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Trophic ecology of groundwater species reveals specialization in a low-productivity environment

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Summary

1. Identifying feeding strategies at lower bounds of habitat productivity is fundamental to understand the relationship between energy availability and trophic specialization. Low productivity is expected to severely constrain trophic specialization because organisms may no longer be able to fulfil their energy requirements by feeding on a reduced set of resources. However, species living in low-productivity habitats often exhibit particular biological traits such as low metabolic rates and high food-finding abilities, which may release constraints on trophic specialization.

2. In the present study, we used carbon (C) and nitrogen (N) stable isotopes to measure the degree of trophic specialization in two species of isopods (*Proasellus valdensis* and *Proasellus cavaticus*) living in groundwater, one of the most energy-limited environments on earth. Fundamental specialization was obtained from a $^{13}C/^{15}N$-labelling experiment in the laboratory: we measured separately the carbon and nitrogen assimilation rates of the two species across the three food sources encountered in their natural cave habitats (fine and coarse particulate organic matter and sedimentary biofilm). Then, for each species, we tested for variation in diet composition among individuals and populations by quantifying the relative contribution of the three food sources to the diet of multiple individuals within 5 cave populations.

3. The labelling experiment showed that both species assimilated about 10 times more carbon and at least 4 times more nitrogen from sedimentary biofilm than from both forms of particulate organic matter. Field samplings showed that sedimentary biofilm made up, on average, 83% of the diet of isopods. Moreover, we found almost no variation in diet among individuals of a cave population as well as among cave populations within species.

4. This study provides the first evidence of a high degree of trophic specialization in a low-productivity cave environment. Both species exhibited a strong fundamental specialization on sedimentary biofilm and most probably fed selectively on this food source in their natural environment. Our findings challenge the prediction that species would adopt generalist feeding strategies at lower bounds of habitat productivity.

Key-words: ^{13}C, ^{15}N, C and N assimilation, diet, fundamental specialization, obligate-cave isopods, particulate organic matter, *Proasellus*, sedimentary biofilm, stable isotopes

Introduction

The identification of feeding strategies is fundamental to the understanding of many ecological processes such as the flow of energy within food webs, the spatial distribution of species and the evolution of competitive interactions in communities (Bolnick *et al.* 2003; Holt 2009; Poisot *et al.* 2011a). Theory distinguishes between generalist and specialist strategies, even though organisms employ a range of feeding strategies between these two extremes (Levins 1968; Futuyma & Moreno 1988; Devictor *et al.* 2010). The degree of trophic specialization of a species has been often

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related to the ecological niche volume occupied by this species (see Devictor *et al*. 2010 for a review). However, niche-based concepts cannot always be applied to understand trophic specialization because performance is needed to interpret the distinctiveness and relative importance of trophic resources for a consumer (Sherry 1990; Irschick, Dyer & Sherry 2005). Indeed, fundamental trophic specialization is defined as the intrinsic property of an organism to maximize its performance under an increasingly narrow subset of possible resources, this degree of fundamental specialization being the outcome of evolutionary mechanisms (Whitfield *et al*. 2009; Devictor *et al*. 2010; Poisot *et al*. 2011a). In parallel, realized specialization integrates the effect of biotic interactions, chance and history on fundamental specialization. Specialization varies across biological organization levels as generalist populations may be composed of many diet-specialized individuals (Roughgarden 1974; Bolnick *et al*. 2003). Acknowledging interindividual and interpopulation variations in specialization is important because these two variation components can have contrasted contributions to the total number of resources used by a species (e.g. Fox & Morrow 1981; Semmens *et al*. 2009).

Species position on the generalist–specialist continuum has been predicted to vary with habitat productivity (Futuyma & Moreno 1988; Poisot *et al*. 2011a,b). The degree of trophic specialization is expected to decrease in habitats of low productivity because organisms may no longer be able to fulfil their energy requirements by feeding on a reduced set of resources (Sherry 1990; Futuyma 1998). However, this prediction of a low degree of specialization in low-productivity habitats has hardly been tested, because of the inherent difficulties to access and study these extreme environments.

In this perspective, obligate-groundwater species offer useful case studies for exploring trophic specialization at lower bounds of habitat productivity because low food availability makes most groundwater environments among the most energy-limited environments on earth (Venarsky *et al*. 2014). There is no photosynthetic primary production in groundwater and a substantial fraction of dissolved and particulate organic matter produced by the surface environment is not available to groundwater food webs because it is intercepted in the mineral soil and the vadose zone (Pabich, Valiela & Hemond 2001). The annual flux of dissolved organic carbon (DOC) in the soil organic horizon ranges from 100 to 400 kg C ha^{-1} $year^{-1}$ (Michalzik *et al*. 2001), whereas that reaching groundwater is typically below 5 kg C ha^{-1} $year^{-1}$ (Datry, Malard & Gibert 2005). Thus, feeding strategies in groundwater are thought to be highly constrained by the scarcity of food sources (Culver 1985; Gibert & Deharveng 2002; Huntsman *et al*. 2011a). From an ecological perspective, the generally held view is that subterranean animals have to feed opportunistically on a wide range of available food sources to satisfy their energy requirements (Racovitza 1950). From an evolutionary perspective, Gibert & Deharveng (2002) suggested that the evolution of subterranean species traits was directed towards 'improving food detection, increasing starvation resistance and broadening the range of food resources, rather than specializing the diet to a particular food supply'. However, the improvement in food-finding ability may itself favour fundamental specialization because it is often directed towards the detection of a particular food source at the expense of the others (Culver 1994; Whitfield *et al*. 2009). Likewise, low metabolic rates of groundwater species (Hüppop 2000; Poulson 2001; Hervant & Renault 2002) may release the constraints on specialization in this low-productivity environment.

Determining the degree of trophic specialization in low-productivity groundwater habitats faced a number of methodological challenges. First, reproductive output and/or survival may not be appropriate to assess fitness advantages of distinct food sources in controlled experiment because of the extremely low fecundity and high resistance to starvation of groundwater species (Henry 1976; Hervant, Mathieu & Barré 1999; Hüppop 2000; Hervant & Renault 2002). Therefore, the fundamental trophic specialization of groundwater organisms has never been assessed. Second, conventional methods of diet analysis such as gut content analysis did not provide time-integrated estimates of the diet of groundwater species. Third, measuring realized specialization by comparing the distribution of resource use and resource availability is riddled with uncertainties because of the difficulty to estimate the relative abundance of different food sources in the groundwater environment.

To overcome these challenges, we provide the first stable isotope analysis of trophic specialization in the groundwater environment. The fundamental trophic specialization of two isopod groundwater species was assessed in the laboratory by measuring separately the assimilation rates of three food sources labelled with ^{13}C and ^{15}N, assimilation serving as a proxy of performance (Lynch 1989; O'Brien *et al*. 2008). Here, measured assimilation rates represented the outcome of both ingestion rate and digestibility for each food source. Then, the diets of the two species in their natural cave habitats were determined by analysing the stable isotope signatures of individuals and their food sources with a Bayesian mixing model (MixSIR; Moore & Semmens 2008). For each species, diet analysis was performed for multiple individuals within 5 populations colonizing distinct caves in order to determine variation in diet composition among individuals and populations of the same species. Under the hypothesis of a trophic specialization of groundwater species, we expected that (i) there would be substantial differences in assimilation rate between food sources; (ii) those food sources that are more easily assimilated in controlled experiments would contribute more to the diet of cave populations; and (iii) all individuals within a specialist species would feed on the same reduced subset of the resources, indicating that there is little difference in diet composition among individuals within cave population as well as among cave populations within species.

Materials and methods

BIOLOGICAL MATERIAL

The present study focused on two obligate-groundwater asellid isopods, *Proasellus valdensis* (Chappuis, 1948) (Fig. 1) and *Proasellus cavaticus* (Leydig, 1871). Five cave populations of each taxon were selected in the Jura and pre-Alps mountains (France) (Table 1). Phylogenetic studies confirmed that populations of *P. valdensis* and *P. cavaticus* belonged to two distinct monophyletic taxa, the five *P. valdensis* populations belonging to the same species, while populations of *P. cavaticus* represented a complex of sibling species (Eme *et al.* 2013, 2014; Morvan *et al.* 2013). For the sake of simplicity, both monophyletic taxa are referred to as species in this article. Both species are detritivores like their surface water relatives *Proasellus meridianus* and *Proasellus coxalis* (Basset & Rossi 1987; Costantini & Rossi 1998; Zimmer *et al.* 2005; Mondy *et al.* 2014), but they complete their entire life cycle exclusively in groundwater. In the laboratory, *P. valdensis* and *P. cavaticus* can apparently be fed with a variety of food sources including dead leaves, woody debris and clayey mud (Henry 1976; Mermillod-Blondin *et al.* 2013), although no study determined on which food source these species performed best. Given this uncertainty, we used the commonly accepted procedure which consists in discriminating between broad food compartments (Simon, Benfield & Macko 2003 or Leberfinger, Bohman & Herrmann 2011). For the cave environment, these compartments correspond to coarse particulate organic matter (CPOM; 1 mm < particle size <6 mm), fine particulate organic matter (FPOM; particle size <1 mm), and microbial biofilm associated to the sediment, to which we referred as sedimentary biofilm. Sampled caves differed widely in elevation, temperature and dominant land use type in the catchment (Fig. 1, Table 1). Streams in the caves also varied in their distance to the soil surface, but none of them were directly connected to the surface by large openings (e.g. sinkholes), which might have resulted in massive inputs of CPOM (leaves and woods) from terrestrial vegetation. The organic carbon content (OC) of cave stream sediment averaged 0·6 ± 0·2 mg C g^{-1} sediment dry weight (n = 10 caves, Table 1), which is one to two orders of magnitude lower than values commonly reported for surface streams (Romani *et al.* 1998; Battin & Sengschmitt 1999; Baker, Valett & Dahm 2000).

FUNDAMENTAL SPECIALIZATION (LABORATORY EXPERIMENT)

In the laboratory, we performed a 60-day long feeding experiment to measure separately the assimilation rate of *P. valdensis* and *P. cavaticus* across the three food sources (FPOM, CPOM and sedimentary biofilm) labelled with ^{13}C and ^{15}N. Individuals were collected delicately from stones using a paintbrush at Les Foules cave (*P. valdensis*) and Baume la Fraîte cave (*P. cavaticus*) and brought back to the laboratory within 3 h of capture in aerated and refrigerated plastic containers filled with water from the caves. These were the only two caves where a sufficient number of individuals (*c.* 150) could be collected alive for the need of the experiment without affecting the cave populations.

Sedimentary biofilm (100 μm < particle size < 1000 μm) was labelled in ^{13}C and ^{15}N using cave sediments incubated in the dark at 10 ± 0·5°C during 1 month in a chemically controlled water (96 mg L^{-1} $NaHCO_3$, 39·4 mg L^{-1} $CaSO_4.2H_2O$, 60 mg L^{-1} $MgSO_4.7H_2O$, 4 mg L^{-1} KCl, 10 mg L^{-1} KNO_3 and 25 mg L^{-1} CH_3CO_2Na). Dissolved acetate [CH_3CO_2Na] was enriched with 1% ^{13}C-acetate ($^{13}C_2H_4O_2$, 99 atom%; ISOTEC, Sigma-Aldrich) and nitrate was enriched with 0·18% ^{15}N potassium nitrate ($K^{15}NO_3$; Cambridge Isotopes Laboratories) to achieve an initial ^{13}C content of 1·352 ± 0·061 atom% and ^{15}N of 0·431 ± 0·012 atom% (means ± standard deviations, n = 4). The CPOM and

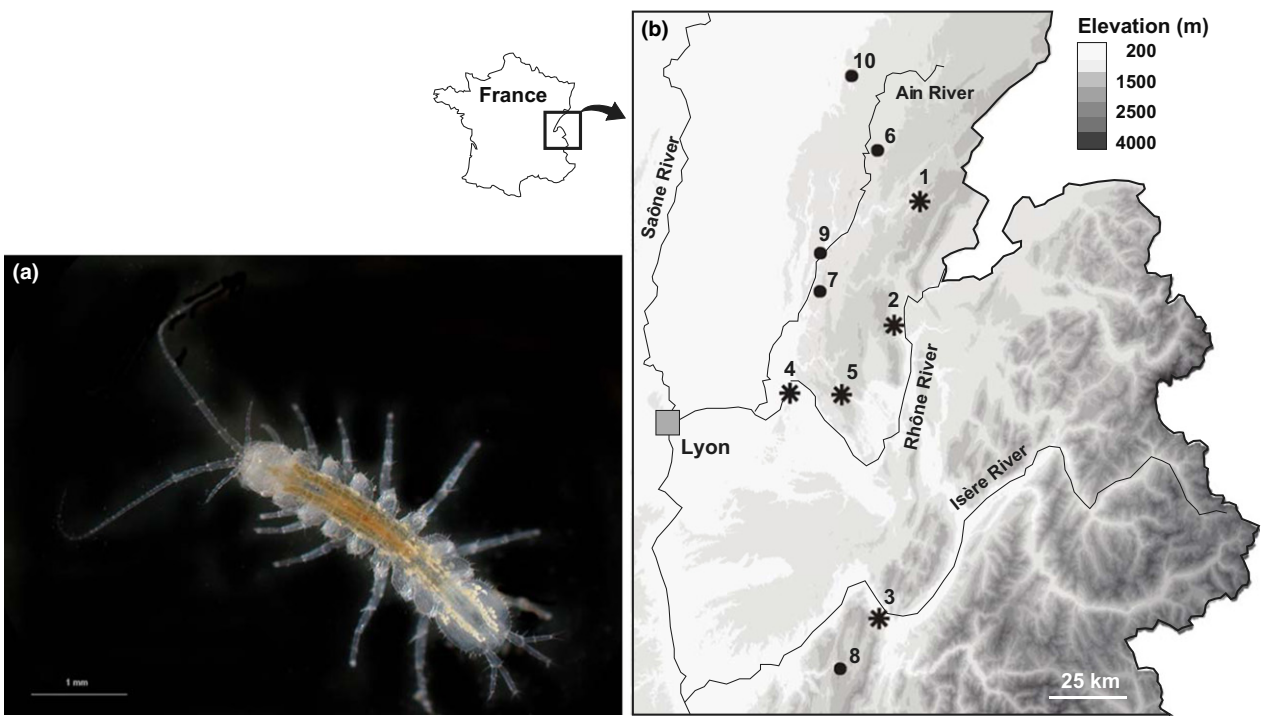

Fig. 1. (a) Photography of *Proasellus valdensis* (Chappuis, 1948) (scale bar represents 1 mm). Courtesy of Claire Morvan. (b) Locations of cave populations for *P. valdensis* (asterisks) and *P. cavaticus* (filled circles) in the French Jura and pre-Alps Mountains. Cave numbers as in Table 1.

Table 1. Location and characteristics of sampled caves

Species	Cave	*N*°	Latitude (decimal degree)	Longitude (decimal degree)	Altitude (m a.s.l.)	OC content of sediment (mg C g^{-1} DW)	Mean annual temperature (°C)	Distance from soil surface (m)	Dominant land use above cave
P. valdensis	Les Foules	1	46·37667	5·89583	683	0·54	9·3	350	Mixed forest/natural grassland
	Huguenots bis	2	46·03194	5·76917	540	0·59	10·1	10	Mixed forest
	Cuves de Sassenage	3	45·20861	5·65861	330	0·50	11·8	290	Coniferous forest
	Grotte de la Balme	4	45·85222	5·33917	225	0·48	11·6	100	Natural grassland
	Falconette	5	45·84215	5·54655	475	0·34	11·2	120	Broad-leaved forest
P. cavaticus	Baume la Fraîte	6	46·52500	5·73389	525	0·79	9·9	30	Arable land
	Moulin de Cramans	7	46·13556	5·47611	590	0·80	9·9	20	Broad-leaved forest
	Grotte Roche	8	45·07167	5·49750	790	0·54	9·8	200	Coniferous forest
	Corveissiat	9	46·24290	5·48355	431	0·57	10·5	80	Discontinuous urban
	Borne aux Cassots	10	46·73667	5·64222	290	1·08	11	120	Broad-leaved forest/ meadows

DW, dry weight; OC, organic carbon.

FPOM were derived from alder leaves. Four alders (*Alnus glutinosa*) were grown during 15 days at the National Institute of Agronomic Research (INRA, Nancy, France) in a controlled climatic chamber with $^{13}CO_2$ and watered with an ammonium nitrate solution enriched in ^{15}N, following the protocol of Pellicer *et al.* (2000). Afterwards, fresh leaves were collected from the alders and dried at 30°C during 1 week in an oven. Values of initial isotopic enrichment in CPOM were found to vary widely among leaves (^{13}C content ranged from 1·247 to 2·902 atom%, and ^{15}N content from 0·402 to 5·06 atom%, $n = 9$ leaves). Thirty dry leaves were ground using a mortar and a pestle, sieved (<1 mm) and homogenized to obtain samples of leaf fragments, which were used as FPOM food source. Isotopic enrichment was homogeneous across FPOM samples ($^{13}C = 2·413 \pm 0·09$ atom% and $^{15}N = 3·168 \pm 0·08$ atom%, means ± standard deviations, $n = 3$). Nine dry leaves were rewetted and then cut into 18-mm-diameter discs, which were used as CPOM food source. During disc preparation, main leaf veins were removed to avoid biases in assimilation rates due to a differential enrichment of vascular tissues. CPOM and FPOM were incubated in river water at 10°C during 1 month to allow their colonization by hyphomycetes (Suberkropp & Chauvet 1995).

Before starting the feeding experiment, 90 individuals per species were starved for 15 days to homogenize physiological conditions among individuals. Then, 9 individuals of each species were collected at day 0 to determine their initial isotopic signature. For each [^{13}C ^{15}N]-labelled food source, 9 groups of 3 individuals were randomly placed in polypropylene beakers with autoclaved gravel as inert substrate. The 54 beakers (3 food sources × 2 species × 9 groups) were placed in a 100-L glass tank filled with chemically controlled water (96 mg L^{-1} $NaHCO_3$, 39·4 mg L^{-1} $CaSO_4.2H_2O$, 60 mg L^{-1} $MgSO_4.7H_2O$ and 4 mg L^{-1} KCl; pH = 7·5; US EPA 1991). They were covered with a 500-µm-mesh net to prevent individual escape, and the tank was continuously aerated to maintain concentration of dissolved oxygen between 9·5 and 10·5 mg L^{-1}. The experiment was conducted under constant darkness and water temperature was set at 10°C ± 0·5 (the overall optimal temperature of these two species, Mermillod-Blondin *et al.* 2013; Eme *et al.* 2014) using a TECO® TC20 water chiller (Ravenna, Italy). Animals were fed *ad libitum* with one of the 3 labelled food sources, and food was renewed every 15 days in order to avoid food limitation. As isotopic signatures were highly variable among leaves, we assigned CPOM discs of the same leaf to each beaker for the whole experiment. Survival was monitored daily, and dead individuals were removed to avoid any microbial development on their bodies and subsequent feeding of the remaining individuals on non-enriched food. The experiment was run for 60 days, with samplings at days 15, 30 and 60. At each sampling date, 3 beakers were removed for each food source treatment and for each species. Individuals and food sources were frozen and freeze-dried for the determination of carbon (C) and nitrogen (N) isotope ratios (see below).

DIET DETERMINATION OF CAVE POPULATIONS

For each of the two *Proasellus* species, we quantified the relative contribution of CPOM, FPOM and sedimentary biofilm to the diet of 5 cave populations (Table 1). In each cave, 5–12 individuals were collected by sight or using a Surber net (mesh size: 200 µm) from a variety of microhabitats including pools fed by percolating water, laminar water flow along vertical walls and stones and gravelly sediments of streams. They were fixed with 96% ethanol in the field and freeze-dried upon return to the laboratory. Previous studies reported only minimal increase in carbon (C) and nitrogen (N) isotope ratios due to ethanol fixation for whole organisms with chitinous tissue (Bosley & Wainright 1999; Sarakinos, Johnson & Zanden 2002). Sediment (100–1000 µm) and organic matter (fragments of leaves and dead wood) were collected alongside with the individuals using a Surber net and brought to the laboratory within 4 h of collection in an isotherm box at 4°C. CPOM, FPOM and sediments were sorted using nets of 3 mesh sizes (100 µm, 1 mm, 6 mm), frozen and freeze-dried upon return to the laboratory for the determination of carbon and nitrogen isotope ratios (see below).

MEASUREMENT OF ISOTOPE RATIOS

Carbon and nitrogen isotope ratios were measured for all isopod and food source samples. Sediments were ground using mortar and pestle and treated with 1N HCl to remove carbonates using the 'rinse method', as described in Brodie *et al.* (2011). CPOM and FPOM were ground using a ball mill grinder (Retsch MM-200). Lipids were extracted from *Proasellus* samples using DCM : MeOH to avoid bias due to variation in fatty acid reserves among individuals (lipids are depleted in ^{13}C compared to bulk organisms, Post *et al.* 2007). Bulk concentrations

and stable isotope compositions of C and N in animals and their food sources (both labelled and collected in the field) were measured using an isotope ratio mass spectrometer coupled in continuous flow with an elemental analyser (PyroCUBE Elementar, Hanau, Germany – Isoprime, Isoprime Ltd, Manchester, UK for CPOM, FPOM and animals; ECS 4010 Costech Analytical, Valencia, CA – Delta Plus XP Thermofinnigan, Bremen, Germany, for the sediment). Measurements were made using sample masses of 180–300 mg, 1–3 mg, 1–3 mg and 200–600 μg of sediment, CPOM, FPOM and isopod tissue, respectively. In-house standards calibrated against IAEA-N1, IAEA-N2, IAEA-CH6, IAEA-C3 and USGS25 reference materials were analysed with the samples, and standard deviations of replicate analyses were lower than 0·10 ‰ (C) and 0·20 ‰ (N). C and N isotope compositions were expressed as δ in ‰ with V-PDB (C) and Air (N) as standards for the samples collected in the field, and in atom% for the food-labelling experiment.

DATA ANALYSIS

Fundamental specialization

The proportion F of carbon and nitrogen incorporated into organism tissues from a labelled food source was calculated from $\delta^{13}C$ and $\delta^{15}N$ as follows (e.g. Cerling *et al.* 2007; O'Brien *et al.* 2008):

$$F = (\delta_t - \delta_{init})/((\delta_{source} + \Delta) - \delta_{init}) \quad \text{eqn 1}$$

where δ_{init} and δ_t are the C or N isotope compositions of organisms at the start ($t = 0$) and at time t of the experiment, respectively, δ_{source} is the isotopic composition of the food source, and Δ is the discrimination factor between the source and animal tissue. The source-tissue discrimination factor was reported to vary with the isotopic composition of the food source (Caut, Angulo & Courchamp 2009). Because of the wide range of isotopic compositions of the different labelled sources (e.g. ^{15}N content ranged from 0·43 atom% for sedimentary biofilm to 5·06 atom% for CPOM), discrimination factors were calculated using the equation of Caut, Angulo & Courchamp (2009) for invertebrates.

The assimilation rates of C and N were calculated for each food source using the reaction progress equation (Cerling *et al.* 2007):

$$(1 - F) = e^{-\lambda t} \quad \text{eqn 2}$$

where λ is the assimilation (or turnover) rate (in day^{-1}) and t is the time in days. Equation 2 can be written as:

$$\ln(1 - F) = -\lambda t \quad \text{eqn 3}$$

and assimilation rates were calculated as the slopes of linear regressions. For each population, C and N assimilation rates were compared among food sources with an analysis of covariance (ANCOVA) followed by a contrast analysis to determine which food treatments differed (pairwise comparisons of the slopes of the linear regressions). Normality of the residuals of linear models was checked using Shapiro tests. For each population, we tested for differences in survival among food sources. We used chi-squared tests to compare the number of organisms collected alive on the different food sources during the whole experiment. Statistical analyses were performed with R 2.13 software (R Development Core Team 2011), and statistical significance was accepted at $\alpha < 0.05$.

Diet determination of cave populations

A Bayesian stable isotope mixing model (MixSIR; Moore & Semmens 2008) was used to estimate the contribution of the three food sources to the diet of *Proasellus* populations. As the diet-tissue discrimination has not been determined for isopod species, we used the commonly accepted values of $+3{\cdot}4 \pm 2‰$ and $+0{\cdot}5 \pm 1‰$ for nitrogen ($\Delta^{15}N$) and carbon isotope discrimination ($\Delta^{13}C$), respectively (e.g. Post 2002). The ranges of isotope signatures of food sources for each cave were narrow enough to avoid the correction of the discrimination values suggested by Caut, Angulo & Courchamp (2009) (maximum range of 7‰ for $\delta^{13}C$ at Falconnette cave – i.e. 0·0025 atom% for ^{13}C content – and 7‰ for $\delta^{15}N$ at Moulins de Cramans cave – i.e. 0·0077 atom% for ^{15}N content –, cf. Results section). MixSIR was run with 10^7 iterations, and results were presented as medians and 5–95% credibility intervals. For each population, the model resampled more than 1000 posterior draws without duplicate, resulting in a robust estimation of the true posterior density.

We partitioned the total trophic niche width (TNW) of populations (and species) into two components: the variation in resource use within (WC) individuals (and within populations) and the variation between (BC) individuals (and between populations) (Roughgarden 1974).

$$TNW_{pop} = WC_{ind} + BC_{ind} \text{ and } TNW_{sp} = WC_{pop} + BC_{pop} \quad \text{eqn 4}$$

where the subscripts pop, ind and sp stand for populations, individuals and species, respectively. The ratios of WC_{ind} to TNW_{pop} and WC_{pop} to TNW_{sp} were used to assess the similarity in diet composition among individuals and populations, respectively. Values approaching 1 indicate strong similarity in resource use among individuals and populations. Variance partitioning was performed with the program IndSpec1 (Bolnick *et al.* 2002) using the Shannon–Weaver index, and the median values of the contributions of the three food sources were calculated with MixSIR for each individual and population.

Results

FUNDAMENTAL SPECIALIZATION (LABORATORY EXPERIMENT)

The $\delta^{13}C$ and $\delta^{15}N$ values of *P. valdensis* and *P. cavaticus* increased during the course of the experiment in all treatments (see Appendix S1 and S2 in Supporting Information), indicating that the two species were able to assimilate CPOM, FPOM and sedimentary biofilm. For both species, the C and N assimilation rates were significantly higher with sedimentary biofilm than with FPOM and CPOM (Fig. 2, Table 2). *P. valdensis* assimilated 9- to 13-fold more carbon and 4- to 6-fold more nitrogen when it fed on sedimentary biofilm rather than on FPOM or CPOM. *P. cavaticus* assimilated 9- to 10-fold more carbon and 9- to 12-fold more nitrogen from sedimentary biofilm than from FPOM or CPOM. Survival rates for the whole experiment were 70% and 74% on sedimentary biofilm, 74% and 78% on CPOM, and 77% and 70% on FPOM for *P. valdensis* and *P. cavaticus*, respectively. There were no significant difference in survival rate among food sources for the two species ($\chi^2 = 0{\cdot}279$, d.f. = 2, $P = 0{\cdot}8697$ for *P. valdensis*; $\chi^2 = 0{\cdot}424$, d.f. = 2, $P = 0{\cdot}8089$ for *P. cavaticus*).

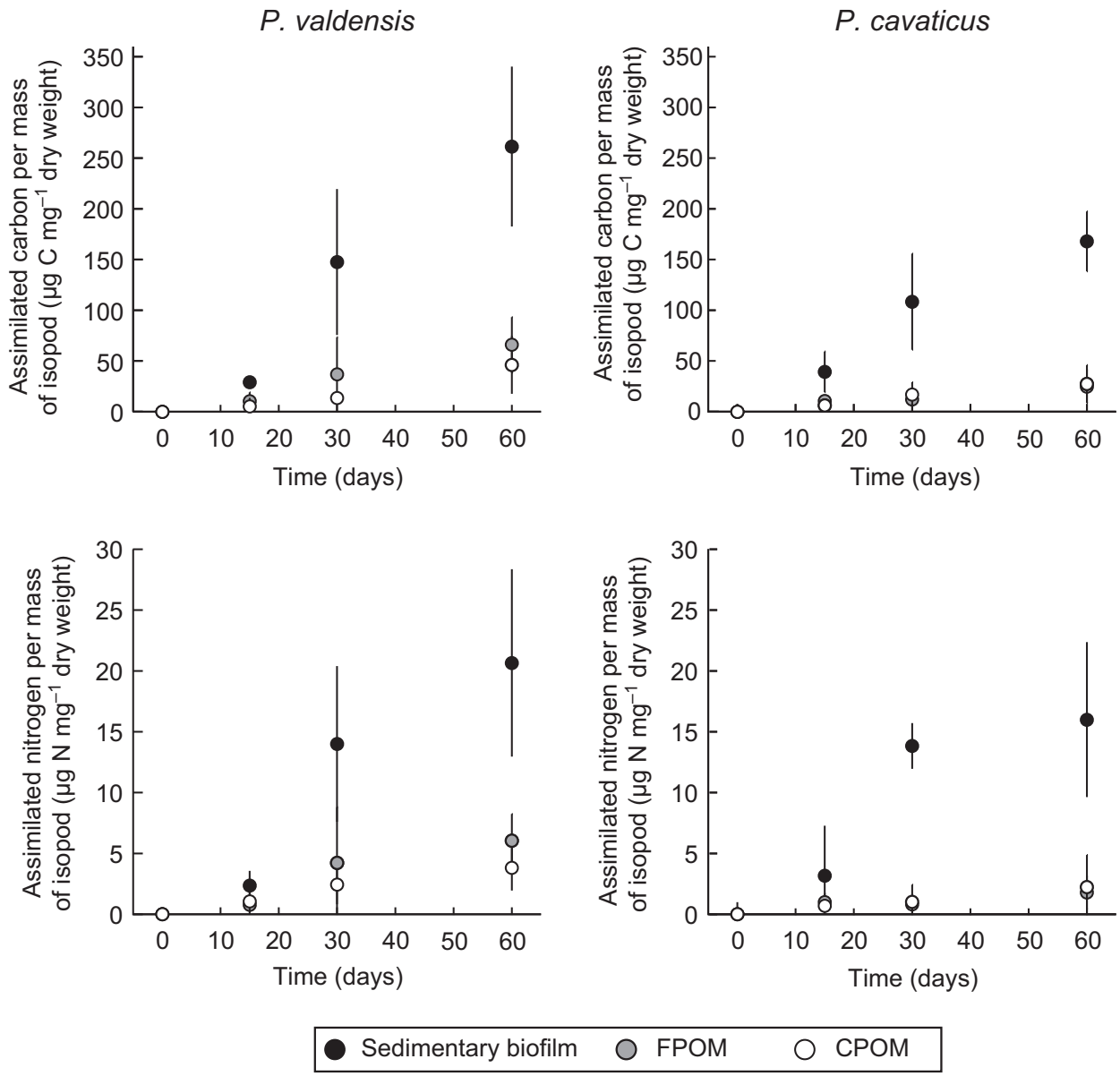

Fig. 2. Assimilated carbon and nitrogen per dry mass of animal (mean ± standard deviation, $n = 3$) measured for three food sources in the two species during the laboratory experiment.FPOM, fine particulate organic matter; CPOM, coarse particulate organic matter.

Table 2. Carbon and nitrogen assimilation rates (turnover rates in day^{-1}) calculated from food-labelling experiment. The difference of assimilation rates between the three food sources is tested by means of an analysis of covariance (ANCOVA) for the two species. FPOM, fine particulate organic matter; CPOM, coarse particulate organic matter

	Assimilation (day^{-1})			
Species	Sedimentary biofilm	FPOM	CPOM	*P*-value
Carbon assimilation				
P. valdensis	0·0293 ± 0·0037	0·0032 ± 0·0008	0·0022 ± 0·0005	$<10^{-9}$
P. cavaticus	0·0103 ± 0·0029	0·0010 ± 0·0003	0·0012 ± 0·0004	<0·001
Nitrogen assimilation				
P. valdensis	0·0059 ± 0·0011	0·0016 ± 0·0005	0·0010 ± 0·0003	<0·0001
P. cavaticus	0·0046 ± 0·0011	0·0004 ± 0·0001	0·0005 ± 0·0003	<0·001

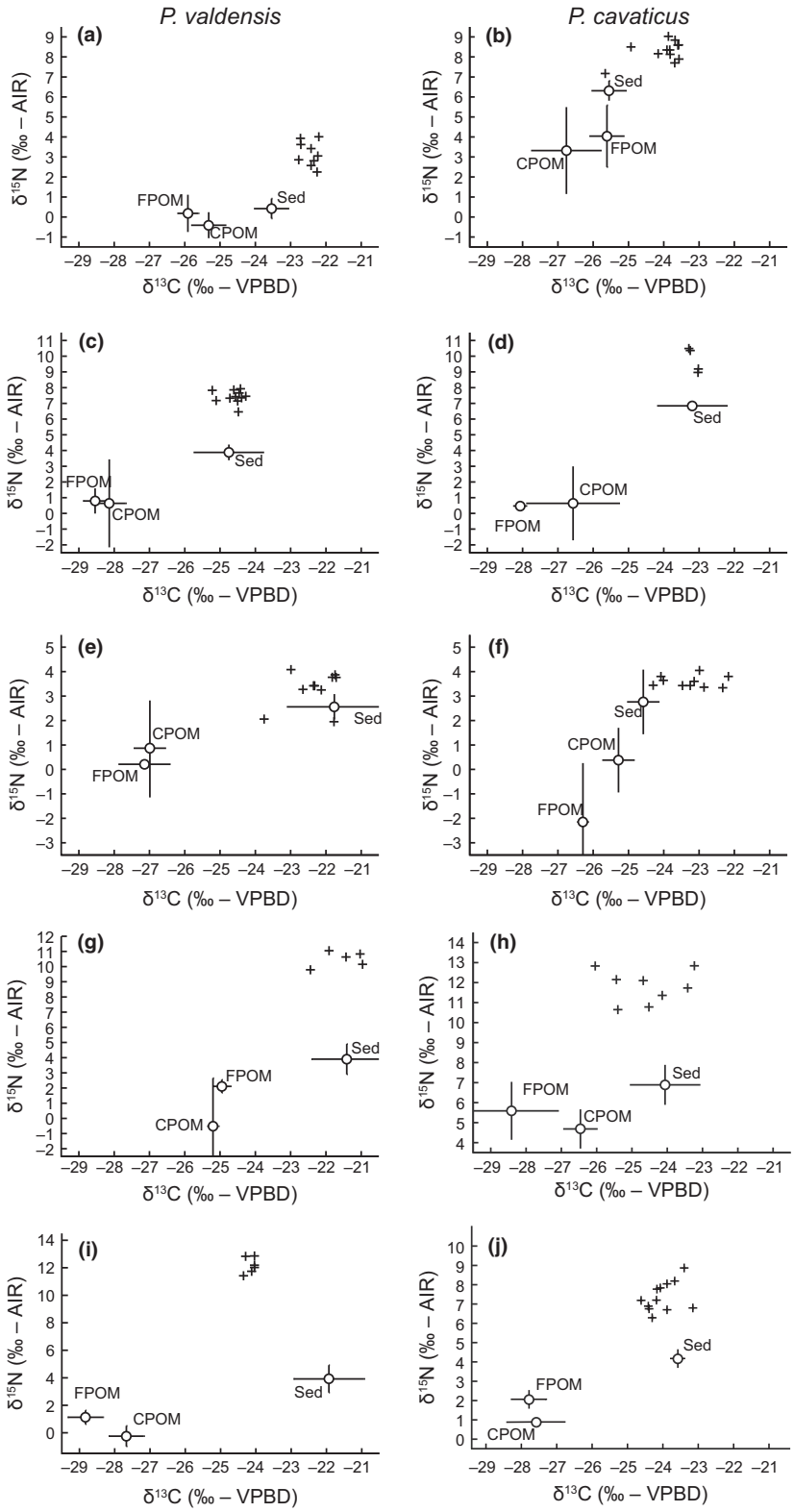

Fig. 3. Carbon and nitrogen stable isotope compositions of food sources (○) and *Proasellus* whole individuals (+; a, c, e, g, i: *P. valdensis*; b, d, f, h, j: *P. cavaticus*) in the 10 sampled caves. Bars represent standard deviations around mean values. Sed: sedimentary biofilm; FPOM: fine particulate organic matter; CPOM: coarse particulate organic matter. (a) Les Foules cave [1]; (b) Baume la Fraîte cave [6]; (c) Huguenot bis cave [2]; (d) Moulin de Cramans cave [7]; (e) Cuve de Sassenage cave [3]; (f) Grotte Roche cave [8]; (g) Grotte de la Balme cave [4]; (h) Corveissiat cave [9]; (i) La Falconnette cave [5]; (j) Borne aux Cassots cave [10]. Numbers in brackets are the cave numbers as in Table 1.

DIET DETERMINATION OF CAVE POPULATIONS

In the 10 studied caves, the C and N stable isotope compositions of particulate organic matter (CPOM and FPOM) were substantially lower than those of sedimentary biofilm (Fig. 3). The mean $\delta^{13}C$ values of CPOM and FPOM varied between −29 and −25‰, whereas that of sedimentary biofilm ranged from −25 to −22‰. In all caves, the mean $\delta^{13}C$ of *Proasellus* individuals were close to the $\delta^{13}C$ values of sedimentary biofilm (*P. valdensis*: −24 to −22‰; *P. cavaticus*: −25·5 to −22·5‰). We measured an increase of 1–5·5‰, except

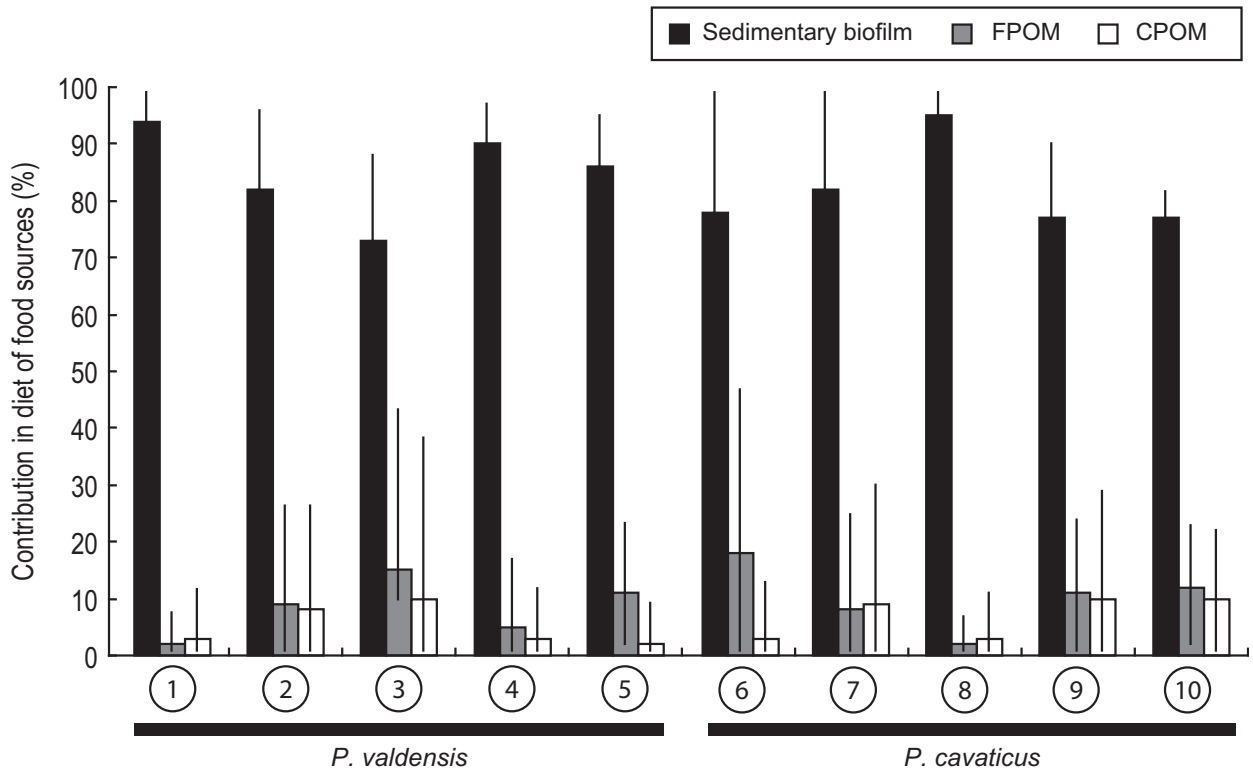

Fig. 4. Contribution of each food source (sedimentary biofilm, FPOM and CPOM) to the diet of the 10 populations of *Proasellus*. Cave numbers as in Table 1. FPOM, fine particulate organic matter; CPOM, coarse particulate organic matter.

in one cave where a higher increase of 8‰ was observed (Fig. 3i), between the mean $\delta^{15}N$ value of sedimentary biofilm and that of *Proasellus* individuals. The C and N stable isotope compositions of *Proasellus* individuals thus indicated a main contribution of sedimentary biofilm to their diet. Results of Bayesian mixing models (MixSIR) showed that individuals of *Proasellus* assimilated carbon and nitrogen primarily from sedimentary biofilm, whatever the population studied (median contribution: from 73% to 95% for the 10 populations, Fig. 4). The contributions of CPOM and FPOM to the diet of *Proasellus* were only 10% in all cave populations, except at Cuve de Sassenage Cave and Baume la Fraîte Cave, where the contribution of FPOM reached 15% and 18%, respectively. We found very little difference in diet composition among individuals within cave populations as well as among populations within species. The WC_{ind}/TNW_{pop} ratio averaged $0{\cdot}99 \pm 0{\cdot}01$ and $0{\cdot}96 \pm 0{\cdot}03$ ($n = 5$ cave populations) for *P. valdensis* and *P. cavaticus* populations, respectively (Table 3). The WC_{pop}/TNW_{sp} ratio was 0·95 and 0·97 for *P. valdensis* and *P. cavaticus*, respectively.

Table 3. Estimations of the similarity in diet composition among individuals in each population. All diets are composed of the same three food sources. Values close to 1 indicate high diet similarity (see text for details). Cave numbers as in Table 1

Species	Cave	*N*°	WC_{ind}/TNW_{pop}	Sample size
Proasellus valdensis	Les Foules	1	0·994	9
	Huguenots bis	2	0·996	11
	Cuves de Sassenage	3	0·980	10
	Grotte de la Balme	4	0·983	5
	Falconette	5	0·996	6
Proasellus cavaticus	Baume la Fraîte	6	0·953	12
	Moulin de Cramans	7	0·999	4
	Grotte Roche	8	0·921	10
	Corveissiat	9	0·948	7
	Borne aux Cassots	10	0·978	12

Discussion

Our findings stand in marked contrast with the prevailing view that subterranean species would tend to exhibit biological traits that enable them to perform equally well on a wide range of food sources (Ginet 1960; Magniez 1975; Gers 1995; Gibert & Deharveng 2002). Instead, we found that *P. valdensis* and *P. cavaticus* maximized their carbon and nitrogen assimilation rates when feeding on sedimentary biofilm. Both species assimilated about 10 times more carbon from sedimentary biofilm than from both forms of particulate organic matter (POM). As all food sources were provided *ad libitum* in the conditions of our experiment, these differences in assimilation rate probably reflected variation in ingestion rate (e.g. morphological traits) and/or efficiency of the digestion of food and

absorption of nutrients (i.e. physiological traits) among food sources. In our study, assimilation rate is considered as a proxy of performance (i.e. a quantitative trait correlated to relative fitness) because the amount of C and N gained per unit of time can maximize the resource available for survival and reproduction. Indeed, assimilation rate was shown to be positively related to reproductive output in other arthropods such as *Daphnia* (Lynch 1989) and *Drosophila* (O'Brien *et al.* 2008). The differences in assimilation rate (among food sources) observed in our laboratory experiment thus indicated a fundamental specialization of both *Proasellus* species on sedimentary biofilm, in terms of ingestion and/or digestibility. This was quite unexpected as earlier observations by Henry (1976) on the feeding behaviour of *P. valdensis* and *P. cavaticus* reared for several years in aquaria with sediment bottom (fine sediment and gravels) suggested they efficiently fed on CPOM because they were able to skeletonize leaves. However, Henry (1976) did not consider the possibility that these species might gain much of their energy by scraping epilithon and/or ingesting fine sediment.

Our field study also did not support the idea that low food availability in the subterranean environment would lead organisms to feed opportunistically on a wide range of food sources (Ginet 1960; Magniez 1975; Culver 1985; Gers 1995; Gibert & Deharveng 2002). Instead, we found that sedimentary biofilm, the food source on which the two species performed best, made by far the highest contribution to the diet of all studied populations. Here, diet is meant in terms of C and N assimilated from the different food sources. Yet, we cannot be entirely conclusive about the realized specialization (i.e. the adjustment of fundamental specialization through biotic interactions, chance and history) of *Proasellus* species because we did not measure the relative abundance of sedimentary biofilm, CPOM and FPOM in the caves. This is a difficult task because estimating their relative proportions ideally implies to measure fluxes of POM (rather than standing stocks) as well as microbial production (Venarsky *et al.* 2014) across all cave areas that can be foraged by the organisms. Yet, there are several arguments indicating that *Proasellus* do not use resources simply in proportion to their relative abundance in the cave environment. First, dietary proportions of cave populations determined in the field were similar to those that could be estimated from assimilation rates measured under unlimited food availability in the laboratory. Indeed, using measured assimilation rates, we calculated that *P. valdensis* would have assimilated 84% and 69% of its C and N from sedimentary biofilm, and *P. cavaticus* would have assimilated 82% and 84% of its C and N from sedimentary biofilm. Secondly, we found almost no variation in dietary proportions among individuals of a population, although they potentially fed on different microhabitats in the cave. Yet, POM is typically more abundant in cave streams where it is transported by floods than in cave pools fed by percolating water and vertical laminar water flows. Thirdly, we also found no variation in dietary proportions among cave populations within species, although we sampled a range of caves differing greatly in their distance to the soil surface and land use cover (Table 1) and thereby receiving potentially contrasted amounts of CPOM (leaves and wood). Several ecological studies performed in caves also indicated poor links between the relative abundance of a given resource and its use by groundwater animals. Indeed, Simon & Benfield (2001) and Venarsky, Benstead & Huryn (2012) showed that leaf litter breakdown rates due to invertebrates were not positively correlated to the abundance of POM in caves (see also Huntsman, Venarsky & Benstead (2011b) for the same kind of relationship for carrion breakdown rates). Using a stable isotope approach, Simon, Benfield & Macko (2003) also demonstrated that variations in CPOM abundance did not influence cave stream food webs. Although these authors did not focus on trophic specialization in cave animals, their results of ^{13}C-acetate tracer additions suggested that dissolved organic matter from surface soils was incorporated into biofilms and supported cave stream food webs, even when CPOM from the surface was abundant. Our results concur with this view because *Proasellus* populations, as subterranean primary consumers, mainly relied on biofilms for the 10 caves investigated in the present study.

Besides this specialization on sedimentary biofilm, it is worth noting that POM always contributed marginally to isopod diets in the field. This small but invariant proportion of POM may complement the diet of *Proasellus* with essential compounds that are absent in sedimentary biofilm ('optimal foraging with nutrients constraints', Lacher, Willig & Mares 1982). For example, Mondy *et al.* (2014) showed that phytosterols occurring in leaf fragments acted as precursors for the synthesis of steroids in *P. meridianus*.

Since we have shown a high degree of fundamental trophic specialization in the two obligate-groundwater species of *Proasellus*, it becomes now crucial to investigate the evolutionary mechanisms which have led to this specialization. We do not know whether this fundamental specialization on sedimentary biofilm is adaptive to the subterranean environment. Yet, their high assimilation rate on sedimentary biofilm relative to that on POM was not anticipated because surface-water species of isopods belonging to the genera *Proasellus* and *Asellus* are known to feed on a wide range of food sources, including CPOM, epilithic biofilm and fungi developed on leaves (Rossi & Fano 1979; Basset & Rossi 1987; Leberfinger, Bohman & Herrmann 2011). However, inputs of leaves and woods (CPOM) to cave streams are extremely heterogeneous across space because they are concentrated in areas that are directly connected to the surface by large openings (Culver 1985; Simon & Benfield 2001). Moreover, CPOM is usually transported over very short distances (<20 m year^{-1}) from their entry points in cave streams (Simon & Benfield 2001). On the contrary, microbial biofilm represents a ubiquitous source of food in the cave environment on which *Proasellus* can efficiently feed

by ingesting fine sediment but also by scraping the surface of rocks. Indeed, laboratory tests showed that individuals of *P. valdensis* and *P. cavaticus* were able to assimilate C and N as efficiently on biofilm developed on glass slides as on sedimentary biofilm (C. Francois, unpublished data). Therefore, specializing on sedimentary biofilm in caves may represent an efficient strategy to minimize food-searching costs. More generally, feeding on a limited number of food sources may enable to optimize digestive costs and assimilation of nutrients (Britt, Hicks & Bennett 2006). As an example, Basset & Rossi (1987) showed that females of *P. coxalis* adopting a diet specialist strategy maximized their reproductive efficiency (number of eggs normalized by the ingestion rate) compared to generalist females. Sedimentary biofilm may also be of better nutritional quality than POM (Cummins & Klug 1979; Lieske & Zwick 2007). In the present study, sedimentary biofilm had a much lower carbon-to-nitrogen molar ratio (C:N ~7) than POM (C:N ~20). According to the ecological stoichiometry theory (Elser *et al.* 2007), the two obligate-groundwater species of *Proasellus* would thus optimize their nutrient balance by feeding on sedimentary biofilm because there is a lower mismatch in C:N ratio between the consumers (C:N of *Proasellus* ~ 5) and that of biofilm than between the consumers and POM.

This study provides the first isotopic evidence of a high degree of trophic specialization in a low-productivity cave environment. We showed that the detritivorous obligate-groundwater isopods *P. valdensis* and *P. cavaticus* maximized their amount of C and N gained per unit of time when feeding on sedimentary biofilm and that they most probably fed selectively on this food source in their natural environment. Our findings challenge the generally held view that subterranean evolution would select for generalist feeding traits and that groundwater species would tend to feed opportunistically on all available resources to cope with low food availability. Indeed, this traditional view of trophic ecology in low-productivity environments fails to consider the eco-evolutionary feedbacks, for example the fact that organisms living in these environments often evolve biological traits such as low metabolic rates and high food-finding abilities which may in turn release the constraints on trophic specialization. Rather, our findings indicated that some obligate-groundwater primary consumers might have evolved specialized feeding strategy to best exploit those resources that are the most ubiquitous in their natural environment. Recent phylogenies of groundwater species-rich clades (Morvan *et al.* 2013) offer useful case studies for testing between these alternative views. We suggest that coupling isotopic studies of fundamental and realized trophic specialization among multiple pairs of closely related surface water–groundwater species can provide a unique opportunity to document and understand patterns in the evolution of specialization at lower bounds of habitat productivity.

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Data accessibility

All data used in this study are present in the manuscript and its supporting information.

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Appendix S1. Changes in $\delta^{13}C$ of individuals and variations of the proportion of C incorporated into their tissues during the course of the labelling experiment.

Appendix S2. Same as Appendix S1 for nitrogen ($\delta^{15}N$).

Appendix S3. Carbon and nitrogen isotope composition of individuals and labelled food sources during the laboratory experiment (mean ± SD).

Appendix S4. Carbon and nitrogen isotope composition of isopods sampled in the ten caves of this study.

Appendix S5. Carbon and nitrogen isotope composition of food sources in the ten caves of this study (mean ± SD).