte) and the epigean (eutroglophile) *N. zagrebensis* S. Karaman. They are morphologically similar since *N. stygius* is not strongly troglomorphic while *N. zagrebensis* is a member of an epigean species group exhibiting the same degree of a fictitious troglomorphy (compare Sket, 2008). *Asellus aquaticus* (Linne) is generally a pan-European species (Birštejn, 1951; Sket, 1965, 1994), split into a series of subspecies and/or a closely related species (Sket, 1958; Verovnik et al., 2005, 2009) that are in-part ecologically different, as some are troglobiotic (Racovitza, 1925; Prevorčnik et al., 2002, 2004). Its most troglomorphic subspecies (probably species) is present in Slovenia.

This study aims to compare metabolic activities of closely related epigean and hypogean species or subspecies (within the same genera) and to investigate their sensitivity to temperature change in order to test the previous findings obtained on phylogenetic distant pairs of species and provide additional information on thermal characteristics of the metabolic enzymes in both types of species or subspecies. To this end, oxygen consumption, ETS activity, and R/ETS ratio were determined in two species of genus *Niphargus* (the epigean species *Niphargus zagrebensis* and the troglobiotic *Niphargus stygius*), and two subspecies of genus *Asellus* (the epigean subspecies *Asellus aquaticus carniolicus* Sket and the troglomorphic subspecies *Asellus aquaticus cavernicolus* Racovitza) at four experimental temperatures.

MATERIALS AND METHODS

Experimental animals

Niphargus stygius (Schioedte 1847) is the type species of the genus. Animals are 10-20 mm long, eyeless, and depigmented. It is strongly troglobiotic, inhabiting systems of fissures in karstified rock and, secondarily, puddles and trickles of seeping water in caves or karst springs primarily in western Slovenia. It is accompanied by other strongly troglobiotic animals. The sample in our study (with mean body mass ± SE = 62.2 ± 5.1 mg wet mass, n = 10) was taken from the cave Unška koliševka at Planina, NE of Postojna in Slovenia. The habitat was a small puddle of percolated water in an artificially modified (for military purposes) cave system.

Niphargus zagrebensis S.Karaman 1950 (syn. *N. valachicus z., N.elegans z.*) was first found near Zagreb (NE Croatia) but is also present in SE Slovenia. It occurs in epigean waters where *Gammarus* spp. are absent and also occurs, albeit rarely, in caves (Delić et al., 2017). In Krakovski gozd at Kostanjevica, SE Slovenia, from where our sample (with 32.5 ± 3.4 mg wet mass, n = 10) originates, *N. zagrebensis* inhabits stagnant or slowly flowing water in puddles and ditches with loamy bottoms and rich deposits of fallen leaves. The water is acidic. Access to caves or interstitial water is here mainly absent. *N. zagrebensis* is accompanied by the characteristic synusia, which also accompany other epigean niphargi in eastern Europe and the Near East: *Asellus aquaticus, Synurella* cf. *ambulans*, *Viviparus* sp., *Tropidiscus* sp., *Pisidium* spp. Ephemeroptera larvae. While *N. zagrebensis* is eyeless (a characteristic of the genus) and only feebly pigmented, all other animals in this environment are represented by strongly pigmented species.

Isopoda are represented by two subspecies of the highly euryoecious *Asellus aquaticus* (Linne). Both are in the Ljubljanica drainage (C-SW Slovenia), sympatric, and closely-related phylogenetically (Verovnik et al., 2004), but not syntopic.

Asellus aquaticus carniolicus Sket 1965 is an epigean subspecies. Our sample (with 48.4 ± 3.1 mg wet mass, n = 10) is from a large and clear residual pool on the bottom of the intermittent karst lake Cerkniško jezero. The 'lake' is highly productive, rich in fauna (including some fish species), but in summer often without any pools. Temperature is highly fluctuating, as is the oxygen content of the water (Gaberščik, 2002).

Asellus aquaticus cavernicolus Racovitza 1925 is a conspicuously troglomorphic subspecies found in sinking rivers. Our sample (with 29.1 ± 4.9 mg wet mass, n = 9) was taken in the cave Zelške jame close to Cerkniško jezero. This subspecies is without eyes and dark pigmentation, with very elongated antennae and pereopods (Sket, 1965). The watercourse is influenced by epigean (sink) stream, which is characterised by slight temperature fluctuation and is comparatively rich in food.

The mean yearly temperature (i.e., also the permanent temperature of ground waters) in 2005-2014 was 9-11°C at Postojna and 10-12°C at Novo mesto (close to Krakovski gozd). The mean monthly temperatures in January and July varied between -1.7 to 1.4 °C and 20.5 to 22.8°C in Novo mesto, while -2.3 to 4.7 °C and 18.5 to 22.3°C in Postojna (Statistični urad RS, 2018).

Collection and maintenance of animals

Animals were caught using a hand net three weeks before experiments were conducted. They were stored in plastic bottles and transported to the laboratory, where they were kept in aquaria in perpetual darkness at 10°C (±0.5°C) in thermo-regulated chambers. Aquaria were previously filled with aerated chemically controlled (synthetic) water, i.e., 294 mg $CaCl_2 \times 2 H_2O$, 123 mg $MgSO_4 \times 7 H_2O$, 65 mg $NaHCO_3$, 6 mg KCl were added per 1 L of bi-distilled water (ISO-standard 6341: 1996). During the period of acclimation to laboratory conditions, *Asellus* were fed *ad libitum* with soaked alder leaves, and *Niphargus* were fed with commercial food (Sera Crabs Natural™, Germany). Water in the aquaria was changed twice a week.

Methods

Experimental procedure

A single experimental animal was transferred to a beaker containing synthetic water and food. The water temperature was then decreased by 1°C every 12 h down to the lowest experimental temperature, i.e., 5°C. Once the desired temperature was reached, the animals were maintained at this temperature for the next five days for acclimation. On the 4th day of the acclimation period, food was removed from beakers for 24 h to ensure that the digestive tract would be empty during subsequent oxygen consumption measurements. After oxygen consumption measurements were taken at the first experimental temperature (5°C), the animals were returned to the beakers, and the same procedure described above was repeated to perform the measurements at 10, 15, and 20°C (i.e., gradual increasing of temperature followed by 5-days of acclimation). After oxygen consumption measurements at all four experimental temperatures, water temperature was gradually decreased to 10°C, where animals were acclimated for five days. Next, prior to the preparation of the homogenate for further analyses, the animals were frozen in liquid nitrogen and stored at -80°C.

Oxygen consumption

Oxygen consumption was estimated by the closed bottle method (Lampert, 1984). The 25-mL ground-glass stoppered bottles were filled with synthetic and aerated water from the same, well-mixed, container. The experimental bottles received animals, while two bottles without animals served as controls. All bottles were closed and kept at 5°C, 10°C, 15°C, or 20°C. We measured the concentration of dissolved oxygen inside the experimental and control bottles with a sensor and a 4-channel fiber-optic oxygen meter (PreSens OXY-4, Germany) after 0.5 h to allow animals time to recover from handling stress (start measurement) and 7.5 h (end measurement). The difference between the concentrations of dissolved oxygen in each experimental bottle at the start and the end of incubation, minus the mean value of control bottles, was taken as the amount of oxygen consumed by animals. Oxygen consumption rate was expressed as mL O_2 per g wet mass per h (mL O_2 g^{-1} h^{-1}).

ETS activity

Respiratory electron transport system (ETS) activity was determined as described by Packard (1971) and improved by G.-Tóth (1999). One animal was homogenised in 6 mL of ice-cold homogenisation buffer (0°C, 0.1 M sodium phosphate buffer pH = 8.4, 75 µM $MgSO_4$, 0.15% (w/v) polyvinyl pyrrolidone, 0.2% (v/v) Triton-X-100) using a glass potter (IKA) and an ultrasonic homogenizer (4710; Cole-Parmer), then centrifuged for 4 min at 0°C at 8500 g (refrigerated centrifuge Sigma 2K15). Two 0.3 mL samples from each supernatant were incubated in 1.5 mL substrate solution (0.1 M sodium phosphate buffer pH = 8.4, 1.7 mM NADH, 0.25 mM NADPH, 0.2% (v/v) Triton-X-100) with 0.5 mL INT solution (2.5 mM 2-p-iodo-phenyl 3-p-nitrophenyl 5-phenyl tetrazolium chloride) and incubated for 30 minutes at 5°C, 10°C, 15°C, and 20°C. The reaction was stopped by adding 0.5 mL of stopping solution (1:1 formalin (conc.): conc. H_3PO_4). Blanks (substrate and INT solution) were incubated and stopped like in the samples, and then 0.3 mL of homogenate was added. Formazan production was determined spectrophotometrically from the absorbance of the sample at 490 nm against the blank (Perkin-Elmer, Lambda 25). ETS activity was measured as the rate of reduction of tetrazolium dye to formazan, converted to equivalent oxygen as described by Kenner and Ahmed (1975), and expressed as mL O_2 per g wet mass per h (mL O_2 g^{-1} h^{-1}).

Weighing

After each of the oxygen consumption measurements and at the end of acclimation to 10°C before freezing, the animals were placed between two sheets of filter paper and gently pressed together to remove the water from their surface. They were then placed on a pre-weighed piece of aluminium foil and weighed on an electro-balance (Sartorius BP 210 S) with 0.1 mg accuracy.

Calculations and statistics

Due to the allometric relationship with body size, oxygen consumption and ETS activity data were normalised to a mean wet mass of genera *Niphargus* and *Asellus,* separately. The size range of our experimental animals was much too narrow to determine a mass exponent b. Therefore, we used b=0.66 for oxygen consumption (Simčič et al., 2012) and 0.65 for ETS activity (Simčič & Brancelj, 2003) determined for freshwater crustaceans to normalise measured data.

To characterize the sensitivity of oxygen consumption and ETS activity to temperature changes, the temperature coefficient, Q_{10}, was calculated according to the equation (Lampert, 1984):

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

where R_1 and R_2 are the measured metabolic rates and T_1 and T_2 the corresponding temperatures. Q_{10}-values were calculated for two temperature ranges, i.e., temperature range 5-10°C, to obtain the response of metabolic activities to the decrease of temperature, and temperature range 10-20°C to estimate the sensitivity to temperature increase (10°C is an average temperature of the habitats).

The thermodynamic response of oxygen consumption and ETS activity over the whole temperature range was shown in terms of the Arrhenius activation energy (E_a), which describes the accelerating influence of temperature on metabolic rate. This calculation was obtained from the slope of linear regression of ln of actual or potential metabolic rate against the inverse of absolute temperature (Mermillod-Blondin et al., 2013) according to the equation:

$$E_a = -R_{gc}S$$

where E_a is expressed in kJ mol^{-1}, R_{gc} is the gas constant (8.31 J mol^{-1} $degree^{-1}$), and S is the slope of the Arrhenius plot.

The normal distribution of data was tested with the Shapiro–Wilk test and equality of variances with Levene's test. In the case of inconsistency, data were log transformed. Prior to pooling the data sets of both sexes in *Niphargus zagrebensis, N. stygius* and *Asellus aquaticus cavernicolus* (specimens of *A. a. carniolicus* were all males), we tested for between-sex variation in oxygen consumption, ETS activity, and R/ETS ratio using an ANCOVA with sex as a factor and mass as a covariate, which showed no significant differences between sexes ($p > 0.05$), therefore we pooled both sexes prior to interspecific comparison.

A two-way mixed model (between/within) ANOVA was carried out to test for differences in oxygen consumption rate, ETS activity, and R/ETS ratio between different populations (between-subject factor) and experimental temperatures (within-subject factor). Tukey's HSD post hoc comparison was used to determine which means differed significantly. For finding interspecific differences in Q_{10} within genera in temperature ranges we applied Student t-tests. We compared the slopes of the linear regressions between epigean and hypogean animals within the same genus using ANCOVA to determine whether they had significantly different E_a. We conducted all statistical analyses with SPSS 20.0 (SPSS Inc. Chicago, Illinois, USA).

RESULTS

Oxygen consumption

The oxygen consumption rate ranged between 0.07 and 0.09 mL O_2 g^{-1} h^{-1} for *N. zagrebensis* and between 0.06 and 0.12 mL O_2 g^{-1} h^{-1} for *N. stygius* (Fig 1a). Oxygen consumption rate increased significantly with temperature (ANOVA; $F_{3,54} = 14.39$; $p < 0.001$), and it was similar between both species (ANOVA; $F_{1,18} = 0.09$; $p > 0.05$). Interaction temperature × population was non-significant (ANOVA; $F_{3,54} = 3.82$; $p > 0.05$). We observed a significant increase in oxygen consumption rate in *N. stygius* at 20°C.

In *Asellus*, the oxygen consumption rate increased significantly with temperature (ANOVA; $F_{3,39} = 14.97$; $p < 0.001$), from 0.03 to 0.11 mL O_2 g^{-1} h^{-1} in *Asellus aquaticus carniolicus* and from 0.02 mL O_2 g^{-1} h^{-1} to 0.08 mL O_2 g^{-1} h^{-1} in *A. a. cavernicolus* (Fig. 1b). Significantly higher oxygen consumption was measured in *A. a. carniolicus* at 20°C in comparison to lower experimental temperatures. The rates of oxygen consumption did not differ between both subspecies (ANOVA; $F_{1,13} = 0.006$; $p > 0.05$). There was a non-significant temperature × population interaction (ANOVA; $F_{3,39} = 2.14$; $p > 0.05$).

In the temperature range 5-10°C, Q_{10} -values of respiration for *N. zagrebensis* were similar to those of *N. stygius* (Student's t-test; $p > 0.05$), while in the temperature range 10-20°C higher Q_{10} values were obtained for *N. stygius* than for *N. zagrebensis* (Student's t-test; $p < 0.05$) (Table 1).

Q_{10}-values of respiration for genus *Asellus* were lower in epigean *A. a. carniolicus* than troglobiotic *A. a. cavernicolus* in the temperature range 5-10°C ($p < 0.05$), but the opposite was observed in the temperature range 10-20°C, where Q_{10} –values of *A. a. carniolicus* were higher than those of *A. a. cavernicolus* ($p < 0.05$) (Table 1).

The Arrhenius activation energy (E_a) of oxygen consumption was lower for epigean *N. zagrebensis* than for hypogean *N. stygius* (ANCOVA; $p < 0.01$). In genus *Asellus*, the same trend of lower E_a in epigean than troglobiotic subspecies was observed, but the values did not differ significantly between the two subspecies (ANCOVA; $p > 0.05$) (Table 2).

ETS activity

ETS activity varied with temperature from 0.29 mL O_2 g^{-1} h^{-1} to 1.68 mL O_2 g^{-1} h^{-1} in *N. zagrebensis* and from 0.38 to 1.45 mL O_2 g^{-1} h^{-1} in *N. stygius* (Fig. 2a). ETS activity did not differ significantly between species of *Niphargus* (ANOVA; $F_{1,17} = 0.21$; $p > 0.05$), but temperature had significant effect on ETS activity (ANOVA; $F_{3,51} = 550.37$; $p < 0.001$). Interaction temperature × population was significant (ANOVA; $F_{3,51} = 11.98$; $p < 0.001$).

ETS activity was similar in both taxa of *Asellus* within the same temperatures ($F_{1,13} = 0.52$; $p > 0.05$) and ranged from 0.21 to 1.15 mL O_2 g^{-1} h^{-1} in epigean *A. a. carniolicus* and from 1.19 to 1.30 mL O_2 g^{-1} h^{-1} in troglomorphic *A. a. cavernicolus* (Fig. 2b). ETS activity increased significantly with temperature (ANOVA; $F_{3,39} = 603.17$; $p < 0.001$). Significant interaction temperature × population was observed (ANOVA; $F_{3,39} = 6.11$; $p < 0.01$).

Q_{10}-values of ETS activity were higher for *N. zagrebensis* than *N. stygius* in the temperature ranges 5-10°C and 10-20°C (Student's t-test; $p < 0.05$) (Table 1).

Higher Q_{10}-values were obtained for *A. a. cavernicolus* than *A. a. carniolicus* in the temperature ranges 5-10°C (Student's t-test; $p < 0.05$), but similar values were observed in the temperature range 10-20°C (Student's t-test; $p > 0.05$) (Table 1).

E_a of ETS activity ranged from 61.1 kJ mol^{-1} for *N. stygius* to 85 kJ mol^{-1} for *A.a. cavernicolus* (Table 2). *N. zagrebensis* had significantly higher E_a than *N. stygius* (ANCOVA; $p < 0.01$), whereas E_a-values of the *Asellus* subspecies did not differ significantly (ANCOVA; $p > 0.05$).

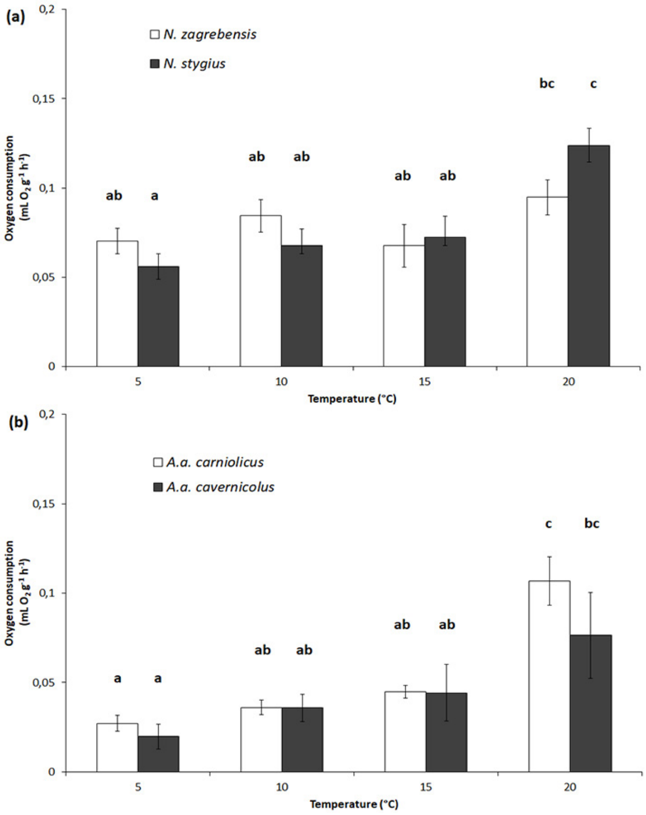

Fig. 1. Oxygen consumption of (a) *Niphargus zagrebensis* and *N. stygius* and (b) *Asellus aquaticus carniolicus* and *A.a. cavernicolus* at four experimental temperatures. Bars labelled with different letters differ significantly — $p < 0.05$ (ANOVA, Tukey's HSD test). Error bars represent mean ± SE (n = 10 for *Niphargus* and n = 5-10 for *Asellus*).

Table 1. Temperature coefficient Q_{10} of oxygen consumption (R) and ETS activity (ETS) calculated for *Niphargus zagrebensis*, *N. stygius, Asellus aquaticus carniolicus* and *A.a. cavernicolus* for temperature ranges 5-10°C and 10-20°C. (mean ± SE; n = 10 for *Niphargus* and n = 5-10 for *Asellus*). Significant differences between the populations of the same genus for R or ETS are marked with * ($p < 0.05$).

T range (°C)	***N. zagrebensis***		***N. stygius***		***A.a. carniolicus***		***A.a. cavernicolus***	
	R	**ETS**	**R**	**ETS**	**R**	**ETS**	**R**	**ETS**
5-10	2.21 ±0.62	4.28* ±0.65	2.50 ±0.82	2.60* ±0.25	3.30* ±1.52	3.18* ±0.29	14.08* ±6.19	6.42* ±0.98
10-20	1.19* ±0.13	3.08* ±0.20	1.91* ±0.17	2.41* ±0.08	5.54* ±1.77	3.12 ±0.10	1.79* ±0.21	2.91 ±0.30

Table 2. Arrhenius activation energy E_a (kJ mol^{-1}) of oxygen consumption (R) and ETS activity (ETS) calculated for *Niphargus zagrebensis*, *N. stygius, Asellus aquaticus carniolicus* and *A.a. cavernicolus* over temperature range 5-20°C. Significant differences between the populations of the same genus for R or ETS are marked with ** ($p < 0.01$).

	E_a	
	R	**ETS**
Niphargus zagrebensis	8.1**	81.7**
Niphargus stygius	35.8**	61.1**
Asellus aquaticus carniolicus	59.7	75.3
Asellus aquaticus cavernicolus	77.9	85.0

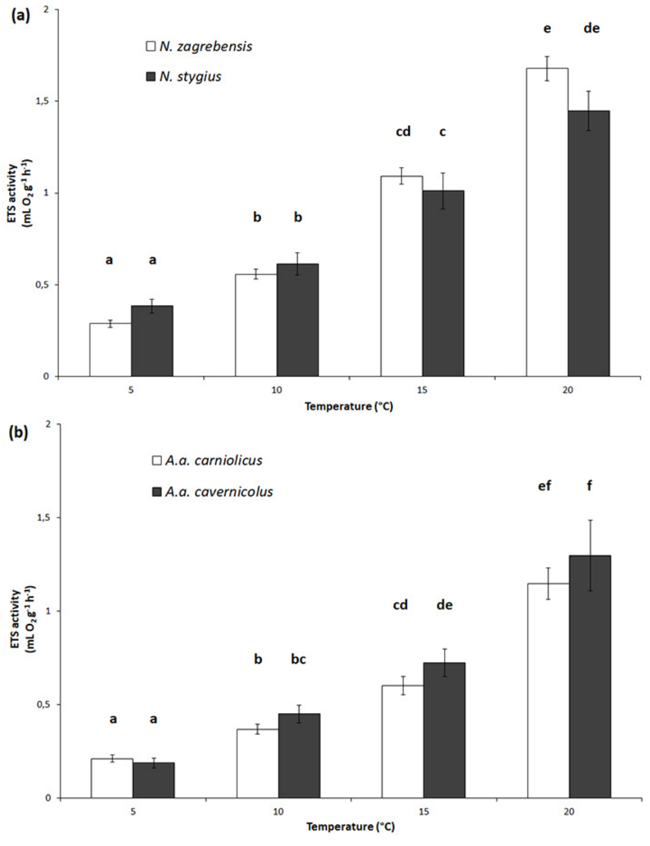

Fig. 2. Electron transport system (ETS) activity of (a) *Niphargus zagrebensis* and *N. stygius* and (b) *Asellus aquaticus carniolicus* and *A.a. cavernicolus* at four experimental temperatures. Bars labelled with different letters differ significantly — $p < 0.05$ (ANOVA, Tukey's HSD test). Error bars represent mean ± SE (n = 9-10 for *Niphargus* and n = 5-10 for *Asellus*).

The relationship between oxygen consumption and ETS activity

The ratio between oxygen consumption and ETS activity (R/ETS), an indicator of exploitation of metabolic potential for actual metabolic activity, ranged from 5.73 to 25.09% in *N. zagrebensis* and between 8.85 and 16.09% in *N. stygius* (Fig. 3a). R/ETS ratio decreased significantly with temperature (ANOVA; $F_{3,51} = 38.54$; $p < 0.001$). Similar values were observed between both species (ANOVA; $F_{1,17} = 0.02$; $p>0.05$) and interaction temperature × population was non-significant (ANOVA; $F_{1,17} = 0.02$; $p > 0.05$).

R/ETS ratios ranged from 8.11 to 12.76 and 6.91 to 15.92 in *A. a. carniolicus* and *A. a. cavernicolus*, respectively (Fig. 3b). The values did not differ significantly between taxa (ANOVA; $F_{1,13} = 0.03$; $p > 0.05$) and temperatures (ANOVA; $F_{3,39} = 2.50$; $p > 0.05$). There was a non-significant interaction temperature × population (ANOVA; $F_{3,39} = 01.75$; $p > 0.05$).

DISCUSSION

The values of studied metabolic traits, i.e., oxygen consumption, metabolic potential (measured as ETS activity), and the ratio R/ETS, did not differ significantly between the epigean and troglobiotic taxa of the same genus from epigean and hypogean habitats, but they responded differently to temperature changes.

In the present study, we measured routine metabolic rate. The routine metabolic rate of cave animals may be reduced due to reduced activity (Hervant et al., 2001). However, our results are in accord with results in previously studied subspecies of *Gammarus minus* as presented by Culver & Poulson (1971), who found that cave ecotypes of *G. minus* showed no lower standard or routine metabolic rate than surface ecotypes (Table 3). Oxygen consumption rate of *N. stygius* in the present study is in accord with that found in the previous study of Simčič et al. (2005), where similar values were reported also for a cave population of the eutroglophile *Niphargus* species, *N. krameri* Schellenberg. Moreover, we obtained a similar oxygen consumption rate in our study for the epigean *N. zagrebensis*. Additionally, oxygen consumption did not differ between both studied *Asellus* subspecies: the highly troglomorphic *A. a. cavernicolus* and epigean *A. a. carniolicus*. This finding means that changes of the metabolism intensity are not necessarily in agreement with the degree of morphological adaptation (troglomorphy). In contrast, all previous studies comparing hypogean and epigean species of different and not closely related families showed lower oxygen consumption for hypogean than epigean animals, suggesting that phylogenetic and historical grounds contribute at least partly to the physiological differences between the more distantly related hypogean and epigean species.

Measurements of ETS activity showed similar metabolic potential for species and subspecies within the genera *Niphargus* and *Asellus*, respectively. This result agrees with the previous finding of Simčič et

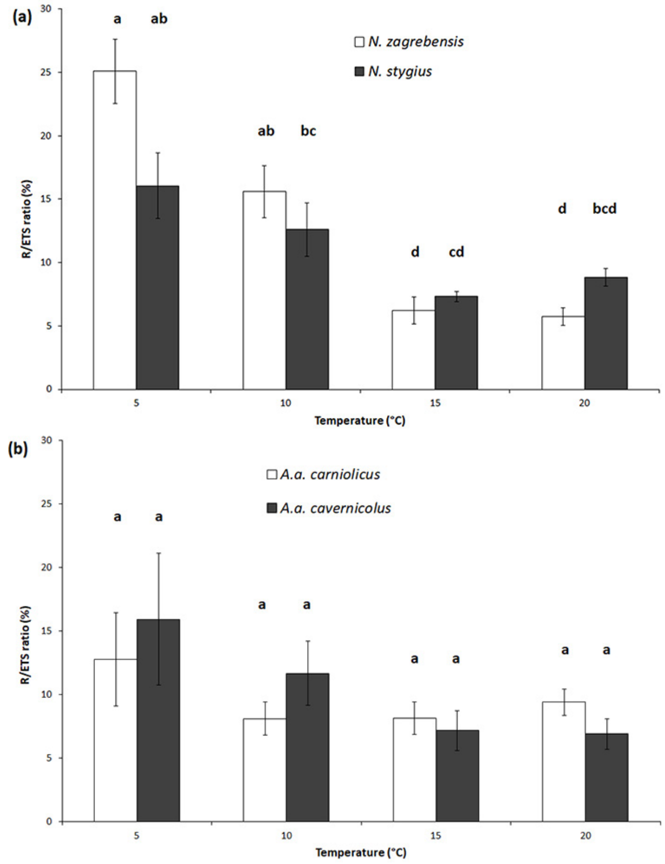

Fig. 3. Exploitation of metabolic potential for actual metabolic activity (R/ETS ratio) for (a) *Niphargus zagrebensis* and *N. stygius* and (b) *Asellus aquaticus carniolicus* and *A.a. cavernicolus*. Bars labelled with different letters differ significantly — $p < 0.05$ (ANOVA, Tukey's HSD test). Error bars represent mean ± SE (n = 9-10 for *Niphargus* and n = 5-9 for *Asellus*).

al. (2005) where ETS activity did not differ between *Gammarus* and *Niphargus* amphipod species. It was assumed that hypogean animals possess relatively high metabolic potential, which can be exploited for energy production immediately following a pulse in the food supply. When food is available after prolonged starvation, it is ecologically advantageous for organisms to quickly and completely restore energy reserves that were depleted during starvation (Hervant & Renault, 2002).

Table 3. Oxygen consumption and electron transport system (ETS) activity in epigean (E) and hypogean (H) crustaceans from various studies. M-intermediate ecotype; data for close to 10°C have been taken.

Taxon	**Location**	**Type**	**Oxygen consumption ($\mu L\ O_2\ g^{-1}\ h^{-1}$)**	**ETS activity ($\mu L\ O_2\ g^{-1}\ h^{-1}$)**	**T (°C)**	**Source**
Niphargus zagrebensis	Krakovski gozd (Slovenia)	E	84	557	10	Present study
Niphargus stygius	Planinsko polje (Slovenia)	H	68	613	10	Present study
Niphargus stygius	Velika Pasjica Cave (Slovenia)	H	58	459	10	Simčič et al. (2005)
Niphargus stygius	Špicberg Cave (Slovenia)	H	85	383	10	Simčič et al. (2005)
Niphargus krameri	Dimnice Cave (Slovenia)	H	65	390	10	Simčič et al. (2005)
Niphargus virei	Spring at Gueux (France)	H	80	-	11	Hervant et al. (1997)
Niphargus rhenorhodanensis	Groundwater in Dombes Forest (France)	H	132	-	11	Hervant et al. (1997)
Gammarus fossarum	River in Pouillyles-Nonains (France)	E	195	-	11	Hervant et al. (1997)
Gammarus fossarum	Hudournik Spring (Slovenia)	E	178	449	10	Simčič et al. (2005)
Gammarus fossarum	River Iščica (Slovenia)	E	165	386	10	Simčič et al. (2005)
Gammarus minus I	Benedict Cave (USA)	H	430	-	13	Culver & Poulson (1971)
Gammarus minus I	Greenbrier Caverns (USA)	H	340	-	13	Culver & Poulson (1971)
Gammarus minus II	Coffman’s Cave (USA)	M	260	-	13	Culver & Poulson (1971)
Gammarus minus III	Fort Spring (USA)	E	210	-	13	Culver & Poulson (1971)
Gammarus minus III	U.S. #219 spring (USA)	E	520	-	13	Culver & Poulson (1971)
Asellus aquaticus carniolicus	Cerkniško jezero (Slovenia)	E	36	368	10	Present study
Asellus aquaticus cavernicolus	Zelške jame (Slovenia)	H	36	450	10	Present study
Asellus aquaticus	Rhône River (France)	E	280	-	11	Hervant et al. (1997)
Stenasellus virei	Groundwater of River Tarn (France)	H	92	-	11	Hervant et al. (1997)

The R/ETS ratio is a useful estimate of organisms’ metabolic state. In the present study, similar ratios were obtained for epigean and hypogean animals within the same genus. This means that the animals from subterranean and surface habitats exploited a similar percentage of the metabolic potential for actual oxygen consumption. The values of R/ETS for species of *Niphargus* at 10°C are similar to those reported in Simčič et al. (2005) for *Niphargus* species from different hypogean habitats that were determined at the same temperature. Moreover, the ratios differed significantly between hypogean amphipod populations of *N. stygius* from different caves and *N. krameri* (Simčič et al., 2005). It was suggested that the reason for different ratios probably lies in differences in the quality and/or quantity of food available in their natural habitats (Simčič et al., 2005). Since animals were used in experiments immediately after being collected in caves, the different food supply had a significant effect on the experimental animals’ oxygen consumption. Moreover, the feeding experiments showed that ETS activity in *N. stygius* did not change with feeding, although oxygen consumption increased significantly in fed animals (Simčič et al., 2005). These results are in agreement with those of Cammen et al. (1990) and Mezek et al. (2010), who found that oxygen consumption declined over time in starved animals, but ETS activity remained relatively constant. Therefore, feeding hypogean animals affected oxygen consumption and ETS activity differently, where fed *N. stygius* had higher exploitation of metabolic potential than the starved ones (Simčič et al., 2005). However, in the present study, the experimental animals were kept in a laboratory for three weeks and fed *ad libitum*. Because of this, the direct effect of quality and/or quantity of food in their natural habitats on oxygen consumption was excluded.

Metabolism in ectotherms is highly dependent on environmental temperature, therefore we also tested both types of species and subspecies for their responses to temperature change. As expected, oxygen consumption and ETS activity increased with temperature (Hochachka & Somero, 2002). However, different responses to temperature changes were observed between oxygen consumption rate and ETS activity. ETS activity is a direct enzymatic process that depends upon enzyme concentration (Bamstedt, 1980) and thermal characteristics of the enzymes (Packard, 1971), whereas oxygen consumption is a complex physiological process, also modulated by changes in substrate concentration and structure of lipid membranes (Angilletta, 2009). In general, the R/ETS ratios decreased with increasing temperature in all studied taxa. This response of R/ETS ratio to temperature changes is in accord with the results of previous studies in invertebrate (Muskó et al., 1995; Simčič & Brancelj, 1997; Simčič, 2005) and vertebrate (Žagar et al., 2015; Simčič et al., 2017) species. Significant interaction between temperature and population observed for both studied genera indicates that the response of ETS activity to particular temperature depends on subspecies or species. This topic is discussed in detail below.

In our study, Q_{10}-values for oxygen consumption were similar for both *Niphargus* species in the temperature range 5-10°C, but Q_{10}-values for ETS activity were higher for *N. zagrebensis* than *N. stygius*. Moreover, in the temperature range 10-20°C, lower Q_{10}-value for ETS activity was also obtained in *N. stygius* than *N. zagrebensis*. However, oxygen consumption increased with temperature more in *N. stygius* than *N. zagrebensis*. It is known that adaptive mechanisms increase metabolic efficiency at low temperature and decrease it at high temperature, thus achieving homeostatic control over their enzyme reaction (Packard et al., 1975), but the ability for capacity acclimation is genetically limited (Prosser, 1991). Low Q_{10} is characteristic of animals that possess enzyme systems with extremely broad temperature optima, which prevents their inactivation during environmental temperature changes (Randall et al., 1997). Therefore, lower Q_{10}-values for ETS activity in *N. stygius* than *N. zagrebensis* indicated more thermally stable enzymatic machinery in *N. stygius* than *N. zagrebensis* in both temperature ranges (Table 1). Contrary to Q_{10} for ETS activity, higher Q_{10}-values for oxygen consumption in *N. stygius* than *N. zagrebensis* in the temperature range 10-20°C indicate a stress response to increased temperature, because increased oxygen consumption rate is one of the indicators of stress (Barton & Schreck, 1987; Simčič & Brancelj, 2007). Moreover, as evidenced by Simčič & Brancelj (2007) and Lukančič et al. (2010) for various invertebrate species, a higher R/ETS ratio at higher temperatures could indicate a stress response. In the present study, we observed a trend of increasing of the R/ETS ratio at the highest temperature for *N. stygius*, but not for *N. zagrebensis*. In the case of genus *Asellus*, we observed the highest Q_{10}-value for oxygen consumption for the hypogean subspecies in the temperature range 5-10 °C and the lowest for the same subspecies at temperature range 10-20°C. Extreme responses to temperature are in agreement with those of Issartel et al. (2005), who reported very low and high Q_{10}-values for hypogean stenothermal *Niphargus virei* as a result of the negative influence of temperature on biochemical and physiological processes (Table 4). On the other hand, Issartel et al. (2005) found that the hypogean *N. rhenorhodanensis* showed characteristics of eurythermal animals, although it lives in strongly buffered habitats. The differences in physiological pattern, regarding temperature, in hypogean N. *virei* and *N. rhenorhodanensis* were likely a result of their biogeographical history (Issartel et al., 2005; Foulquier et al., 2008). Therefore, other environmental factors, besides seasonal temperature variation in the habitats, could affect the thermal sensitivity of animals. Additionally, Di Lorenzo & Galassi (2017) obtained the controversial results for a stygobiotic copepod species, *Diacyclops belgicus* (Kiefer), where Q_{10} was 0.39 considering the mean values of the oxygen consumptions and 0.99 considering the median values.

According to the previous studies (e.g., Van Dijk et al., 1999; Simčič & Brancelj, 2004), populations having high E_a would be more susceptible to temperature change than organisms with low E_a. Therefore, much lower E_a of oxygen consumption for *N. zagrebensis* indicated lower temperature sensitivity to temperature changes in comparison to related hypogean species *N. stygius*. However, E_a of ETS activity, and E_a for respiration rate reportedly differed (del Giorgio, 1992; Simčič & Brancelj, 1997); ETS activity reflects the purely mechanistic nature of the *in vitro* enzymatic ETS reaction as opposed to the complex and tightly controlled process of respiration. Contrary to E_a of oxygen consumption, E_a of ETS activity was higher for *N. zagrebensis* than *N. stygius*. This indicates that ETS activity of *N. stygius* increased less strongly with rising temperature, meaning that the metabolic potential of *N. stygius* was less responsive to temperature changes than that of *N. zagrebensis*. The reason for relatively stable metabolic potential could be the reduction of the cost for mitochondrial biosynthesis and degradation. Moreover, the cost of maintenance of a higher number of mitochondria, e.g., maintenance of proton gradients, and aerobic enzyme capacities, would contribute to a rise of basal metabolism (Rolfe et al., 1999; Pörtner, 2002). Consequently, this increase would cause a shift in energy budget unfavourable to the accumulation of energy reserves for growth and for reproduction (Lannig et al., 2003). This assumption is in accord with the reports of previous studies on the metabolic responses of hypogean and epigean animals to long-term starvation and the subsequent resumption of feeding where hypogean species have a faster and more efficient assimilation of available nutrients during recovery from starvation (Hervant et al., 1999a, 2001; Hervant & Renault, 2002). The rapid recovery from nutritional stress of troglobiotic organisms was explained by lower exploitation of metabolic potential for current metabolic activity, i.e., lower R/ETS ratio (Simčič et al., 2005). Thus, the optimal relationship between the existing metabolic potential and its

Table 4. Temperature coefficient (Q_{10}) and activation energy (E_a) of oxygen consumption in epigean (E) and hypogean (H) crustaceans from various studies.

Taxon	Type	T range (°C)	Q_{10}	E_a (kJ mol^{-1})	Source
Niphargus zagrebensis	E	5 - 10	2.21	8.1	Present study
	E	10 - 20	1.19		Present study
Niphargus stygius	H	5 - 10	2.50	35.8	Present study
	H	10 - 20	1.91		Present study
Niphargus rhenorhodanensis	H	-2 - 14	7.13	-	Issartel et al. (2005)
	H	21 - 26	0.68	-	Issartel et al. (2005)
Niphargus virei	H	-2 - 14	16.13	-	Issartel et al. (2005)
	H	21 - 26	0.36	-	Issartel et al. (2005)
Gammarus fossarum	E	-2 - 14	5.33	-	Issartel et al. (2005)
	E	21 - 26	0.66	-	Issartel et al. (2005)
Asellus aquaticus carniolicus	E	5 - 10	3.30	59.7	Present study
	E	10 - 20	5.54		Present study
Asellus. aquaticus cavernicolus	H	5 - 10	14.08	77.9	Present study
	H	10 - 20	1.79		Present study
Proasellus valdensis (PV1)	H	-	-	86.4	Mermillod-Blondin et al. (2013)
Proasellus valdensis (PV2)	H	-	-	42.4	Mermillod-Blondin et al. (2013)
Proasellus n. sp. 1	H	-	-	53.0	Mermillod-Blondin et al. (2013)
Proasellus n. sp. 2	H	-	-	62.2	Mermillod-Blondin et al. (2013)

exploitation for current energy production, capable of providing the sufficient amount of energy with minimal maintenance costs, is probably realised in subterranean populations at a specific temperature.

E_a-values of oxygen consumption for both subspecies of *Asellus* were found to be in the range of values reported by Mermillod-Blondin et al. (2013) for three isopod species of genus *Proasellus* colonizing groundwater habitats characterized by annual temperature amplitude of less than 1°C (Table 4). Contrary to the findings for genus *Niphargus*, E_a-values did not differ significantly between epigean *A. a. carniolicus* and troglomorphic *A. a. cavernicolus*. One of the reasons for different respond to temperature between both genera is probably the various amount of food that is available for *N. stygius* and *A. a. cavernicolus* in comparison to their relative epigean taxa. *N. stygius* is exposed to food depletion in its habitat, while *A. a. cavernicolus* is not. However, Mermillod-Blondin et al. (2013) demonstrate that populations of three groundwater species could exhibit distinct responses to temperature variation, although they all colonized habitats showing little seasonal temperature fluctuations. Thus, the metabolic responses to temperature change in populations that originate either from thermally stable or variable habitats are heterogeneous, probably due to different combinations of all intrinsic and extrinsic factors that are unique for each population.

In conclusion, the results of this study indicate that metabolic rates in closely related epigean and hypogean species/subspecies are not necessarily dissimilar. Our measurements reveal that either (1) subterranean environment alone does not dictate a decrease of metabolic activity in animals; or (2) both, epigean and subterranean habitats are variable to such an extent that only statistical evaluation of a higher number of populations could tell us what these significant differences are. Moreover, higher sensitivity of oxygen consumption to temperature changes and relatively more stable metabolic potential, as observed in the troglobiotic *N. stygius,* indicates different thermal metabolic characteristics between hypogean and epigean species. On the other hand, the responses of metabolic activities to temperature changes did not differ significantly between both subspecies of genus *Asellus*. However, the metabolic activity of animals is regulated on several biological levels within their genetic limits that influence how an organism will respond to a particular environmental condition. It is likely that some species/populations are adapted in different ways, which might compensate for the adaptation in metabolic intensity. Further studies of physiological and biochemical traits of closely related animals from various surface and subterranean habitats are needed to provide a more comprehensive picture of the metabolic adaptive mechanisms to the underground environmental conditions.

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