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NOTE AND COMMENT

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Trophic plasticity among spring vs. cave populations of *Gammarus minus*: examining functional niches using stable isotopes and C/N ratios

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Abstract In some environments, species may exhibit trophic plasticity, which allows them to extend beyond their assigned functional group. For *Gammarus minus*, a freshwater amphipod classified as a shredder or detritivore, cave populations have been observed consuming heterotrophs as well as shredding leaves, and therefore may be exhibiting trophic plasticity. To test this possibility, we examined the C and N stable isotope and C/N ratios for cave and spring populations of *G. minus*. A 15-day feeding experiment using leaves and *G. minus* from a spring population established that the diet-tissue discrimination factor was 3.2 ‰ for δ^{15}N. Cave *G. minus* were 8 ‰ higher in δ^{15}N relative to cave leaves, indicating they did not derive nitrogen from leaves, whereas field collected spring populations were 2–3 ‰ higher than spring leaves, indicating that they did. Cave *G. minus* were 2.6 ‰ higher in δ^{15}N than the cave isopod, *Caecidotea holsingeri*. Relative to spring populations, Organ Cave *G. minus* were ^{15}N enriched by 6 ‰, suggesting they occupied a different trophic level, or incorporated an isotopically distinct N source. While stable isotopes cannot tell what the cave *G. minus* are eating, the isotopes certainly show that *G. minus* are not eating leaves and are trophically distinct form the surface populations. Differences in C/N ratios were observed, but reflect the size of the *G. minus* examined and not feeding group or habitat. The isotope data strongly support the hypothesis that cave populations of *G. minus* have become generalist or omnivorous by including animal protein in their diet.

Keywords Trophic plasticity · *Gammarus minus* · Stable isotopes · Functional niches

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Introduction

Trophic plasticity is hypothesized to be a common adaptation in organisms that experience low quantity or low quality of resources or highly variable sources of nutrition (MacNeil et al. 1997). If nitrogen is contained in a high carbon matrix such as leaves or other low protein plant material and a low carbon matrix such as detritus with bacterial films or animal tissue, organisms may diet-switch to the source with lower C/N ratio (Denno and Fagan 2003). Arthropod predators in a nitrogen-limited environment, for example, may prey on other nitrogen rich predators in addition to its normal prey (Denno and Fagan 2003). Low abundance of preferred dietary items may also result in switching from one trophic niche or functional feeding group to another; for example, a shredder to a generalist omnivore (Fagan et al. 2002). In some invertebrates, the nitrogen content can vary three-fold among individuals of the same species, indicating that a species assigned to a single trophic niche may include different diets that differ greatly in nitrogen content (Elser et al. 2000). This process may cloud the assignment of an exclusive "functional feeding group" to a particular species (MacNeil et al. 1997), especially if little studied species are grouped with related, better studied ones, or if the species inhabits nutritionally constrained environments, such as those in caves.

One relatively well-studied species that may experience nutritional constraint and severe nitrogen limitation is the freshwater amphipod crustacean *Gammarus minus*. This species inhabits both surface spring runs and cave stream environments throughout the Appalachians (Culver et al. 1995), with different cave populations having evolved independently from surface populations (Carlini et al. 2009). Surface populations of *G. minus*, usually classified as "detritivore" or "shredder", readily skeletonized leaves but derived their nutrition from the bacteria and fungi that have colonized the decaying leaves (Kostalos and Seymour 1976; Francois et al.

2015). Cave populations of *G. minus* may obtain part of their nutrition in a similar way because they also skeletonize leaves in the laboratory (Culver et al. 1995; Fong personal observation). However, they have been observed to also prey on other invertebrates in cave streams, such as oligochaetes, isopods, and amphipods in the genus *Stygobromus* as well as to cannibalize smaller or injured individuals (Culver et al. 1991; Fong 2011). Although the abundance and quality of food resources vary greatly among subterranean systems in general, cave systems in the Appalachians seem to be nitrogen-limited but not carbon-limited compared to surface systems (Culver and Pipan 2009). In this study we examine whether *G. minus* has expanded or shifted its trophic status from a detritivore on the surface to an omnivore or predator in the nitrogen limited cave stream.

Materials and methods

Study sites

Gammarus minus specimens were collected from two spring runs, Taylor Spring (TS) and Ward Spring (WS) and one subterranean stream in Organ Cave (OC) in Greenbrier County, West Virginia, USA (see Carlini et al. 2009 for a map of the area). Organ Cave receives drainage waters from an 8.1 km^2 basin that injects a mix of allochthonous organic material from the surface, such as twigs, leaves, and dissolved organic matter, with no evidence of autochthonous primary production via photosynthesis or chemosynthesis (Simon et al. 2003). Water from Organ Cave resurfaces at Organ Spring on Second Creek about 0.7 km upstream from Ward Spring, thus the Organ Cave and Ward Spring populations belong to the same hydrologic unit. We used Ward Spring because it is more easily accessible than is Organ Spring, and the two populations of *G. minus* are genetically similar (Carlini et al. 2009). Organ Cave amphipods, however, are on average significantly larger in body size than Ward Spring amphipods, with average and standard error of head capsule length of sexually mature males at 1.08 ± 0.04 mm compared to 0.67 ± 0.03 mm (N = 25–30, Fong, unpublished data). Taylor Spring is located about 30 km north of Organ Cave and Ward Spring. We included Taylor Spring because its amphipods are similar in size to those of Organ Cave, at 0.96 ± 0.06 mm, as a control for a difference in body size rather than habitat or any difference in trophic status.

Collections

Thirty *G. minus* amphipods were hand-collected with an aquarium net from each site along with samples of submerged leaf-litter and frozen until ready for processing. All collections were during the fall of 2012 (15 *G. minus* from each site) and fall 2013 (all remaining samples). Leaf samples were taken from both spring (Ward) and cave environments where the *G. minus* were collected. Organ Cave sediments were also collected in order to assess the isotope signature of cave organic material. Additionally, possible prey invertebrates from Organ Cave were collected: four samples of the amphipod *Stygobromus emarginatus* and the isopod *Caecidotea holsingeri*. Thawed specimens were dried at 60 °C for three days in individual open glass vials. Dried samples were homogenized with a mortar and pestle. Individual amphipods were weighed and placed in 5 × 9 mm pressed tin capsules (between 0.5 and 1 mg). Sediments were split for $\delta^{13}C$ and $\delta^{15}N$ analysis. For $\delta^{15}N$, 17 to 29 mg of sediments were weighed into tin capsules after drying. For $\delta^{13}C$, the sediments were acidified to remove carbonates. Six 40 ml beakers were filled with 30 mg of sediments. These were then stirred in 1 N HCl for 24 h to ensure that all CO_2 generation had ceased. The pH of the solution was then raised to neutral using KOH pellets. Then each beaker's contents were vacuum filtered through binder-free, 47 mm diameter, 1.2 μm glass fiber filters (Whatman). Approximately 23–30 mg were then scraped form the filters for analysis. For leaves, approximately 5–7 mg were weighed for analysis.

Feeding experiments

In order to assess the diet-tissue fractionation associated with *Gammarus minus* consuming leaf material, approximately 150 *G. minus* were collected from Apple Cave Spring in Greenbrier County, WV where they are known to be abundant. The amphipods were captured using dip nets and were transported back to the laboratory on ice. They were then placed in holding containers in a refrigerated chamber at 10 °C and allowed to acclimate for 24 h. The next day they were transferred in groups of 25 into containers of purified spring water with a diet of prepared leaf litter (see below) and kept in the refrigerated chamber. The broad-leaf deciduous diets were prepared in the laboratory by (1) boiling for 1 day to remove tannins, changing the water each time it turned brown, (2) transferring cooled leaves to two modified 5-gallon Deer Park water jugs equipped with air hoses, (3) adding 10 L of microbe-laden water taken from lab populations of *G. minus* and (4) storing at room temperature for 2 weeks in order to produce a rich biofilm on the leaves (D. Fong, personal communication). When the leaves were added to the bins holding the *G. minus* they began skeletonizing the leaves. Care was taken to ensure that prepared leaf material was abundant in the holding container throughout the experiment. The subsampling for isotope analysis occurred daily over a period of 15 days. Fifteen days was judged to be adequate for the amphipods to approach "isotope equilibrium" with their food because the half-life for similar sized brine shrimp (*Artemia* < 2.5 cm) is

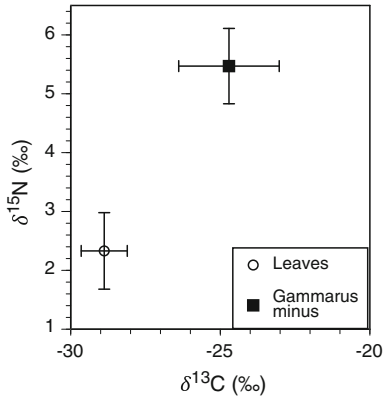

Fig. 1 Diet tissue δ^{15}N and δ^{13}C discrimination for *Gammarus minus* from a surface spring (Apple Cave Spring, WV USA) fed broad-leaf deciduous diets (leaves). Mean δ^{15}N of *G. minus* was 5.5 ± 0.6 ‰ (N = 89) and that of leaves was 2.3 ± 0.7 ‰ (N = 6); a difference of 3.2 ‰. This is within the estimated trophic fractionation or diet tissue discrimination reported in most literature (3.0 to 3.5 ‰). Mean δ^{13}C for *G. minus* was −24.7 ± 1.9 ‰ (N = 89), and that of leaves was −28.9 ± 0.8 ‰ (N = 6)

4 days (Fry and Arnold 1982). Even for the larger juvenile penaeid shrimp, half-life of muscle tissue was 8 days (Parker et al. 1991). Therefore, 15 days is a reasonable time to allow the *G. minus* to approach isotope equilibrium with its diet. This is particularly true since the diet was C3 leaves, which was the same diet the *G. minus* were consuming in the spring environment. Subsamples of six amphipods (only adult, non-ovigerous animals were used) were set aside in individual containers with clean water and placed in the 10 °C refrigerator for 48 to 72 h. Once guts were fully evacuated (visually determined), each individual was rinsed with pure spring water and placed in a glass vial to dry (60 °C for 48 h). The dry mass of each individual was recorded prior to preparation for isotope analysis in order to ensure mass was consistent among the animals for the duration of the experiment.

Isotope and C/N analysis

Samples were shipped to the UC Davis stable isotope laboratory where δ^{13}C and δ^{15}N were determined using a PDZ Eurpoa ANCA-GSL elemental analyzer coupled to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Standards were Pee Dee Belemnite for carbon and N (air) for nitrogen. The laboratory standards (calibrated to the international standards) used were Bovine liver, USGS-41 Glutamic Acid, nylon 5 and Peach leaves. For nylon 5 (N = 17), δ^{13}C and δ^{15}N reproducibility was ±0.09 and ±0.11 ‰. For Peach leaves (N = 9), δ^{13}C and δ^{15}N reproducibility was ±0.07 ‰. Computation of the δ (delta) value follows the same procedure for all stable isotopic measurements, as follows

$$\delta^{x}E = [({}^{x}E/{}^{y}E)\text{sample}/({}^{x}E/{}^{y}E)\text{standard}] - 1)$$

where E is the element analyzed (C or N) and x is the atomic weight of the heavier isotope, and y is the atomic weight of the lighter isotope (x = 13 or 15 and y = 12 or 14 for C and N respectively).

C/N ratios were also obtained using elemental mass. Standard deviations of laboratory standards Nylon 5 and Peach leaves were 0.03 and 0.26 respectively.

Statistics

Tests for differences among groups were made using nonparametric multiple comparison Dunn tests with Control for Joint Ranks, with Bonferroni adjustment (alpha = 0.05).

Results

Gammarus minus feeding experiments (15 days with a subsample of 6 per day) to determine diet-tissue discrimination showed mean δ^{15}N of was 5.5 ± 0.6 ‰ (N = 89) and that of leaves was 2.3 ± 0.7 ‰ (N = 6); a difference of 3.2 ‰ (Fig. 1). Prepared leaves fed to the *G. minus* during the feeding experiment were skeletonized as they were consumed, so care was taken to ensure an abundance of fresh leaf material was available. There was no change in mass among the sampled pool of *G. minus* (Fig. 2, regression R^2 of 0.0189, F ratio 1.72, P = 0.19), further suggesting that the amphipod population was consuming the leaves (not starving). There was no change in *G. minus* δ^{15}N over the 2 week feeding experiment (Fig. 3, regression R^2 = 0.04, F ratio 3.7 P = 0.06). This indicated that the animals were in "isotope equilibrium with their food, and there was no trend towards a ^{15}N enrichment over time which would have suggested protein sparring (Castellini and Rea 1992). Therefore all samples were pooled for the analysis of the diet-tissue discrimination factor. The observed δ^{15}N difference, given above, was within the estimated trophic fractionation (diet tissue discrimination) reported in most literature (3.0–3.5 ‰). Mean δ^{13}C for *G. minus* was −24.7 ± 1.9 (N = 89), while that of leaves was −28.9 ± 0.8 ‰ (N = 6) (Fig. 1). The 4.2 ‰ difference between leaf material and *G. minus* is larger than the often-reported 1–1.5 ‰ trophic shift between consumer and diet. This is due to the leaf carbon δ^{13}C signal reflecting carbon that is not incorporated by the *G. minus* consumer. The *G. minus* shredding leaves actually incorporates the bacterial or fungal films that coat the leaves, not the carbon of the leaf itself.

Among the field collections, Organ Cave (OC) *G. minus* were significantly enriched in δ^{13}C, by approximately 3 ‰, and in δ^{15}N, by about 6 ‰, relative to Ward Spring (WS) or Taylor Spring (TS) animals (Fig. 4; Table 1, 2). WS and TS *G. minus* did not differ in

Table 1 Average ± SD (N) for stable isotopes plus C/N ratios for groups examined

	δ^{13}C (‰)	δ^{15}N (‰)	C/N
Organ Cave: *Gammarus minus*	−25.08 ± 0.57 (N = 30)	10.81 ± 0.94 (N = 30)	5.4 ± 0.7 (N = 30)
Ward spring: *G. minus*	−27.39 ± 1.13 (N = 30)	4.40 ± 0.62 (N = 30)	6.1 ± 0.9 (N = 30)
Taylor spring: *G. minus*	−27.90 ± 1.39 (N = 30)	4.38 ± 1.40 (N = 30)	5.4 ± 0.8 (N = 30)
Organ Cave: *Stygobromus emarginatus*	−24.90 ± 0.53 (N = 4)	13.96 ± 1.32 (N = 4)	4.3 ± 0.3 (N = 4)
Organ Cave: *Caecidotea holsingeri*	−25.78 ± 0.94 (N = 4)	8.24 ± 1.20 (N = 4)	4.1 ± 0.3 (N = 4)
Organ Cave sediments	−25.14 ± 0.43 (N = 6)	6.00 ± 0.19 (N = 6)	8.2 ± 0.7 (N = 6)
Organ Cave leaves	−29.08 ± 1.83 (N = 4)	2.57 ± 3.48 (N = 4)	51.9 ± 36.4 (N = 4)*
Ward spring leaves	−29.01 ± 0.44 (N = 16)	2.43 ± 0.88 (N = 15)	40.7 ± 10.1 (N = 15)*

* There was considerable variation in C/N ratios among leaves as more degraded leaves contained less N

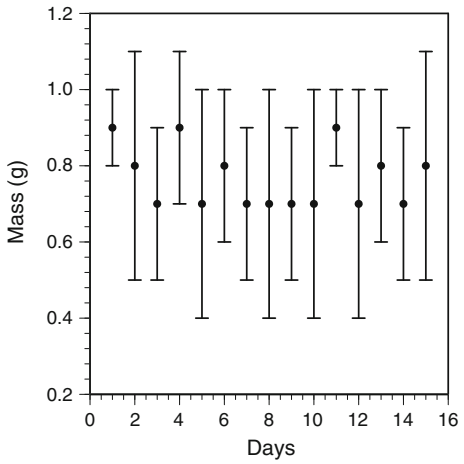

Fig. 2 Masses for *Gammarus minus* from a surface spring (Apple Cave Spring, WV USA) fed broad-leaf deciduous diets (leaves). There was no significant mass change over time (regression R^2 of 0.0189, F ratio of 1.72, prob >0.19)

δ^{13}C and δ^{15}N values (Table 1). Isotope ratios for the second species of amphipod (*Stygobromus emarginatus*) were not significantly different than OC *G. minus*. The isopod *Caecidotea holsingeri* was not statistically different than OC *G. minus* either, although their mean δ^{15}N was 2.6 ‰ lower (Tables1, 2).

Within OC, the leaf material was 8 ‰ lower in δ^{15}N and 2 ‰ lower in δ^{13}C than the *G. minus*, differences that were statistically significant (Table 2). Sediment and leaf material from OC were also significantly different in δ^{13}C and δ^{15}N, with sediments being approximately 4 and 3 ‰ heavier than leaves (respectively) (Table 2). Leaf material from both springs were 2–3 ‰ depleted in δ^{15}N, but showed roughly the same δ^{13}C value as the average *G. minus* from the both springs (Table 1; Fig. 4).

C/N ratios for all invertebrates ranged between 4.5 and 7.8, with low standard deviations (Table 1; Fig. 5). WS *G. minus* had significantly higher C/N ratios (average 6.1) than TS and OC animals (both 5.4) (Table 1, 2 ; Fig. 5). Leaf material from the WS showed much higher C/N ratios than invertebrates, ranging from 30 to 59. C/N ratio of leaf material from OC ranged up to 90, but the mean was substantially lower (Table 1).

Discussion

Surface populations *G. minus* are generally placed in the "leaf shredder" functional feeding group and derive nutrients from microbes colonizing leaves (Kostalos and Seymour 1976; Simon et al. 2003). Conventionally, there is a 3–3.3 ‰ increase in δ^{15}N values between trophic levels, and an approximate 1 ‰ increase in δ^{13}C values, in a variety of terrestrial and aquatic ecosystems (Minagawa and Wada 1984; reviewed in Lajtha and Michener 2007). The feeding experiment reported here shows a 3.2 ‰ (δ^{15}N) diet tissue discrimination for spring *G. minus* fed spring leaves. Since these diet-tissue discrimination values are observed among both invertebrate and vertebrate species, they can be used to include or exclude possible nutrient sources if their isotopes are known. Another component of deciphering food webs using isotopes includes the assimilation rates of nutrients. An organism's tissues might not reflect the isotope signature of a recently arrived food source (a migratory prey item for example) if there is insufficient time for the nutrients to be incorporated. In the systems examined here, the *G. minus* and other cave invertebrates, as well as their potential food items, are not migratory. Additionally, the nutrient assimilation rate for small invertebrates is rapid, even for non-growing adults (Fry and Arnold 1982). For brine shrimp (*Artemia*), which are approximately the same length of *G. minus* in this study (<2.5 cm), assimilation half-lives can be 4 days (Fry and Arnold 1982), and even for larger invertebrates (penaeid shrimp) half-life was approximately 8 days (Parker et al. 1991). Therefore, it is reasonable to consider the isotope characterization of the *G. minus* and possible foods in these environments as reflecting "isotope equilibrium". The Ward Spring populations of *G. minus* were approximately 2 ‰ higher in δ^{15}N than leaves from the springs where they were captured, and Taylor Spring *G. minus* were isotopically indistinguishable from the Ward Spring population. While this "trophic shift" or "diet-tissue discrimination" is a bit lower than would be expected for organisms incorporating N from bulk leaves (which is 3.2 ‰ for *G. minus*), it is consistent with the lower δ^{15}N trophic enrichment that can occur in environments where nitrogen is limited and diet-tissue fractionation for nitrogen is minimized

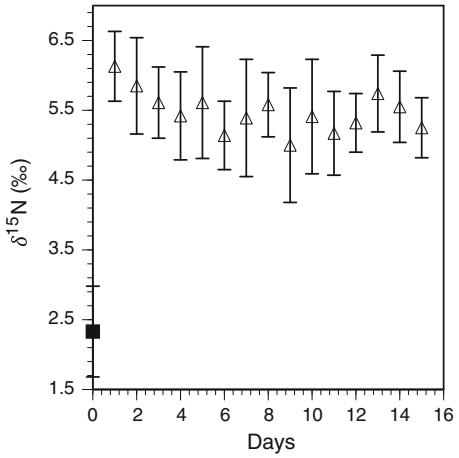

Fig. 3 Daily δ^{15}N average values with standard deviations during the feeding experiment. The δ^{15}N value of the leaf diet are placed at day 0 in the figure. Each day 5–6 *Gammarus minus* were collected, placed in clean water, and kept in 10 °C refrigeration chambers for gut evacuation (48 to 72 h). Once guts were fully evacuated (visually determined), individual *G. minus* were prepared for isotope analysis as described in the methods. A regression of δ^{15}N vs. time showed no significance (R^2 was 0.0396, F ratio 3.7 P = 0.06)

(Hobson et al. 1993). Our results indicate that the surface animals do not obtain their N from animal protein. Although the bulk leaf tissue δ^{15}N reflects plant tissue N, it also reflects the nutritionally important biofilm (Francois et al. 2015), which is also ^{15}N-depleted. Regardless whether leaf tissue or biofilm is their N source, the data clearly indicates surface populations of *G. minus* are not predaceous.

The Organ Cave *G. minus* δ^{15}N and δ^{13}C values were 6 and 2.5 ‰ higher than the Ward Spring and Taylor Spring *G. minus* values. Organ Cave *G. minus* were also more than 8 ‰ higher in δ^{15}N and 2.5 ‰ higher in δ^{13}C than Organ Cave leaves (Table 1). Organ Cave *G. minus* carbon isotope values were not significantly different from sediment organic carbon, although the sediment was significantly lower in δ^{15}N (4.8 ‰). This suggests that sediment organic material (probably fungal) may have been a contributing nutrient source for Organ Cave *G. minus*, but would have to be augmented with more ^{15}N enriched sources. The data clearly show that the cave and surface populations are not relying on the same nitrogen and carbon sources, and that the Organ Cave *G. minus* are not deriving N from cave leaves, but from some more ^{15}N enriched sources. Cave ecologists have observed *G. minus* consuming other invertebrates (Culver et al. 1991; Fong 2011), and while one potential prey species, *Stygobromus emarginatus*, were too ^{15}N enriched to have been a nitrogen source, the isopods *Caecidotea holsingeri* were ^{15}N depleted (2.6 ‰) and would be consistent with expected prey or scavenged species isotope values. Indeed, Culver et al. (1991) hypothesized that *Gammarus* could be a predator of *Caecidotea holsingeri* specifically. However, there could easily be a different ^{15}N enriched nitrogen food item which was not collected. Taken as a whole, the data strongly support the hypothesis that the cave population has become generalist or omnivorous by including animal protein in its diet. Furthermore, the two spring populations differ in body size yet show similar δ^{15}N values, indicating that the cave population became predaceous not because it became larger and thus more capable of handling prey, but rather because of an expansion of its feeding niche after colonizing the nitrogen-limited cave environment. Expansion of feeding niche after colonizing the cave environment has also been demonstrated in a predaceous salamander that included bat guano in its diet in a cave (Fenolio et al. 2005), among cave amphipods in the Edwards Aquifer (TX, USA) (Hutchins et al. 2014), and in a guild of marine sediment inhabiting amphipods that included only detritivorous species in open areas while a majority of the species in caves were carnivorous (Navarro-Barranco et al. 2013).

Mulholland et al. (2000) examined nitrogen sources for stream invertebrates including *G. minus*. They report that *G. minus* appeared to derive nutrition from fine

Table 2 Comparisons that were significantly different as shown by the Dunn tests with Control for Joint Ranks with Bonferroni adjustment (the P value takes the adjustment into account)

	P value
δ^{13}C comparison	
Organ Cave *Stygobromus emarginatus* vs. spring leaves	0.0005
Organ Cave *S. emarginatus* vs. Organ Cave leaves	0.0339
Organ Cave *Gammarus minus* vs. spring leaves	< 0.0001
Organ Cave sediments vs. spring leaves	0.0017
Organ Cave *G. minus* vs. Organ Cave leaves	0.0337
Organ Cave *Caecidotea holsingeri* vs. spring leaves	0.0424
G. minus: Ward spring vs. Organ Cave	< 0.0001
G. minus: Taylor spring vs. Organ Cave	< 0.0001
Taylor spring *G. minus* vs. Organ Cave sediments	0.0085
Taylor spring *G. minus* vs. Organ Cave *S. emarginatus*	0.0184
δ^{15}N comparison	
Organ Cave *S. emarginatus* vs. spring leaves	< 0.0001
Organ Cave *G. minus* vs. spring leaves	< 0.0001
Organ Cave *S. emarginatus* vs. Organ Cave leaves	0.0159
Organ Cave *C. holsingeri* vs. spring leaves	0.0073
Organ Cave *G. minus* vs. Organ Cave leaves	0.0066
Organ Cave sediments vs. spring leaves	0.0065
G. minus: Ward spring vs. Organ Cave	< 0.0001
G. minus: Taylor spring vs. Organ Cave	< 0.0001
Taylor spring *G. minus* vs. Organ Cave *S. emarginatus*	0.0029
Ward spring *G. minus* vs. Organ Cave *S. emarginatus*	0.0021
C/N comparison	
Organ Cave sediments vs. Organ Cave *C. holsingeri*	0.0008
Ward spring *G. minus* vs. Organ Cave *C. holsingeri*	0.0243
Ward spring *G. minus* vs. *S. emarginatus*	0.0396
Organ Cave sediments vs. Organ Cave *G. minus*	0.0311
Ward spring *G. minus* vs. spring leaves	0.0008
Taylor spring *G. minus* vs. Organ Cave sediments	0.0246
Organ Cave *G. minus* vs. Organ Cave leaves	0.0134
Organ Cave *G. minus* vs. spring leaves	< 0.0001
Taylor spring *G. minus* vs. Organ Cave leaves	0.0109
Taylor spring *G. minus* vs. spring leaves	< 0.0001
Organ Cave *S. emarginatus* vs. Organ Cave sediments	0.0014
Organ Cave *S. emarginatus* vs. Organ Cave leaves	0.0005
Organ Cave *S. emarginatus* vs. spring leaves	< 0.0001
Organ Cave *C. holsingeri* vs. Organ Cave leaves	0.0003
Organ Cave *C. holsingeri* vs. spring leaves	< 0.0001

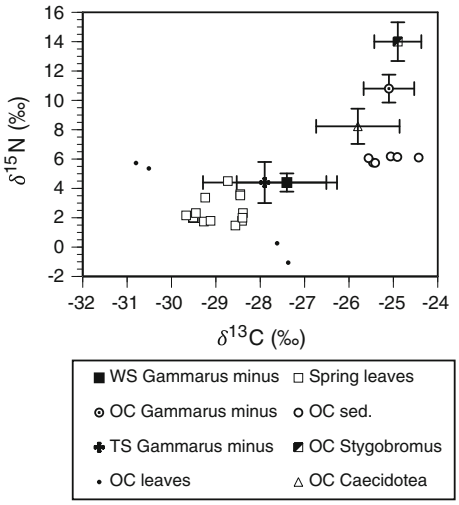

Fig. 4 δ^{13}C vs. δ^{15}N for *Gammarus minus* from Organ Cave (OC), Taylor Spring (TS), and Ward Spring (WS). Also included are leaves from OC and WS, sediments from OC, as well as *Stygobromus emarginatus* and *Caecidotea holsingeri* from OC. OC *G. minus* are significantly enriched in δ^{13}C and δ^{15}N relative to *G. minus* from either spring. Spring *G. minus* are not significantly different from each other in δ^{13}C or δ^{15}N. Spring leaf δ^{15}N is significantly lower than that of *G. minus* from either spring. Ward Spring δ^{13}C is significantly higher than spring leaf δ^{13}C. Organ Cave *G. minus* do not obtaining nitrogen from the same sources as the spring *G. minus*, and δ^{15}N values are consistent with generalists or omnivores consuming animal protein

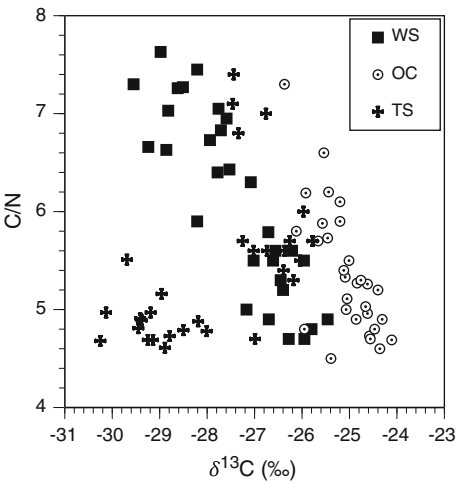

Fig. 5 C/N ratios vs. δ^{13}C values for *Gammarus minus* from the three sites. Significant differences in C/N ratios exist between the Ward Spring and other two sites. C/N ratios for the leaf litter are not shown, however for Organ Cave, litter was 52 ± 36 (N = 4) and spring litter was 41 ± 10 (N = 15)

benthic organic matter or epixylon based on δ^{15}N (assumed diet tissue discrimination (trophic shift) of 2–4 ‰). Simon et al. (2003) examined Organ Cave trophic structure using the natural abundance δ^{15}N and an acetate-tracer δ^{13}C, but did not include surface populations and species in their study. They found that *G. minus* appeared to consume fine particulate organic matter (FPOM) based on tracer data, but they were too ^{15}N enriched (approximately 5-6 ‰) for FPOM to be the N source. Their *G. minus* δ^{15}N data suggested that ^{15}N-enriched epilithon was a food source (based on the expected 3–3.5 ‰ trophic shift), however the tracer data did not indicate this was the case. In fact, the *G. minus* continued to ^{13}C enrich well after the tracer spike, which we suggest may indicate they were receiving C as it passed through a longer food chain than that represented by one with FPOM or epilithon as the direct C or N source. Perhaps the slowly enriching δ^{13}C values of the *G. minus* combined with their high δ^{15}N values (between 8 and 12 ‰) indicated they were generalists or omnivores consuming animal tissue. Francois et al. (2015) also conducted a stable isotope labeling study, but in a laboratory environment. They found that sedimentary biofilms were much more important source of carbon and nitrogen than particulate organic matter (fine or coarse) when invertebrates were presented with all three foods.

The Ward Spring *G. minus*, which are consistently 35 % smaller than either the Taylor Spring or Organ Cave *G. minus*, had significantly higher C/N ratios. This may result from the smaller Ward Spring amphipods having a higher amount of C rich chitin relative to internal volume, which increases their C/N ratio relative to the larger amphipods. Generally, carnivorous organisms have more N per unit mass than organisms lower on the food chain (Elser et al. 2000; Denno and Fagan 2003) with predators having 15 % on average more N per gram than herbivores (Fagan et al. 2002). The larger Organ Cave and Taylor Spring amphipods do not show a difference in C/N ratio although one could expect N to be concentrated per unit mass in the Organ Cave *G. minus* if they were deriving nutrition from animal tissue, as our δ^{15}N and δ^{13}C data suggest.

In the highly N limited cave environment, and perhaps even in the surface spring environment, the amphipods may retain N regardless of whether their source of N was animal or plant based. An emphasis on nitrogen retention would account for the reduced δ^{15}N trophic shift observed between both spring *G. minus* populations vs. Ward spring leaf material. It is reasonable to hypothesis that nitrogen is even more limited in the cave, which not only pushed the cave *G. minus* population into omnivory, but also encouraged N retention.

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References

Carlini DB, Mannng J, Sullivan PG, Fong DW (2009) Molecular genetic variation and population structure in morphologically differentiated cave and surface populations of the freshwater amphipod *Gammarus minus*. Mol Ecol 18:1932–1945

Castellini MA, Rea LD (1992) The biochemistry of natural fasting at its limits. Experientia 48:575–582

Culver DC, Pipan T (2009) The biology of caves and other subterranean habitats. Oxford University Press, New York City
Culver DC, Fong DW, Jernigan RW (1991) Species interactions in cave stream communities: experimental results and microdistribution effects. Am Midl Nat 126:364–379
Culver DC, Kane TC, Fong DW (1995) Adaptation and natural selection in caves. The Evolution of Gammarus minus. Harvard University Press, Cambridge
Denno RF, Fagan WF (2003) Might nitrogen limitation promote omnivory among carnivorous arthropods? Ecology 84:2522–2531
Elser JJ, Fagan WF, Denno RF, Dobberfuhl DR, Folarin A, Huberty A, Interland S, Kilham SS, McCauley E, Schultz KL, Siemann EH, Sterner RW (2000) Nutritional constraints in terrestrial and freshwater food webs. Nature 408:578–580
Fagan WR, Siemann E, Mitter C, Denno RF, Huberty AF, Woods HA, Elser JJ (2002) Nitrogen in insects: implications for trophic complexity and species diversification. Am Nat 160:784–802
Fenolio DB, Graening GO, Collier BA, Stout JF (2005) Coprophagy in a cave-adapted salamander: the importance of bat guano examined through nutritional and stable isotope analyses. Proc R Soc Lond [Biol] 273:439–443
Fong DW (2011) Management of subterranean fauna in karst. In: Van Beynen P (ed) Karst management. Springer, N.Y, pp 201–224
Francois CM, Mermillod-Blondin F, Malard F, Fourel F, Lecuyer C, Douady CJ, Simon L (2015) Trophic ecology of groundwater species reveals specialization in a low-productivity environment. Funct Ecol. doi:10.1111/1365-2435.12484
Fry B, Arnold C (1982) Rapid 13C/12C turnover during growth of brown shrimp (*Penaeus aztecus*). Oecol 54:200–204
Hobson KA, Alisauskas RT, Clark RG (1993) Stable-nitrogen isotope enrichment in avian tissues due to fasting and nutritional stress: implications for isotopic analysis of diet. Condor 95:388–394
Hutchins BT, Schwartz BF, Nowlin WH (2014) Morphological and trophic specialization in a subterranean amphipod assemblage. Freshw Biol 59:2447–2461
Kostalos M, Seymour RL (1976) Role of microbial enriched detritus in the nutrition of *Gammarus minus* (Amphipoda). Oikos 27:512–516
Lajtha K, Michener RH (2007) Stable isotopes in ecology and environmental science, 2nd edn. Blackwell Scientific Publications, Cambridge
MacNeil C, Dick JTA, Elwood RW (1997) The trophic ecology of freshwater *Gammarus* spp. (Crustacea:Amphipoda): problems and perspectives concerning the functional feeding group concept. Biol Rev 72:349–364
Minagawa M, Wada E (1984) Stepwise enrichment of ^{15}N along food chains: further evidence and the relation between $\delta^{15}N$ and animal. Geochim Cosmochim Acta 48:1135–1140
Mulholland PJ, Tank J, Sanzone DM, Wollheim WM, Peterson BJ, Webster JR, Meyer JL (2000) Food resources of stream macroinvertebrates determined by natural-abundance stable C and N isotopes and a 15N tracer addition. JNABS 19:145–157
Navarro-Barranco C, Tierno-de-Figueroa JM, Guerra-Gracia JM, Sanchez-Tocino L, Garcia-Gomez JC (2013) Feeding habits of amphipods (Crustacea: Malacostraca) from shallow soft bottom communities: comparison between marine caves and open habitats. J Sea Res 78:1–7
Parker PL, Anderson RK, Lawrence AL (1991) Stable isotope methodology for evaluation of nutritional requirements of shrimp. In: DeLoach P, Dougherty WJ, Davidson MA (eds) Frontiers in Shrimp Research. Elsevier Science Publishers, Amsterdam, pp 157–171
Simon KS, Benfield EF, Macko SA (2003) Food web structure and the role of epilithic biofilms in cave streams. Ecology 84(9):2395–2406