Freshwater Biology

Freshwater Biology (2015) **60,** 426–435 doi:10.1111/fwb.12509

Metabolic rates of a hypogean and an epigean species of copepod in an alluvial aquifer

T. DI LORENZO*, WALTER D. DI MARZIO[†], D. SPIGOLI[‡], M. BARATTI*, G. MESSANA*, S. CANNICCI[‡] AND DIANA M. P. GALASSI[§]

**Istituto per lo Studio degli Ecosistemi - CNR, Sesto Fiorentino, Firenze, Italy*

[†]Programa de Investigación en Ecotoxicología, Departamento de Ciencias Básicas, Universidad Nacional de Luján - Comisión Nacional de Investigaciones Científicas y Técnicas CONICET, Luján, Argentina

[‡]Department of Biology, University of Florence, Sesto Fiorentino, Italy

[§]Department of Life, Health and Environmental Sciences, University of L'Aquila, L'Aquila, Italy

SUMMARY

1. Reduced metabolic rates of groundwater taxa, compared to those of surface water species, have long been inferred to be an adaptive trait where there is a low and discontinuous food supply and unpredictable shifts between hypoxic and normoxic conditions. However, there have been neither measurements of the respiratory rate of groundwater copepods nor a comparison of rates between closely related groundwater and surface water species.

2. We measured the metabolic rates of two species of Cyclopoida: Cyclopidae, the stygobiotic (hypogean) copepod *Diacyclops belgicus* and the epigean *Eucyclops serrulatus*, which co-occur in the same alluvial aquifer. We expected the metabolic rate of the hypogean to be lower than that of the epigean species, irrespective of the ontogenetic stage, which would be consistent with the hypothesis that there is a generally lower metabolic rate in groundwater species.

3. The metabolic rate of *D. belgicus* was significantly lower than that of the epigean *E. serrulatus*, irrespective of the ontogenetic stage. We found an allometric relationship between oxygen consumption and body mass for *E. serrulatus*, an isometric one for *D. belgicus* juveniles and a rate of oxygen consumption that apparently does not change systematically with body mass for *D. belgicus* adults.

4. The low metabolic rate of *D. belgicus* may be advantageous in oligotrophic groundwater habitats, where large fluctuations in oxygen availability occur. However, these physiological adaptations can put hypogean species at risk of replacement by more metabolically active epigean taxa, whenever the availability of organic matter increases, as happens with organic pollution. Moreover, the low metabolic rate of the hypogean species may entail an inability to cope with toxicants, rendering them more sensitive to pollutants. A higher metabolic rate in juvenile *D. belgicus* compared to that of adults allows copepodids to mature quickly when food is briefly abundant.

Keywords: alluvial aquifer, copepods, groundwater, metabolism, oxygen

Introduction

Crustaceans represent about 10% of total invertebrate species known from fresh waters globally (Balian *et al.*, 2008). With more than 1100 species living in ground water (Galassi, 2001; Galassi, Huys & Reid, 2009), copepods are by far the most abundant and species-rich group in ground water and ecosystems dependent upon ground water (Hatton & Evans, 1998). The orders Cyclopoida and Harpacticoida have most successfully invaded inland ground water (Boxshall & Defaye, 2008), while the Calanoida are less represented there, occurring in the plankton of subterranean lakes (Brancelj & Dumont, 2007). Accordingly, more than one-third of the known species of copepods are 'stygobiotic', that is strictly confined to ground water and with specific morphological and physiological adaptations to this environment (Galassi, 2001). Other copepod species may

Correspondence: Stefano Cannicci, Department of Biology, University of Florence, Via Madonna del Piano 6, 50019 Sesto Fiorentino, Italy.
E-mail: stefano.cannicci@unifi.it

be found in ground water, being either 'stygophiles' or 'stygoxenes'. Stygophile copepods can live and reproduce in subterranean habitats, as well as in some marginal surface water environments, and they may, or may not, possess some affinities and morphological adaptations to ground water. Stygoxene copepods are epigean (surface water) species, often characterised by wide ecological tolerance (Malard, Plénet & Gibert, 1996), that are occasionally found in ground water. They enter ground water accidentally, via passive transport, or actively, with infiltrating water (Brunke & Gonser, 1997; Di Lorenzo, Stoch & Galassi, 2013). Although stygoxenes do not show affinities or adaptations to ground water, they can be found in this environment, sometimes with egg sacs and copepodids. However, their populations typically do not proliferate in ground water (Gibert *et al.*, 1994), except where it is organically enriched, often due to pollution (Malard *et al.*, 1994, 1996).

While some information is available on morphological adaptations of stygobiotic copepods, little is known about their other biological traits. From the few studies available (see Galassi, 2001 for a review), stygobiotic copepods seem to produce fewer (though larger) eggs than epigean species, possibly thus securing an endogenous food supply for larvae in an environment where food availability is highly stochastic. A general trend towards prolongation of the post-embryonic development in stygobiotic cyclopoids and harpacticoids has been observed, as well as a longer life span compared to epigean relatives (Rouch, 1968; Glatzel, 1990). Time for egg development is also longer than that of epigean relatives among the cyclopoids (Lescher-Moutoué, 1973, 1974; Bjornberg & Por, 1986). These developmental traits, which have been interpreted as adaptations (by conserving energy) in an oligotrophic environment (Hervant & Renault, 2002; Wilhelm, Taylor & Adams, 2006), have been related to the low metabolic rate reported for stygobiotic taxa (Culver, 1982; Hüppop, 1985; Hervant, Mathieu & Messana, 1997; Hervant *et al.*, 1998; Wilhelm *et al.*, 2006). However, to our knowledge, neither measurements of respiratory metabolism of groundwater copepods, nor comparison of metabolic rates among closely related groundwater and surface water copepod species, have ever been performed.

We measured the metabolic rates of two species of Cyclopoida Cyclopidae, the stygobiotic (hypogean) copepod *Diacyclops belgicus* and the stygoxene *Eucyclops serrulatus*, which co-occur in the same alluvial aquifer. We expected the metabolic rate of the stygobiont to be lower than that of the epigean species, irrespective of the ontogenetic stage (juveniles and adults), which would be consistent with the more general hypothesis that there is an overall difference between these two groups.

Methods

Collection of copepods for respiration experiments

The specimens used were collected from two wells (W1 and W2), located 300 m apart (coordinates: W1 43°49′02.61″ N 11°11′59.79″ E; W2 43°48′59.43″ N 11°11′51.87″ E). Both wells (water table depth >2 m) are situated in the shallow Quaternary alluvial aquifer of Medio Valdarno (Tuscany, Italy). Prior to respirometric trials, we examined ground water from both wells for dissolved organic carbon (DOC) and 32 chemicals, including ammonium, nitrites, nitrates, heavy metals, inorganic pollutants, PAHs, pesticides and organochlorines. Chemical analyses indicated that ground water from the two wells contained no contaminants at concentrations of concern according to the European Directives 2006/118/EC and 2000/60/EC. A phreatobiological net-sampler (mesh size 60 μm, modified after Cvetkov, 1968) was used to collect copepods from the bottom and the water column of the wells. After collection, samples were kept in a cooler and immediately transferred to the laboratory. In the laboratory, copepods were separated into two morphological groups (*Eucyclops* group and *Diacyclops* group, respectively), based on external macro-characters assessed at 12× magnification. In a preliminary survey, and on one occasion only, 25% of the specimens collected in each group was identified to species at 100× magnification, after mounting and dissection on a glass slide, to check the groupings made at 12×. The specimens required for the further respirometric trials were identified at 12× magnification from then on. Two different and highly localised species were identified: the stygoxene *E. serrulatus* occurring in the well W1 and the stygobiotic *D. belgicus* occurring in the well W2. DNA barcoding analysis was carried out on live specimens of the two species to exclude the presence of cryptic species belonging to the same genera (see also Di Lorenzo *et al.*, 2014a).

Experimental design

To ensure the same experimental conditions for both species, the specimens required for the respirometric trials were freshly collected 3 days before the beginning of each trial. In the laboratory, specimens were counted and the organisms required for each trial were acclimated for three days in the laboratory prior to testing in a commercial water: (pH: 7.4, electrical conductivity: 415 μS cm^{-1}, DOC: not detected; HCO_3^-: 301, Ca^{2+}: 48.6, Mg^{2+}: 28.2, SiO_2: 15.2, NO_3^-: 8.5, Na^+: 5.8, SO_4^{3-}: 4.1, K^+: 1, F^- <0.15, all expressed in mg L^{-1}), that was slightly different in chemical composition from ground water collected from the two wells (W1: pH: 7.6; electrical conductivity: 524 μS cm^{-1}; DOC: 1.1; HCO_3^-: 437; Ca^{2+}: 85.6, Mg^{2+}: 17.0, NO_3^-: 11.4, Na^+: 6.8, SO_4^{3-}: 7.4, K^+: 3.0, all expressed in mg L^{-1}; W2: pH: 7.6; electrical conductivity: 512 μS cm^{-1}; DOC: 1.6; HCO_3^-: 445; Ca^{2+}: 83.8, Mg^{2+}: 19.0, NO_3^-: 11.4, Na^+: 6.9, SO_4^{3-}: 7.9, K^+: 3.2, all expressed in mg L^{-1}). However, copepods seemed not to be stressed by the water change (no evident variations in movement and behaviour). The use of commercial water was necessary to ensure the same experimental conditions for both species and for further comparative studies with other species. The specimens not used in the trials were returned alive to the wells within 2 h of collection. During acclimation, copepods were deprived of food to allow for complete gut clearance. The digestive system [oesophagus (foregut), midgut, hindgut] was clearly visible under a stereomicroscope at 80× magnification. An empty gut was required to prevent the overshoot in oxygen consumption due the release of faecal pellets (Kreibich *et al.*, 2008; Köster & Paffenhöfer, 2013) during the trials. Only actively swimming individuals were selected. Trials were carried out with juveniles (copepodids C1-C4) and non-egg-carrying (non-ovigerous) adult females of both species. Due to the low abundances of the two species in each well, we were unable to collect a sufficient number of males of both species and ovigerous females of *D. belgicus*, thus the effect of sex and fecundity were not analysed.

Measurements of respiration rate

As a proxy of metabolic activity, routine metabolism according to Ikeda (1985) and Ikeda *et al.* (2001) (measured as standard respiratory rates with uncontrolled but minimum motor activity) of juveniles and non-ovigerous individuals of *D. belgicus* and *E. serrulatus* was measured in four separate trials with four replicates each. Respiration measurements were carried out between January 2013 and March 2013 and maximum of one trial per week was performed. In each trial, standard respiratory rate of copepods was measured simultaneously in five sealed glass respirometers (four replicates plus one control) each containing 2 mL of oxygen-saturated standard water. The respirometers were placed in a 15°C water bath and kept dark. Oxygen in each respirometer was measured with a PSt3 (PreSens, Regensburg, Germany) optical oxygen sensor glued to the inside wall of the respirometer and connected to a single-channel oxygen transmitter Fibox 3 (PreSens) via an optical fibre. Data were recorded using the FibSoft v.1.0 software (Loligo Systems ApS, Tjele, Denmark). Prior to measurements, sensors were calibrated in air-equilibrated water (100% saturation) and in a sodium thiocyanate saturated solution in water (0%). Pilot experiments were run to determine the number of individuals, of each ontogenetic stage of both species, that would give a measurable decrease in oxygen in the respirometers but not cause saturation to fall below 70% over 3-h incubation (Schurmann & Steffensen, 1992). Based on the pilot trials, we determined that, for respiration experiments, the chambers should contain 25–30 juveniles and 20–25 non-ovigerous females of *E. serrulatus*, and 30–35 juveniles and 25–30 non-ovigerous females of *D. belgicus*. An empty chamber was run within each trial as a control, to account for background oxygen depletion. For each replicate in a trial, the oxygen consumed in each chamber was corrected by the oxygen depletion in the control to account for chamber losses. Measurements were taken every 30 min during 3-h incubation. At the end of each trial, the respirometers were opened and copepods were counted under a stereomicroscope at 12× magnification and checked for mortality (no movement after gentle stimulation by means of a sorting needle). No deaths were observed. To obtain standard respiratory rate in μg O_2 ind^{-1} h^{-1}, the loss of oxygen in each respirometer was divided by the number of individuals in the chamber during the experiment.

Measurements of size and mass

At the end of each incubation, prosome length (L_{ind}, mm) and width (W_{ind}, mm) of each individual, for a total of 455 specimens, were measured dorsally under a stereomicroscope fitted with an eyepiece micrometer at 50× magnification. L_{ind} was measured from the anterior tip of the cephalic shield to the end of the major body articulation (leg-4-bearing somite), while W_{ind} was measured at the leg-4-bearing somite. The cumulative dry mass (DM) of the pool of individuals for each replicate in a trial was measured in pre-massed aluminium foil baskets (5 mm in diameter), using an analytical balance at 1 μg of precision. They were then dried at 60°C for 24 h, cooled for 30 min in a desiccator at room temperature and placed on the balance three times consecutively to obtain a mean dry mass. We calculated the dry mass of each individual (DM_{ind}) in the pool according to Svetlichny *et al.* (2012) measured as:

$DM_{ind} = CF\ L_{ind} * W_{ind}^2$, where CF is stage- and species-specific condition factor calculated as $CF = DM_p/L_m * W_m^2$, where DM_p is the dry mass of a pool, and L_m and W_m are the mean values of pool-specific size metrics.

Statistical analyses

To test for differences in standard respiratory rate between species and between developmental stages, we used a permutational analysis of covariance (permutational ANCOVA; Anderson, 2001), with individual dry mass as the covariate and 'species' (fixed and orthogonal) and 'developmental stage' (fixed and nested within 'species') as grouping factors. The permutational analysis was performed on the basis of a Euclidean distance similarity matrix and using square-root-transformed data, applied after performing a Levene test on the original data set. *Post hoc t*-tests were applied when appropriate to test for differences between levels within factors. These tests were performed using PRIMER v.6 and PERMANOVA + routines for PRIMER (Anderson, Gorley & Clarke, 2008).

To provide generalised relationships between the metabolic rate (standard respiratory rate) and the body dry mass at 15°C of the two species, data from metabolic experiments were fitted according to the Power law after Ikeda (1985). Standard respiratory rate (Y) was given as a function of dry body mass (X1 = mg), which was taken as an independent variable. The relationship between Y and X1 is known empirically as $Y = \alpha X1^{\beta}$ (where α and β are constants). According to Ikeda (1985), we assumed that β is independent from ambient temperature. A nonlinear regression analysis was performed to obtain α and β parameters, using the loss function (observed data - predicted data)^2, and the quasi-Newton estimation method. The leave-one-out cross-validation technique was used to estimate the model prediction capability. The model prediction capability is something different from the model fitness capability, that is the ability of the model to estimate the response for objects that do not participate in the calculated model. Predictive residual sums of squares (PRESS), sums of squares (SS) and squared cross-validated correlation coefficient parameters (Q2) were calculated. Q2 is the coefficient of determination for the adjusted model in prediction. Prediction intervals were computed. All analysis were carried out using Statistica V8 (StatSoft) and specially designed SOLVER spreadsheets using Microsoft Excel (Sparks, 2000; Di Marzio *et al.*, 2001; Kito *et al.*, 2004; WHO, 2007; Pratim *et al.*, 2009; Zar, 2010).

Two-way analyses of variance were used to test for differences in L_{ind}, W_{ind} and DW_{ind} among juveniles and non-ovigerous females and between the two species, with factor 'stage' nested in factor 'species'. The assumptions of normal distributions and equal variances were verified using Shapiro's and Bartlett's tests, respectively. Biological data were square-root-transformed if necessary. *Post hoc* Tukey tests were performed when appropriate. Bonferroni corrections were applied to all tests (whose original significance threshold was set at $P < 0.05$), leading to a threshold of $P < 0.008$. All analyses were performed by R software, version 3.01 (http://www.R_project.org).

Results

Total oxygen consumed during incubations was never more than 20% of the initial values. Respiration rate increased with body dry mass in *E. serrulatus*, but not in

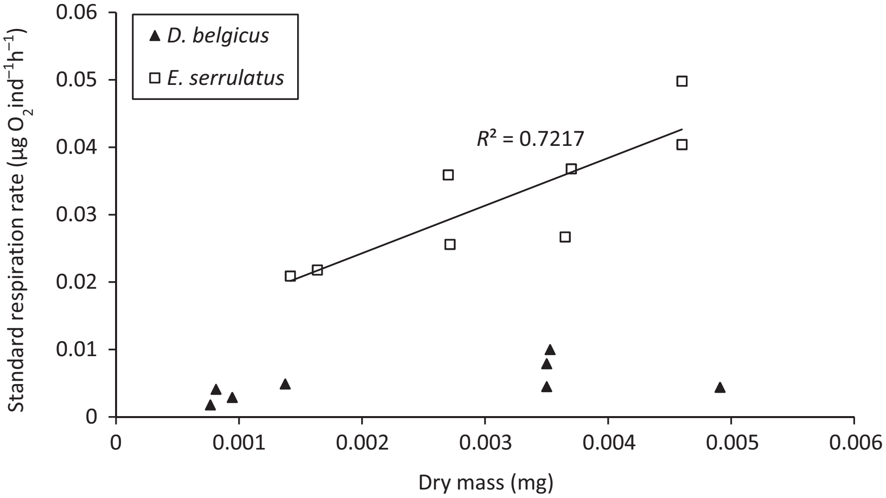

Fig. 1 Respiration rates recorded in the laboratory at standard temperature conditions of *Eucyclops serrulatus* and *Diacyclops belgicus* from Medio Valdarno alluvial aquifer (Florence, Italy). Relationship between respiration rate and body dry mass (DM) was significant in *E. serrulatus*, but not in *D. belgicus* (pseudo $F = 11.91$; $P < 0.01$ and pseudo $F = 3.02$; $P = 0.13$, respectively, permutational ANCOVA). For *E. serrulatus*, the regression line and the relative R^2 value, between respiration rates and dry mass (DM), are shown.

D. belgicus (pseudo $F_{1,8} = 11.91$; $P < 0.01$ and pseudo $F_{1,5} = 3.02$; $P = 0.13$, respectively, permutational ANCOVA; Fig. 1), and no differences were recorded between stages in the two species (pseudo $F_{2,11} = 2.63$; $P = 0.11$). The standard respiratory rate of the stygobiotic species *D. belgicus* was significantly lower than that of the epigean *E. serrulatus* at all the ontogenetic stages (pseudo $F_{1,11} = 79.97$; $P < 0.001$; Fig. 1).

Standard respiration rate (SRR) of *E. serrulatus* (cumulative data for juveniles and females) followed the power law $\mathrm{SRR} = a\mathrm{DM}^b$, where DM is dry mass (in mg), a is a constant, and b is the scaling constant or the slope in this allometric relationship (Lucas, 1996; Brey, 2010). For *E. serrulatus*, the allometric curve is showed in Fig. 2(a); the b value of 0.6011 was significant ($P < 0.01$). For the stygobiotic *D. belgicus* (cumulative data for juveniles and females), the slope did not differ from zero at $P = 0.12$ (Fig. 2b), indicating that the respiration rates did not vary with dry mass. However, by analysing the standard respiratory rate of *D. belgicus* for adults and juveniles separately, the slope for copepodids was significant for juveniles, yielding an isometric relationship with a b value of 1.012 ($P < 0.005$).

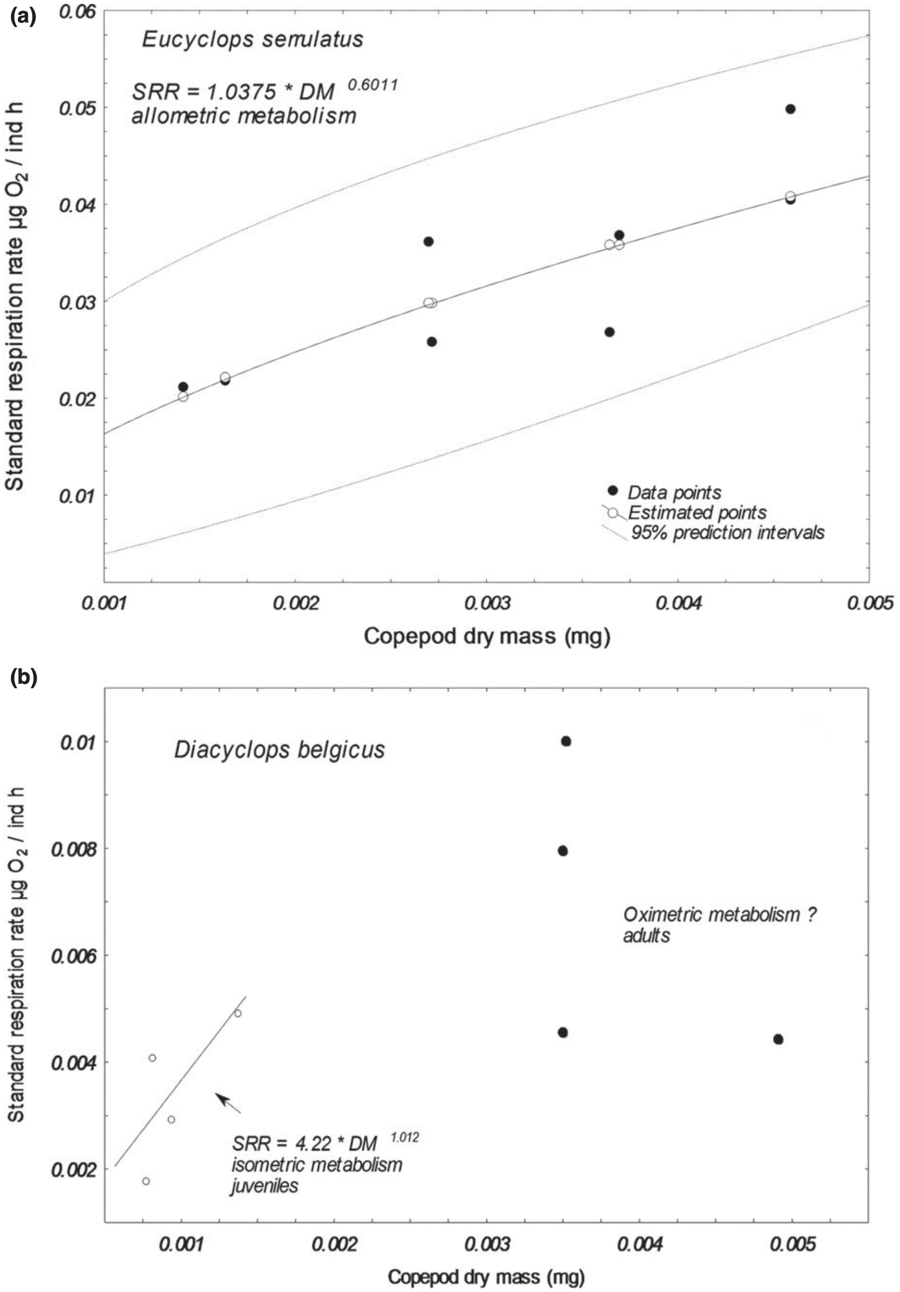

Fig. 2 Relationships between standard respiration rates (SRR) and dry mass (DM) at 15°C for the epigean *Eucyclops serrulatus* (showing 95% prediction intervals) (a) and the hypogean *Diacyclops belgicus* (b) from Medio Valdarno alluvial aquifer (Florence, Italy) fitted according to the Power law after Ikeda (1985). Relationship between respiration rate and body dry mass (DM) was significant in *E. serrulatus* and in juveniles of *D. belgicus* (white dots), but not in adults of *D. belgicus* (black dots).

L_{ind}, W_{ind} and DM_{ind} (Table 1) were significantly different between juveniles and non-ovigerous females within each species (Table 2; Fig. 3). Juveniles of *D. belgicus* were significantly different in L_{ind}, W_{ind} and DM_{ind} from those of *E. serrulatus* (Table 1; Fig. 3). Non-ovigerous females of *D. belgicus* were different in L_{ind} and W_{ind} from those of *E. serrulatus* (Table 1; Fig. 3). However, there were no significant differences in DM_{ind} of non-ovigerous females between the two species ($P = 0.56$, *post hoc* Tukey test).

Discussion

Overall, the standard respiration rate of *E. serrulatus* was higher than that of *D. belgicus*, which is consistent with the view that groundwater species generally have a lower metabolic rate to cope with low and discontinuous food supply and with unpredictable shifts between hypoxic and normoxic conditions in ground water (Culver & Poulson, 1971; Sket, 1996; Poulson, 2001; Bishop, Kakuk & Torres, 2004; Mathieu & Hervant, 2004; Simčič, Lukančič & Brancelj, 2005). Recently, Bishop & Iliffe (2012) have measured the respiration rate of the shrimp *Barbouria cubensis*, a recent cave coloniser found in both surface anchialine pools and deeper ground waters. The respiration rate of *B. cubenis* was lower than that reported for other epigean crustaceans. Similarly, Wilhelm *et al.* (2006) observed that the rate of oxygen consumption by the stygobiotic amphipod *Gammarus acherondytes* was 4.5 times lower than that of the epigean *Gammarus troglophilus*. Finally, oxygen consumption by the hypogean amphipod *Niphargus stygius* is lower than in the epigean *Gammarus fossarum* (Simčič & Brancelj, 2007). Evidence for a generally lower metabolic rate in groundwater species is thus accumulating, but a formal meta-analysis of such species comparisons has not yet been carried out.

A low metabolic rate may be advantageous for species living in oligotrophic habitats, such as deep alluvial aquifers (Henry & Danielopol, 1999; Malard & Hervant, 1999). However, this physiological adaptation puts stygobionts at risk of replacement by more metabolically active epigean taxa, whenever the amount and the frequency of organic matter increase, as happens in the event of organic pollution. Such pollution may profoundly alter the aquifer community and lead to the disappearance of native, and often strictly endemic, taxa (Sket, 1973; Malard *et al.*, 1994; Simon & Buikema, 1997; Wilhelm *et al.*, 2006). Moreover, the low metabolic rate of hypogean species may entail an inability to cope with toxicants, as suggested by Avramov, Schmidt & Griebler (2013), rendering them more sensitive to pollutants, as observed by Di Marzio *et al.* (2009), Reboleira *et al.* (2013) and Di Lorenzo *et al.* (2014a,b).

Eucyclops serrulatus showed an allometric increase in metabolic rate with body mass. In agreement with our

Table 1 Standard respiration rate (SRR) and morphological characteristics of *Diacyclops belgicus* and *Eucyclops serrulatus* at 15°C

	Diacyclops belgicus		*Eucyclops serrulatus*	
	J	NOF	J	NOF
SRR (µg O_2 ind^{-1} h^{-1})	0.0034 ± 0.0013	0.0067 ± 0.0027	0.0260 ± 0.0069	0.0384 ± 0.0095
L_m (mm)	0.2122 ± 0.1361	0.4159 ± 0.0196	0.3570 ± 0.1143	0.6637 ± 0.1333
W_m (mm)	0.1219 ± 0.0220	0.1641 ± 0.0309	0.1894 ± 0.0417	0.333 ± 0.0679
DM_{ind} (mg)	0.0010 ± 0.0010	0.0039 ± 0.0007	0.0021 ± 0.0007	0.0041 ± 0.0005

Values are represented as mean ± SD. J, C1 – C4 copepodids; NOF, non-ovigerous females and C5 copepodids; L_m, prosome length; W_m, prosome width; and DM, dry mass.

Table 2 Results of ANOVA test for differences in prosome length (L), prosome width (W) and dry mass (DM) across species and life stage. All factor species were fixed; factor life stage was nested in factor species

	L				W				DM			
Source	df	MS	*F*	*P*	df	MS	*F*	*P*	df	MS	*F*	*P*
Species – Sp	1	0.1541	258.1	<0.0001	1	1.3444	783.2	<0.0001	1	$2.011*10^{-6}$	6.066	0.0299
Stage (Sp)	2	0.1356	227.2	<0.0001	2	1.0536	306.9	<0.0001	2	$1.246*10^{-5}$	37.600	0.0001
Res	12	0.0006			12	0.7399			12	$3.310*10^{-7}$		
Total	15				15				15			

df, Degrees of freedom; MS, mean square values; *F* values; and probability levels are shown.

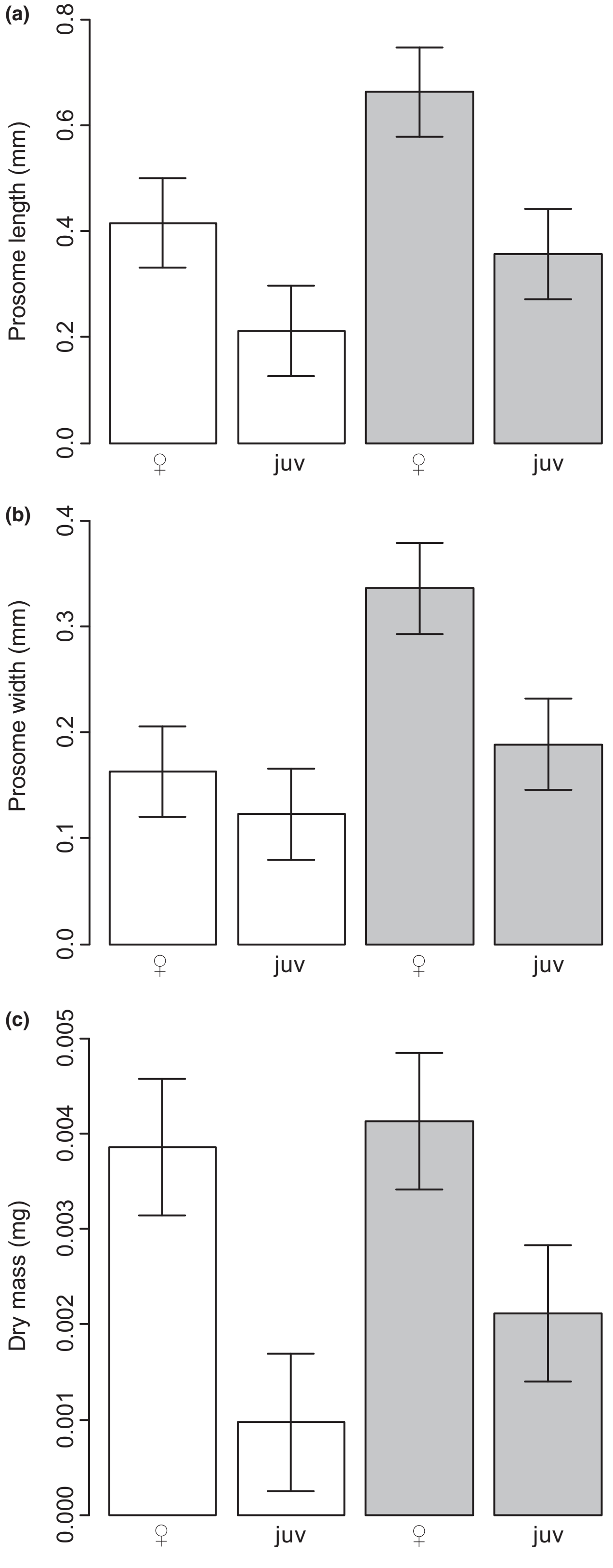

Fig. 3 Mean (±SE): (a) prosome length, (b) prosome width, (c) dry mass of non-ovigerous females (♀) and juveniles (juv) of the hypogean *Diacyclops belgicus* (white bars) and the epigean *Eucyclops serrulatus* (grey bars).

results, Wilhelm *et al.* (2006) found that oxygen consumption increased allometrically with the dry mass of the epigean crustacean amphipod *G. troglophilus*, where the slope was 0.682 for individuals collected during February and May (winter–spring seasons).

Lack of a significant positive slope in the regression of oxygen consumption rate on body dry mass precluded us from providing a generalised metabolic model for adults of *D. belgicus*. Similarly, Wilhelm *et al.* (2006) found that standard respiratory rate of the stygobiotic *Gammarus acherondytes* did not increase with body mass. They pointed out that the lack of a significant positive slope in the regression between the oxygen consumption rates on body mass might be a novel result for cave amphipods (Wilhelm *et al.*, 2006). A constant low metabolism, which we might call a potentially 'oximetric' relationship [using as prefix the word οχι (oxi) from the Greek that means 'not', or simply 'ametric'], could be a physiological adaptation in such a food-limited environment. The hypothesis that a similar pattern might apply to stygobiotic taxa, at least to hypogean crustaceans, should be considered and further data are needed to test this possibility fully.

The standard respiratory rate of juvenile *D. belgicus* increased isometrically (the rate increases proportionately with body mass during growth). Peters (1983) found that routine metabolic rate also scales isometrically in larval fish ($DM^{1.00}$), whereas in juvenile and adult fish, it scales allometrically with mass ($DM^{0.8}$). This suggests that, as organisms pass from one life stage to the next, the metabolic exponent decreases as the overall body size of the organism increases (Giguère, Côtè & St-Pierre, 1988). Such a relationship has been observed both inter- and intraspecifically (Zeuthen, 1953; Johansen, Brix & Lykkeboe, 1982). With isometric metabolism, stygobiotic copepod larvae (e.g. copepodids) could maximise the exploitation of transient food patches, which can occur in alluvial aquifers. Although temperature and other physicochemical variables show less pronounced changes in deep and isolated alluvial aquifers, food availability may be very restricted in terms of quantity and timing (Brunke & Gonser, 1997; Foulquier *et al.*, 2010, 2011). Under this scenario, juveniles of stygobiotic taxa can develop faster when food is available, thus quickly reaching sexual maturity.

The dry mass of non-ovigerous females of *E. serrulatus* was not significantly different from that of *D. belgicus*, although on average non-ovigerous females of *E. serrulatus* were 1.6 times longer and 2.02 times wider than those of *D. belgicus*. However, hypogean species are known to have larger body stores than their epigean relatives (Hervant & Renault, 2002). Groundwater species display a remarkable resistance to long-term starvation (Poulson, 2001; Hervant & Renault, 2002), by means of fat reserves, which is adaptive in environments where fasting periods are frequent (Hervant & Renault, 2002). However, food quality may also be low in ground water. Food quantity and quality are known to affect survival, development and reproductive rates of *E. serrulatus*¸ which enhances its reproductive rates on a rich and diversified diet (McLaren, 1963; Hopp & Maier, 2005; Nandini & Sarma, 2007). In the ground water, microbial biofilm production, which is the main source of food, is fuelled by dissolved organic matter (DOC) carried by infiltrating surface water. However, in alluvial aquifers, DOC is often scarce, amounting to about 1 mg L^{-1} or less (Brunke & Gonser, 1997; Foulquier *et al*., 2011). The low food availability (DOC: 1.1 mg L^{-1}) and the permanent darkness of the well where we found *E. serrulatus* might play a role in affecting the biomass of this species.

Beginning with these data, further studies should address the variation in metabolic rate of both *E. serrulatus* and *D. belgicus* under stress (e.g. temperature increase, groundwater pollution) to assess whether defence and repair may increase metabolic costs. The relevance of assessing the physiological response to toxicants of stygobionts may serve to gauge their potential as early warning systems in routine groundwater monitoring and to demonstrate their role as indicators of groundwater quality.

Acknowledgments

We thank Alessio Iannucci and Anna Aurora Dedonno (University of Florence) for assistance in field and laboratory activities. We are indebted to two anonymous reviewers and to Prof. Alan Hildrew, who provided comments that improved the manuscript. This work was supported by project RIPARI (Regione Toscana, POR-FERS 2007-2013) and by Italia MIUR funds (ex 60%).

References

Anderson M.J. (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, **26**, 32–46.

Anderson M.J., Gorley R.N. & Clarke K.R. (2008) *PERMANOVA + for PRIMER: guide to software and statistical methods*. PRIMER–E Ltd, Plymouth.

Avramov M., Schmidt S.I. & Griebler C. (2013) A new bioassay for the ecotoxicological testing of VOCs on groundwater invertebrates and the effects of toluene on *Niphargus inopinatus*. *Aquatic Toxicology*, **130–131**, 1–8.

Balian E.V., Segres H., Lévêque C. & Martens K. (2008) The freshwater animal diversity assessment: an overview of the results. *Hydrobiologia*, **595**, 627–637.

Bishop R.E. & Iliffe T.M. (2012) Ecological physiology of the anchialine shrimp *Barbouria cubensis*: a comparison of epigean and hypogean populations. *Marine Biodiversity*, **42**, 303–310.

Bishop R.E., Kakuk B. & Torres J.J. (2004) Life in the hypoxic and anoxic zones: metabolism and proximate composition of Caribbean troglobitic crustaceans, with observations on the water chemistry of anchialine caves. *Journal of Crustacean Biology*, **24**, 379–393.

Bjornberg M.H.G.C. & Por F.D. (1986) Comparative notes on the development of two species of *Bryocyclops* (Copepoda, Cyclopoida). In: *Proceedings of the Second International Conference on Copepoda* (Eds G. Schriever , H.K. Schminke & C.-T. Shih), *Syllogeus* **58**, 229–231.

Boxshall G.A. & Defaye D. (2008) Global diversity of copepods (Crustacea: Copepoda) in freshwater. *Hydrobiologia*, **595**, 195–207.

Brancelj A. & Dumont H. (2007) A review of the diversity, adaptations and groundwater colonization pathways in Cladocera and Calanoida (Crustacea), two rare and contrasting groups of stygobionts. *Archiv für Hydrobiologie*, **168**, 3–17.

Brey T. (2010) An empirical model for estimating aquatic invertebrate respiration. *Methods in Ecology and Evolution*, **1**, 92–101.

Brunke M. & Gonser T. (1997) The ecological significance of exchange process between rivers and groundwater. *Freshwater Biology*, **37**, 1–33.

Culver D.C. (1982) *Cave Life Evolution and Ecology*. Harvard University Press, Cambridge, Massachusetts, USA.

Culver D.C. & Poulson T.L. (1971) Oxygen consumption and activity in closely related amphipod populations from cave and surface habitats. *American Midland Naturalist*, **85**, 74–84.

Cvetkov L. (1968) Un filet phreatobiologique. *Bulletin de l'Institut de Zoologie et Musée, Académie Bulgare des Sciences*, **27**, 215–218.

Di Lorenzo T., Cifoni M., Lombardo P., Fiasca B. & Galassi D.M.P. (2014b). Threshold value for groundwater quality in the EU may not protect groundwater fauna: evidence from an alluvial aquifer in Italy. *Hydrobiologia*, doi: 10.1007/s10750-014-2018-y.

Di Lorenzo T., Di Marzio W.D., Sáenz M.E., Baratti M., Dedonno A.A., Iannucci A. *et al*. (2014a) Sensitivity of hypogean and epigean freshwater copepods to agricultural pollutants. *Environmental Science and Pollution Research*, **21**, 4643–4655.

Di Lorenzo T., Stoch F. & Galassi D.M.P. (2013) Incorporating the hyporheic zone within the river discontinuum: longitudinal patterns of subsurface copepod assemblages in an Alpine stream. *Limnologica*, **43**, 288–296.

Di Marzio W.D., Castaldo D., Pantani C., Di Cioccio A., Di Lorenzo T., Sáenz M.E. *et al.* (2009) Relative sensitivity of hyporheic copepods to chemicals. *Bulletin of Environmental Contamination and Toxicology*, **82**, 488–491.

Di Marzio W.D., Galassi S., Todeschini R. & Consolaro F. (2001) Traditional versus WHIM molecular descriptors in QSAR approaches applied to fish toxicity studies. *Chemosphere*, **44**, 401–406.

Foulquier A., Malard F., Mermillod-Blondin F., Montuelle B., Dolédec S., Volat B. *et al.* (2011) Surface water linkages regulate trophic interactions in a groundwater food web. *Ecosystems*, **14**, 1339–1353.

Foulquier A., Simon L., Gilbert F., Fourel F., Malard F. & Mermillod-Blondin F. (2010) Relative influences of DOC flux and subterranean fauna on microbial abundance and activity in aquifer sediments: new insights from 13C-tracer experiments. *Freshwater Biology*, **55**, 1560–1576.

Galassi D.M.P. (2001) Groundwater copepods: diversity patterns over ecological and evolutionary scales. *Hydrobiologia*, **454–453**, 227–253.

Galassi D.M.P., Huys R. & Reid J.W. (2009) Diversity, ecology and evolution of groundwater copepods. *Freshwater Biology*, **54**, 691–708.

Gibert J., Vervier P.H., Malard F., Laurent R. & Reygrobellet J.L. (1994) Dynamics of communities and ecology of karst ecosystems: example of three karsts in Eastern and Southern France. In: *Groundwater Ecology* (Eds J. Gibert, D.L. Danielopol & J.A. Stanford), pp. 425–450. Academic Press, New York.

Giguère L.A., Côtè B. & St-Pierre J.F. (1988) Metabolic rates scale isometrically in larval fishes. *Marine Ecology Progress Series*, **50**, 13–19.

Glatzel T. (1990) On the biology of *Parastenocaris phyllura* Kiefer (Copepoda, Harpacticoida). *Stygologia*, **5**, 131–136.

Hatton T. & Evans R. (1998) *Dependence of ecosystems on groundwater and its significance to Australia*. CSIRO (Land and Water), Canberra.

Henry K.S. & Danielopol D.L. (1999) Oxygen dependent habitat selection by *Gammarus roeseli* Gervais (Crustacea, Amphipoda): experimental evidence. *Hydrobiologia*, **390**, 51–60.

Hervant F., Mathieu J., Barré H., Simon K. & Pinon C. (1998) Comparative study on the behavioral, ventilator, and respiratory responses of hypogean and epigean crustaceans to long-term starvation and subsequent feeding. *Comparative Biochemistry and Physiology*, **118A**, 1277–1283.

Hervant F., Mathieu J. & Messana G. (1997) Oxygen consumption and ventilation in declining oxygen tension and posthypoxic recovery in epigean and hypogean crustaceans. *Journal of Crustacean Biology*, **18**, 717–727.

Hervant F. & Renault D. (2002) Long-term fasting and realimentation in hypogean and epigean isopods: a proposed adaptive strategy for groundwater organisms. *Journal of Experimental Biology*, **205**, 2079–2087.

Hopp U. & Maier G. (2005) Survival and development of five species of cyclopoid copepods in relation to food supply: experiments with algal food in a flow-through system. *Freshwater Biology*, **50**, 1454–1463.

Hüppop K. (1985) The role of metabolism in the evolution of cave animals. *National Speleological Society Bulletin*, **47**, 13–146.

Ikeda T. (1985) Metabolic rates of epipelagic marine zooplankton as a function of body mass and temperature. *Marine Biology*, **85**, 1–11.

Ikeda T., Kanno Y., Ozaki K. & Shinada A. (2001) Metabolic rates of epipelagic marine copepods as a function of body mass and temperature. *Marine Biology*, **139**, 587–596.

Johansen K., Brix O. & Lykkeboe G. (1982) Blood gas transport in the cephalopod, *Sepia officinalis*. *Journal of Experimental Biology*, **99**, 331–338.

Kito S., Satsuma A., Ishikura T., Niwa M., Murakami Y. & Hattori T. (2004) Application of neural network to estimation of catalyst deactivation in methanol conversion. *Catalysis Today*, **97**, 41–47.

Köster M. & Paffenhöfer G.-A. (2013) Oxygen consumption of fecal pellets of doliolids (Tunicata, Thaliacea) and planktonic copepods (Crustacea, Copepoda). *Journal of Plankton Research*, **35**, 323–336.

Kreibich T., Saborowski R., Hagen W. & Niehoff B. (2008) Short-term variation of nutritive and metabolic parameters in *Temora longicornis* females (Crustacea, Copepoda) as a response to diet shift and starvation. *Helgoland Marine Research*, **62**, 241–249.

Lescher-Moutoué F. (1973) Sur la biologie et l'écologie des copépodes cyclopides hypogés (crustacés). *Annales de Spéléologie*, **28**, 429–502.

Lescher-Moutoué F. (1974) The number of naupliar instars in *Eucyclops serrulatus*. *Annales de Limnologie*, **10**, 263–274.

Lucas A. (1996) *Bioenergetics of aquatic animals*. Taylor & Francis, London.

Malard F., Crague G., Turquin M.-J. & Bouvet Y. (1994) Monitoring karst groundwater: the practical aspect of subterranean ecology. *Theoretical and Applied Karstology*, **7**, 115–126.

Malard F. & Hervant F. (1999) Oxygen supply and the adaptations of animals in groundwater. *Freshwater Biology*, **41**, 1–30.

Malard F., Mathieu J., Reygrobellet J.-L. & Lafont M. (1996) Biomonitoring groundwater contamination: application to a karst area in southern France. *Aquatic Sciences*, **58**, 158–184.

Malard F., Plénet S. & Gibert J. (1996) The use of invertebrates in groundwater monitoring: a rising research field. *Ground Water Monitoring and Remediation*, **16**, 103–116.

Mathieu J. & Hervant F. (2004) Adaptation: physiological. In: *Encyclopedia of caves and karst Sciences* (Ed J. Gunn), pp. 11. Fitzroy Dearborn, an inprint of Taylor and Francis Group, New York-London.

McLaren I.A. (1963) Effects of temperature on growth of zooplankton and the adaptive value of vertical migration. *Journal of the Fisheries Research Board of Canada*, **20**, 685–727.

Nandini S. & Sarma S.S.S. (2007) Effect of algal and animal diets on life history of the freshwater copepod *Eucyclops serrulatus* (Fischer, 1851). *Aquatic Ecology*, **41**, 75–84.

Peters R.H. (1983) *The ecological implications of body size*. Cambridge University Press, New York.

Poulson T.L. (2001) Morphological and physiological correlates of evolutionary reduction of metabolic rate among amblyopsid cavefishes. *Environmental Biology of Fishes*, **62**, 239–249.

Pratim R.P., Paul S., Mitra I. & Kunal R. (2009) On two novel parameters for validation of predictive QSAR Models. *Molecules*, **14**, 1660–1701.

Reboleira A.S., Abrantes N., Oromì P. & Gonçalves F. (2013) Acute toxicity of copper sulfate and potassium dichromate on stygobiont *Proasellus*: general aspects of groundwater ecotoxicological and future perspective. *Water, Air, & Soil Pollution*, **224**, 15–50.

Rouch R. (1968) Contribution à la connaissance des harpacticides hypogés (Crustacés-Copépodes). *Annales de Spéléologie*, **23**, 5–167.

Schurmann H. & Steffensen J.F. (1992) Lethal oxygen levels at different temperatures and the preferred temperature during hypoxia of the Atlantic cod, *Gadus morhua* L. *Journal of Fish Biology*, **41**, 927–934.

Simčič T. & Brancelj A. (2007) The effect of light on oxygen consumption in two amphipod crustaceans–the hypogean *Niphargus stygius* and the epigean *Gammarus fossarum*. *Marine and Freshwater Behaviour and Physiology*, **40**, 141–150.

Simčič T., Lukančič S. & Brancelj A. (2005) Comparative study of electron transport system activity and oxygen consumption of amphipods from caves and surface habitats. *Freshwater Biology*, **50**, 494–501.

Simon K.S. & Buikema A.L. Jr (1997) Effects of organic pollution on an Appalachian cave: changes in macro-invertebrate populations and food supplies. *America Midland Naturalist*, **138**, 387–401.

Sket B. (1973) Gegenseitige beeinflussung der wasserpollution und das höhlenmilieus. In: *Procedings of the International Congress of Speleology*, 6th, Vol. 5. pp. 253–262.

Sket B. (1996). Biotic diversity in hypogean habitats in Slovenia and its cultural importance. In: *International Biodiversity Seminar UNESCO XIV* (Eds A. Cimerman & N. Gunde-Cimerman), pp. 59–74. Meeting, Ljubljana.

Sparks T. (2000) *Statistics in Ecotoxicology*. John Wiley & Sons, New York, NY, USA.

Svetlichny L.S., Khanaychenko A., Hubareva E. & Aganesova L. (2012) Partitioning of respiratory energy and environmental tolerance in the copepods *Calanipeda aquaedulcis* and *Arctodiaptomus salinus*. *Estuarine, Coastal and Shelf Science*, **114**, 199–207.

WHO (2007) Guidance document on the validation of (quantitative) structure-activity relationships [(Q)SAR] models. WHO, ENV/JM/MONO, pp. 2–135.

Wilhelm F.M., Taylor S.J. & Adams G.L. (2006) Comparison of routine metabolic rates of the stygobite, *Gammarus acherondytes* (Amphipoda: Gammaridae) and the stygophile, *Gammarus troglophilus*. *Freshwater Biology*, **51**, 1162–1174.

Zar J.H. (2010) *Biostatistical Analysis*. Pearson, Boston, MA, USA.

Zeuthen E. (1953) Oxygen uptake as related to body size in organisms. *Quarterly Review of Biology*, **28**, 1–12.

(Manuscript accepted 22 October 2014)